APPARATUS AND METHOD FOR DETERMINING &/OR MONITORING ELECTROPHYSIOLOGICAL PROPERTIES OF ION CHANNELS

The present invention provides an apparatus and a method for making reliable electrical measurements on ion channel containing objects such as cell membranes and artificial membranes, which is capable of high throughput and substantially automated operation, and is capable of using substantially smaller amounts of test compound, than presently known apparatus. In particular, the invention provides an apparatus in which is provided a cell detection means which detects the presence of the cell in a flow system at a position remote from the measurement site, that detection means being in communication with the location means so as to actuate the location means when a cell is detected.

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APPARATUS AND METHOD FOR DETERMINING &/OR MONITORING ELECTROPHYSIOLOGICAL PROPERTIES OF ION CHANNELS

This invention relates to an apparatus or method for determining and/or monitoring electrophysiological properties of ion channels in ion channel containing objects, by establishing an electrophysiological measuring configuration. The invention is particularly directed towards an apparatus or method for determining and/or monitoring a current flow through a cell membrane forming part of a cell by forming a high resistance seal around a measuring electrode.

The apparatus of the invention may form part of a measuring system for studying electrical events in cell membranes, such as an apparatus for carrying out patch clamp techniques to study ion transfer channels in biological membranes. More particularly, the invention relates to an apparatus for such a measuring system having high throughput and utilising only small amounts of test compounds and liquid carrier, and being capable of carrying out many tests in a short period of time by performing parallel tests on a number of cells simultaneously and independently.

It is known to electrically insulate a patch of membrane and study the ion channels in that patch under voltage-clamp conditions (the ‘patch-clamp’ technique) (Hamill et al., (1981): “Improved patch-clamp techniques for high resolution current recordings from cells and cell free membrane patches”, Pflügers Arch. 391, 85-100).

Ion channels are transmembrane proteins that catalyse transport of inorganic ions across cell membranes. These ion channels participate in processes as diverse as, for example, generating and timing action potentials, synaptic transmission, secretion of hormones and contraction of muscles. Many
drugs exert their specific effects via modulation of ion channels. Examples include the antiepileptic compounds phenytoin and lamotrigine which block voltage dependent Na\(^+\) channels in the brain, the antihypertensive drugs nifedipine and diltiazem which block voltage dependent Ca\(^{2+}\) channels in smooth muscle cells, and stimulators of insulin release like glibenclamide and tolbutamide which block an ATP regulated K\(^+\) channel in the pancreas.

In addition to chemically induced modulation of ion channel activity, the patch clamp technique has enabled scientists to perform manipulations with voltage dependent channels. These techniques include adjusting the polarity of the electrode in the patch pipette and altering the saline composition to moderate the free ion levels in the bath solution.

The patch clamp technique represents a major development in biology and medicine, since this technique allows measurement of ion flow through single ion channel proteins, and further allows the study of the responses of single ion channels to drugs.

Standard patch clamp techniques use a thin (approx. 0.5-2\(\mu\)m in diameter) glass pipette. The tip of this patch pipette is pressed against the surface of the cell membrane so that the tip seals tightly to the cell and isolates a few ion channel proteins in a tiny patch of membrane, while forming a high resistance seal between the interior of the pipette and the surrounding solution. Such a seal is known as a ‘gigaseal’.

The activity of these channels can be measured individually (“single channel” recording) by removing the remaining parts of the cell or, alternatively, the patch can be ruptured (e.g. by applying subatmospheric pressure in the pipette) to give high conductance access to the cell interior, so allowing measurements of the channel activity of the entire cell.
membrane ("whole cell" recording). In an intermediary patch clamp method between the single channel and whole cell, ("cell-attached" method), the cell is attached to the pipette with a gigaseal but the patch of membrane inside the pipette remains intact.

During both single channel recording and whole cell recording, the activity of individual channel subtypes can be characterised by imposing a "voltage clamp" across the membrane, so that there is a constant membrane potential. This is achieved because the amplifier supplies exactly the current which is necessary to keep the membrane potential at a predetermined level. Hence, currents resulting from opening and closing of ion channels are not allowed to influence the membrane potential.

The time resolution and voltage control in such experiments are often in the millisecond or even microsecond range. However, a major obstacle to the use of the patch clamp technique as a general method in pharmacological screening has been the limited rate at which compounds could be tested (typically no more than one or two per day). This is compounded by the slow rate of solution change that can be accomplished around cells and patches.

Major limitations determining the throughput of the patch clamp technique result from the need for localisation and clamping of cells and pipette, and the macroscopic nature of the solution feed system which leads the dissolved compound to the cells or patches.

In standard patch clamp set-ups, cells are placed in experimental chambers which are continuously perfused with a physiological salt solution. The establishment of the cell-pipette connection in these chambers is time-consuming and troublesome. Compounds are applied by changing the inlet
to a valve connected to a small number of feed bottles. The required volumes of the supporting liquid and the sample to be tested are high.

High throughput systems for performing patch clamp measurements have been proposed, which typically consist of a substrate with a plurality of sites adapted to hold cells in a measuring configuration where the electrical properties of the cell membrane can be determined.

WO 99/66329, (Cenes), discloses an apparatus comprising a substrate with perforations arranged in wells and electrodes provided on each side of the substrate. The substrate is made by perforating a silicon substrate with a laser and may be coated with anti-adhesive material on the surface. The substrate is adapted to establish gigaseals with cells by positioning the cells on the perforations using suction creating a liquid flow through the perforations, providing the anti-adhesion layer surrounding the perforations, or by guiding the cells electrically. The cells can be permeabilised by electromagnetic (EM) fields or chemical methods in order to provide a whole cell measuring configuration.

All perforations, and hence all measurable cells in a well, share one working electrode and one reference electrode. This means that measurements on individual cells can not be performed.

WO 99/31503, (Vogel et al.), discloses a measuring device with an aperture arranged in a well on a substrate (carrier) and separating two compartments. The measuring device comprises two electrodes positioned one on either side of the aperture and adapted to position a cell at the aperture opening. The substrate may have hydrophobic and hydrophilic regions in order to guide the positioning of the cells at the aperture opening. Cell positioning
by means of electrophoretic movement of cells towards the aperture is also disclosed.

WO 01/48474 (AstraZeneca) discloses a measuring device comprising a first channel in which is located an aperture which separates the first channel from a second channel. An electrode is in contact with liquid in each channel. Cells are caused to flow through the first channel to the vicinity of the aperture, where they may be located at the aperture by entrainment in flow through the aperture, or by other means, for example electrophoresis or dielectrophoresis. The aperture is adapted to allow formation of a high resistance seal between the cell and the region surrounding the aperture. The use of impedance measurements between the electrodes in the first and second channels to determine if a cell is at the aperture is disclosed.

While the devices of the prior art offer means of making measurements on ion channel containing objects in a potentially more automatic way, they suffer from the disadvantage that the location means which locates the cell at the measurement site is not itself controlled. This means that the location means either operates continuously, or at a predetermined time only, so relying on a cell to be tested to be within range by chance at a given time. If the concentration of cells in the suspension is low, or the location means exerts only a weak force, the location process is found in practice to be time-consuming and uncertain. This is a significant limitation on the capabilities of the prior art apparatus.

Additional problems arise with the prior art devices due to the operation of the location means itself. In general, in each of the devices of the prior art it is advantageous to limit the amount of time during which the cell location means operates. In the case that the measurement site comprises an aperture
separating two solutions, the cell suspension solution (the 'front-side' solution) will usually have a different composition from that of the solution at the opposite side of the aperture (the 'rear-side' solution). The front-side solution has a composition suitable for the external cell environment while the rear-side solution is intended to match the internal composition of the cell for good results in whole cell configuration measurements. This means that the more front-side solution that is drawn through the aperture, the longer it takes for re-equilibration of the rear-side composition by diffusion before measurements occur. Alternatively, active exchange of the rear-side solution is needed, so increasing the complexity of the design.

In the case that cell location is by electrophoresis, as for example in WO 99/31503, it is desirable also that the operating time is minimised. Capture by electrophoresis has the disadvantage that the DC potential difference between the electrodes will cause a DC current to flow, leading to redox reactions at the electrodes which can be problematic owing to the limited redox capacity of reversible electrodes such as Ag/AgCl. In addition gas consumption or generation may occur if irreversible electrodes such as Pt are used. The limited capacity of small reversible electrodes makes prolonged operation in electrophoresis mode particularly disadvantageous if the electrodes have to be very small indeed, for example if a measuring electrode is to be surrounded by a gigaseal.

The operating time needed by the location means to locate a cell might be reduced by increasing the concentration of cells in the suspension. However, this increases the risk of blockage of the system through accumulation of agglomerates of cells, many types of which have a tendency to cluster when at high concentration. This also increases the risk of more than one cell being located at the measurement site simultaneously, so compromising the measurement. Operation with fairly dilute cell
suspensions has advantages, provided that the pumping time for capture can be kept low.

Irrespective of which cell location means is used, it is advantageous to activate the location method only when a suitable cell for test is within close enough range of the measurement site that the problems arising from overlong operation of the location mechanism are avoided.

The devices of the prior art comprise means for detection of cells in the vicinity of the measuring site, but have the disadvantage that the cell is only detected when it is close to the measuring site. WO 99/31503 for example discloses that measurement of the resistance between the aperture and the bulk of solution outside the aperture is used to detect the presence of the cell in the vicinity of the aperture.

It is well known that the resistance between the inside of a narrow orifice and a remote point in a conducting solution outside the orifice is not much affected by the presence of a non-conducting body or plane outside a distance similar to the diameter of the orifice (Korchev et al., (1997) *Biophys J.* 73 p653-8).

Measurement of resistance between electrodes on either side of the aperture, disclosed in WO 99/31503, is therefore useful only for detection of the cell when it is already close to the aperture, probably within a maximum distance of 5 times the diameter of the aperture. This is useful to control the location process for example to avoid the use of too great a location force at the point at which the cell reaches the aperture, so reducing the risk of the cell being drawn through the aperture.
Such measurements cannot detect a cell positioned further than the maximum distance from the aperture, so are not useful to initiate the location process to capture a cell from a dilute suspension while avoiding the disadvantageous effects described above.

Electrical detection of cells, or measurement of their properties, in a flowing medium is well known, and in the prior art has been used to control sorting or manipulation of the cells at a downstream location.

It is known to use the Coulter principle for the detection of cells in a flowing stream and for the measurement of their sizes. This method was first disclosed in US 2 656 508 (and see for example US 6 175 227 and further references therein), which describes how the resistance of a volume of electrolyte is increased when a cell lies within it, because the cell membrane is of higher resistivity than the electrolyte. The increase in resistance is proportional to the ratio of the cell volume to the volume of the electrolyte under consideration.

The measurement is commonly accomplished by measuring the impedance of an aperture through which a cell suspension flows; electrodes are located in larger compartments on each side of the aperture, and the detection volume is the volume within the aperture, which being small gives high sensitivity. The instruments operating by this principle are commonly referred to as ‘Coulter counters’. This principle has been extended to more complex AC measurements of the impedance of the cell and cell / suspension interfaces from which information on the properties of the cells can be deduced.

The use of an aperture allows large electrodes to be used, and relaxes constraints on their fabrication and location. It is clear from the general
principle though that if small electrodes are used, enclosing a small volume of electrolyte of typical dimension the same order as that of the cell, then an aperture is not necessary and the simple presence of the cell between the electrodes will give a measurable rise in resistance between them.

US 6 169 394 (Frazier et al., University of Utah) discloses a microsystem for characterisation of cells or other particulates suspended in a medium by means of impedance measurements, where the suspension is flowed through a microchannel with one or more pairs of electrodes located transversely across the channel. The space between the electrodes then constitutes the Coulter aperture. Optionally electrodes may also be provided in reservoirs at the inlet and outlet ends of the microchannel in analogy with conventional Coulter counter systems.

US 6 122 599 (Mehta) describes an advanced Coulter principle particle measurement apparatus which is designed to allow characterisation of cells and other particles while avoiding the errors which can arise in simpler Coulter apparatus. This disclosure refers to many previous developments in the field and describes their shortcomings. The apparatus provides further sets of electrodes in addition to the two usually found one each side of the aperture, in order to increase the reliability of measurement.

Blankenstein et al. {Biosensors and Bioelectronics, vol. 13 no 34, (1998) 427-438} describe a micro-fabricated Coulter counter fabricated in planar layers on the surface of a substrate, though this is not used to control downstream analysis processes. Similarly Satake et al. {Sensors and Actuators B 83 (2002) 77-81} describe a Coulter principle blood cell analyser micro-fabricated on a chip surface, again used to analyse particles but not to control a downstream process. Both of these devices use a planar flow channel with a constriction which constitutes the Coulter aperture, and
electrodes in larger upstream and downstream areas each side of a constriction.

According to a first aspect of the present invention there is provided an apparatus for determining and/or monitoring of electrophysiological properties of ion channels in ion channel containing objects the apparatus comprising:

(i) a substrate comprising a first measuring site for holding ion channel containing objects, the site comprising a passage in the substrate, a first end of the passage defining a first aperture in a first upper surface of the substrate and being in contact with a first domain, the first upper surface of the substrate in the vicinity of the aperture being adapted to form a seal with an ion channel containing object held at the site, and a second end of the passage being in contact with a second domain;

(ii) an inlet channel in liquid communication with the first domain and the first aperture;

(iii) a first measurement electrode in electrical contact with the first domain;

(iv) a second measurement electrode in electrical contact with the second domain;

(v) measuring means electrically connected to the first and second measurement electrodes and adapted to make electrical
measurements on an object sealed to the sealing region at the
measurement site;

(vi) location means for causing movement of the objects towards
the measuring site to cause an object to form a seal with the
first aperture;

wherein the apparatus further comprises a Coulter detector for detecting the
presence of an object in the inlet channel, the detector controlling operation
of the location means, such that movement of an object towards the
measuring site is caused by the location means in response to signals
emitted from the detector.

According to a second aspect of the present invention there is provided an
apparatus for determining and/or monitoring of electrophysiological
properties of ion channels, in ion channel containing objects, the apparatus
comprising:

i) a substrate comprising a first measuring site for holding ion
channel containing objects, the substrate having a first surface in
contact with the first domain, the measuring site comprising a first
measurement electrode; a sealing region surrounding the first
measurement electrode; and a conductive track for connecting the
first measurement electrode to a measuring instrument;

ii) an inlet channel in liquid communication with the first
measuring site and the first domain;
iii) a second measurement electrode which, in use is in electrical contact with the first domain;

iv) measuring means electrically connected to the first and second measurement electrodes and adapted to make electrical measurements on an object sealed to the sealing region at the measurement site;

v) location means for causing movement of an object towards and onto the measurement site;

vi) wherein the apparatus further comprises Coulter detection means which determines the presence of the object in the inlet channel which detection means outputs a signal which controls the location means whereby the flow of an object towards the measuring site is caused by the location means in response to signals emitted from the detector.

The term “Coulter detector” is used herein to describe a detector that detects cells using the Coulter principle.

An important feature of the present invention is that not only does the detector detect when an object is in the inlet channel, but it also detects movement of the object through the inlet channel. This means that the detector which is operably connected to the location means will in use, activate the location means when an object is detected in the inlet channel, and then also control the location means such that the movement of the object towards the measurement site is accurately controlled. This will reduce, if not remove the chances of more than one cell arriving at the
aperture or the working electrode at the same time, which might prevent a
gigaseal from forming correctly.
Although the invention has been described with respect to an ion channel
containing object it is to be understood that this term embraces any object
comprising an ion channel containing structure such as a cell or artificial
vesicle. A cell is an example of such of structure and for clarity is used as
such in this specification.

The seal formed between the cell and the aperture or working electrode is a
gigaseal. The term “gigaseal” refers to a high resistance seal conventionally
having a resistance of at least 1G Ohm, although for certain types of
measurements where the test current is large, lower values may be
sufficient.

Preferably the first and second domains comprise first and second ionic
solutions respectively. The cell is preferably moved to the measurement
site in a suspension flowing through the inlet channel.

Advantageously the detector comprises first detection means comprising a
pair of detection electrodes forming a detection region within the inlet
channel, the volume of the detection region being sufficiently small that the
resistance of the solution increases measurably when a cell is within the
solution.

Preferably, the cross sectional dimension of the Coulter detector is less than
20 times the dimension of the cell and yet more preferably less than 5 times.
Preferably in the second aspect of the invention the first measurement electrode has a diameter of less than 5 µm, and more preferably less than 3 µm.

Preferably, in the second aspect of the invention the first and second measurement electrodes are formed on or proximal to the first surface.

The detection region may be defined between two electrodes located in the inlet channel, either transversely across the channel or adjacent to one another.

Advantageously however, the inlet channel comprises a constriction forming a narrowest region within the channel, and the detection region is located in the narrowest region of the channel. Detection electrodes are placed in the inlet channel on either side of the constriction so that in use they contact the solution in the channel so as to establish a conducting path between the electrodes through the constriction.

This means that the constriction comprises the region of highest electrical resistance between the electrodes.

Advantageously the constriction has dimensions comprising a height and/or width of less than 200µm, and/or a length of less than 1mm, and yet more preferably dimensions of a height and/or width of less than 100µm, and/or a length of less than 500µm.

The measurement site may be positioned within the detection region. Alternatively it is positioned downstream of the detection region. This means that when the detector detects the presence of a cell passing through the detection region, the detector causes the location means to operate
before the cell has travelled towards the location means. In other words the location means are used to draw the cell towards the measurement site. The location means are activated just after the cell has entered or passed the channel, depending on whether the cell velocity is also used to detect some suitable cells.

 Preferably, the measurement site is located at a downstream end of the constriction in the channel. This means that when the concentration of cells is sufficiently low that only one cell on average is within the narrow portion at any one time, that cell can be detected and located at the measurement site without other cells which may be within the vicinity of the measurement site being located either instead of or together with the detected cell.

 It has been found advantageous to detect the speed with which the cell passes through the inlet channel. Owing to the parabolic flow profile of the suspension medium in the channel, cells in the centre will flow substantially faster than those near the side walls. It is desirable to avoid locating slow moving cells at the measurement site. This is because, due to the parabolic flow profile, the slow moving cells will have followed paths close to the edges of the channel. This could mean that the cells have touched the sides of the channel and therefore it may be difficult to form a gigaseal between such a cell and the aperture. It is advantageous therefore to activate the location means only when cells moving at a particular speed are detected. Speed of the cell can be calculated from the time it spends inside the detection region.

 Advantageously the detector comprises a plurality of detection regions at a pre-determined separation distance, for determining the speed of the object from the time taken to pass from one detection region to the next; or a
single detection region further comprising a plurality of electrode pairs at a
pre-determined separation distance, for determining the speed of the cell
from the time taken to pass from one electrode pair to the next.

The detection region must be made sufficiently long in the direction of flow
so that the time that the cell spends between the electrodes can be measured.

Preferably the electrodes forming the electrode pair have a length in the
range 10-500μm (i.e. in the range 5 to 10 cell diameters). If more than one
pair of electrodes is used, 5 to 10 cell diameters should be the spacing
between adjacent pairs of electrodes.

Advantageously, the apparatus comprises a plurality of measuring sites
which may be used simultaneously. In such an apparatus, the detector and
location means will be designed to give a minimum degree of interrogation
and consequent signal processing to allow the selection of appropriate cells
from a suspension.

The apparatus may comprise a plurality of location means. For example in
the case where the measurement site comprises an aperture, an initial
location stage involving electrophoresis or dielectrophoresis may be used to
move a cell towards the inlet channel. This may be followed by a final
location of the cell by suction through the aperture.

Advantageously, the signal from the Coulter detector may be used for
discriminating between the various types of cell and other objects or
particles found within a suspension; and also for selecting only the type of
cell of interest – e.g. single healthy cells. The Coulter signal, i.e. the
fractional change in resistance (R) of the detection region, depends on the
volume of the cell relative to that of the detection region according to
\[ \Delta R / R = V(\text{cell}) / V(\text{detection region}) \]

In general, cells will be present in the suspension in a variety of sizes, and only those within a given size range will be desired for tests. Analysis of the Coulter signal (for example, size and shape of the pulse) can be used to select cells of a given size and nature, so excluding debris (in general smaller than the cells of interest) and agglomerations of cells (bigger than the upper size range). Where the test objects are cells, the electrical properties of dead and unhealthy cells are different from those of live cells e.g. the cell membrane is often more permeable and of lower resistivity. Analysis of the Coulter signal allows the selection of healthy cells for subsequent analysis.

Either DC, pulsed DC or AC interrogation of the detection region can be used. Using pulsed DC or AC excitation will give a higher degree of discrimination between different types of cell and their properties, e.g. the condition of the cells.

The use of DC is preferred in small multi-channel systems, owing to the simpler electronics and the minimisation of cross-talk between the detection system and measurements that might be running simultaneously in other parts of the system.

When a DC measurement arrangement is used with a single pair of electrodes, DC detection means that a net current is passed between electrodes which can lead to electrochemical changes of the electrode surface which in turn can cause an undesirable drift in the electrode potential. The signal is expected to be small, and so a high gain is needed in the system to give a useful output. However, drift then becomes a problem
especially with design and manufacturing constraints leading to small electrode areas.

Preferably therefore the apparatus further comprises a measurement system which comprises a self balancing DC bridge, with the resistance of the inlet channel in one arm, which can compensate for drift in the electrode potentials at steady state current with time, together with signal conditioning to give a useful output. However it is to be understood that different types of circuitry are also appropriate to the present invention.

The location means may comprise means for causing pumped flow through an aperture, the pumping being achieved by any desired means. Slight counterflow can be provided through the aperture as is the case in conventional electrophysiology experiments, to maintain a clear aperture and surrounding sealing region, until such time as a suitable cell is detected. The detector then controls the location means in combination with, or in place of the counterflow, thereby to achieve location of the cell with optimally low probability of debris interfering with the process.

The location means may make use of electrophoresis, dielectrophoresis, magnetic attraction of a magnetically tagged cell or any other force in order to move a cell towards the measurement site.

Where pumping is used this may be achieved by any means known in the art. For example, pumping may be by means of electroosmotic flow, or other electrokinetic effects such as electrocapillarity, control of pressure from a pressurised fluid, creation of vapour or gas in a region in contact with the solution by chemical reaction or boiling, pumps driven by piezoelectric effects or other means known in the art.
with the solution by chemical reaction or boiling, pumps driven by piezoelectric effects or other means known in the art.

Preferably, the sealing region at the measuring site comprises the surface of the substrate itself and is preferably patterned to give a localised sealing region in the vicinity of the measuring electrode or the aperture so as to minimise the possibility of a cell adhering to a site without forming a seal that entirely surrounds the measuring electrode or aperture.

Although the seal between the cell and the aperture is described as being formed on the first upper surface of the substrate, it is to be understood that the seal may extend into the passage, or alternatively, may be formed completely within the passage.

The apparatus according to the first and second aspects of the present invention may comprise a plurality of test structures for testing cells as described herein above.

The apparatus of the invention can be used in such a way that the location means operates while the solution in the inlet channel is in motion, or with the solution stationary, according to the exact way in which the location means is designed to work in a given embodiment. When a suitable cell for testing is within the detection region the location means is actuated.

If the test structure is designed such that that flow of the solution slows over time, the location means can be actuated when a cell is detected travelling slowly enough for the location means to be effective.
(i) supplying a first conductive solution containing at least one object through an inlet channel to a first surface of a substrate comprising a measuring site having an aperture, such that the first solution contacts the first surface, the first surface being in fluid communication with a second surface of the substrate via the aperture;

(ii) supplying a second conductive solution to the second surface so as to establish liquid contact between the first and second surfaces;

(iii) measuring electrical continuity to test that liquid contact has been achieved between first and second electrodes respectively located on or proximal to the first and second surfaces;

(iv) detecting the presence of an object using a Coulter detection means provided in the inlet channel;

(v) using the output of the detector to control a location means to drive a cell in the first solution towards a measurement site having a sealing region surrounding the aperture;

(vi) performing electrical measurements on the object.

According to a fourth aspect of the present invention there is provided a method of making electrical measurements on an ion channel containing object, comprising the steps of:

(i) supplying a first conductive solution comprising at least one ion channel containing object through an inlet channel, to a first surface of a substrate comprising a measurement site having a first
21 measurement electrode located on the first surface, and a sealing region surrounding the first electrode;

(ii) providing a second measurement electrode in electrical connection with the first solution;

(iii) detecting the presence of an object using a Coulter detector provided in the inlet channel;

(iv) using the output of the detector to control a location means to drive a membrane containing object to the measurement site to form a seal around the first measurement electrode;

(v) performing electrical measurements on the object.

Preferably the method comprises the further step of testing the resistance of the seal at the measurement site on the substrate by measuring the electrical impedance between said first and second electrodes immediately after performing step (iv).

Even more preferably the method comprises the further step of establishing a measurement configuration for the cell by means of one or more electrical pulses applied between the first and second electrodes immediately before performing step (v).

Embodiments of the invention will now be further described by way of example only with reference to the accompanying figures in which:-

Figure 1a is a first cross section of an embodiment of a test structure forming part of an apparatus according to the invention;
Figure 1b is a plan view of part of the test structure shown in Figure 1a;

Figure 2a is a representation of an inlet channel of the test structure shown in Figures 1a and 1b;

Figure 2b is a representation of an inlet channel of the test structure shown in Figures 1a and 1b with different dimensions from that in Figure 2a;

Figure 3 is a representation of a measuring system comprising the test structure shown in Figures 1a and 1b;

Figure 4a-c are plan views of part of alternative embodiments of the inlet channel forming part of the test structure of Figure 1a;

Figure 5 shows resistances in the solution path between the detection electrodes in the test structure shown in Figure 1a;

Figure 6 is a representation of an electronic measuring system according to the invention suitable for operation with the test structure of Figure 1a;

Figure 7 is a representation of a second electronic system suitable for operation with the test structure of Figure 1a;

Figure 8a is a graphical representation of the variation in output voltages of the Coulter detector forming part of the invention, with cell size;

Figure 8b & c show results from tests using the test structure of Figure 1a with the electronic system of Figure 7;
Figures 9a, 9b, 10a, 10b, 11a, 11b, are representations of various inlet channels suitable for forming part of the test structure of Figure 1a;

Figures 12 and 13 are representations of further embodiments of the invention.

Referring to Figures 1a and 1b, an apparatus in the form of a test structure according to the present invention is shown.

A test structure 10 comprises a substrate 12, a housing component 14. The substrate defines a passage 16 forming apertures 600, 610 in top and bottom surfaces of the substrate respectively. Cells to be tested can be located at the aperture 600. The substrate 12 is formed from an inorganic material, for example silicon with one or more insulating coatings. The housing compartment 14 serves to form one or more liquid pathways between the exterior of the test structure and the substrate and is preferably formed from a polymer.

The test structure comprises a measurement site 18 comprising the passage 16 extending through the substrate 12. The aperture 600 is adapted to receive a cell to be tested and is surrounded by a sealing region to which the cell can adhere so as to form a gigaseal.

A front-side channel 20, which defines a first domain adapted to receive a liquid containing cells to be tested, contacts the aperture 600. A second, or rear-side domain 22 contacts the aperture 610. In the embodiment shown in figure 1a, only a single measurement site and a single second domain are shown. However, it is to be understood that a plurality of measurement sites and associated structures may be provided in a single test structure, and more than one test structure may be provided in each apparatus.
The substrate 12 is mounted in the housing in such a way as to achieve a high resistance seal between the first and second domains.

The front-side channel 20 is in liquid communication with housing inlet pathway 28 and housing outlet pathway 32, the sense of inlet and outlet being defined as the usual direction of motion of the cells during the test procedure. The housing inlet pathway 28 in turn communicates with an inlet well or reservoir 30 into which solution can be added from outside the device, and the housing outlet pathway 32 communicates with an outlet well 34. The rear-side compartment 22 similarly communicates with rear side inlet pathway 29, and then with a rear side inlet well 31; also with rear side outlet pathway 33 and then with rear side outlet well 35. Electrode 36 is provided which in use, contacts liquid in the housing inlet pathway 28. Electrode 38 is provided to contact liquid in the housing outlet pathway 32, and electrode 40 is provided to contact liquid in second domain 22. Contact to the electrodes from outside the device is by means of suitable tracking provided within the housing component 14 to external contacts as known in the art. A detection amplifier (not shown) is connected between electrodes 36 and 38 to allow detection of cells in the front-side channel according to the Coulter principle.

In the embodiment shown in Figures 1a and 1b, the electrodes are provided within the housing; in an alternative embodiment one or more of the electrodes may be provided as part of the supporting instrumentation system and may make contact with the liquids in the test structure by dipping into the wells.

Figure 1b shows a diagrammatic plan view of the test structure of Figure 1a. The front-side channel 20 is shown communicating with the housing inlet
pathway 28 through the channel 52, and similarly with the housing outlet pathway through the channel 54. The front-side channel has a constriction 50 formed as a region where the front-side channel narrows while maintaining constant depth and in this embodiment the measurement site 18 lies centrally within a constriction. The current path between electrodes 36 and 38 in this embodiment comprises the liquid contained within the housing inlet and outlet channels, the front channel and the channels 52, 54 connecting them. The detection region is located at constriction 50. This means that the resistance between the electrodes 36 and 38 will increase as the cell enters a constriction.

The apparatus shown in figure 1a and 1b can be fabricated in different ways from a variety of different materials, such that the material adjacent the aperture in the sealing region is suitable to form a gigaseal to a cell comprising a lipid membrane. Such materials include silicon, plastics, pure silica and other glasses such as quartz or borosilicate, or silica doped with one or more dopants selected from the group of Beryllium (Be), Magnesium (Mg), Calcium (Ca), Boron (B), Aluminium (Al), Gallium (Ga), Germanium (Ge), Nitrogen (N), Phosphorus (P), Arsenic (As) and oxides from any of these. The adhesion material may itself form the upper surface of the substrate 12, or may be deposited over or implanted into the upper surface.

The minimum dimensions of the device are similar to those of the cells which are to be tested, so for example for typical mammalian cells of diameter of order 10 \( \mu \text{m} \) the diameter of the gigaseal will preferably be 5 \( \mu \text{m} \) or less. Therefore the diameter of the aperture where present is preferably 5 \( \mu \text{m} \) or less, more preferably 2 \( \mu \text{m} \) or less; the outside diameter of the adhesion region is preferably 10 \( \mu \text{m} \) or less. These dimensions are
varied according to the size of the cell to be tested. Larger cells require larger dimensions and smaller cells require smaller dimensions.

Methods of fabrication of the embodiment in figure 1 are based on standard micro-fabrication processing technology as known in the art, preferably using a silicon substrate as the basis for the substrate 12 as described in our earlier application WO 02/29402.

The front-side channel 20 may be formed on the surface of the aperture substrate 12 using a photolithographic process in order to define the shape of the channel and a constriction in a layer of material 56. The front-side channel is therefore conveniently a constant height and a constriction is formed by a narrow region in the channel as shown, though by patterning more than one layer of material 56 the height may also be varied.

Such a structure is conveniently made using the photo-patternable epoxy SU8 (Microchem Inc.).

The substrate 12 is mounted adjacent the housing component 14 in such a way as to establish liquid connections between the front channel 20 and housing inlet and outlet pathways 28 and 32, by means of the vertical channels 52 and 54. Such mounting means include liquid sealant and compressible gaskets.

The constriction in the front-side flow channel preferably has a maximum dimension of height or width of 200 μm, and more preferably is less than 100 μm, with a minimum dimension set by the dimension of the cells to be tested. A front channel constriction dimension of 50 μm x 50 μm is effective.
The length of a constriction is preferably less than 1 mm, and more preferably is less than 500 μm. The dimensions of the remainder of the front-side flow channel and of the housing inlet and outlet pathways 28 and 32 defining the current path between the electrodes 36 and 38 are not critical, but the overall impedance of the solution in the portion of the electrical pathway between the electrodes outside a constriction is preferably less than that inside a constriction in order for sensitivity of detection to be maintained.

Figures 2a and 2b show a substrate 12 before being mounted in the housing, with front channels formed on the upper surface of the substrate from photo-patterned SU8 50 μm thick and of the form shown diagrammatically in figure 1b. In Figure 2a, the constriction 50 in the channel is 50 μm wide and in figure 2b it is 200 μm wide.

Figure 3 shows a diagram of an apparatus of the invention incorporating the test structure shown in figures 1 and 2. The test structure is used in conjunction with other parts of the apparatus that serve to deliver the solutions, test cells and compounds to be tested. Figure 3 represents only sufficient of the apparatus to illustrate the principle of the function of the test structure. In addition to the components shown, the apparatus comprises solution dispensing means, an instrument which holds the test structure and makes pressure and/or electrical connections to the test structure, and control means as appropriate to run the necessary sequence of operations.

In the embodiment shown in figure 3, the location means causes flow through the test aperture. The test structure 10 is provided with a front-side inlet well 30 and a rear-side inlet well 31. The wells are adapted to receive a pressure-tight connection, which allows liquids to be driven through the
structure under pressure from gas or liquid. The front-side inlet well 30 is open to the atmosphere in order that solutions can be dispensed into the well by dispensing means (not shown).

The front-side outlet well 34 is connected via a pressure connection 100 to a source of negative pressure (Pf). The rear-side outlet well 35 is connected via a pressure connection 102 to a source of pressure (Pr1) and rear-side inlet well 31 is connected via a connector 104 to a source of pressure (Pr2). The front-side outlet electrode 38 is connected to impedance measuring means 112, which is also connected to rear-side electrode 40. The front-side inlet electrode 36 is also connected to a detection amplifier 110, which is also connected to the front-side outlet electrode 38. Outputs from the impedance measuring means 112 and the detection amplifier 110 are passed to the location control means 114, which controls pressure outputs (Pf), (Pr1) and (Pr2).

The apparatus performs the following steps when in use:

1. The rear-side of the test structure is first primed by dispensing rear-side solution into well 31 and applying a positive pressure (Pr2) > (Pr1) to drive liquid through the rear-side channel. (Pr2) and (Pr1) are then equalised at positive pressure (Pr1 and Pr2 > atmospheric pressure) to force the solution to the rear-side of the aperture and to remove any air bubbles that may become trapped there.

2. Front-side solution is then dispensed into the inlet well 30 and negative pressure (Pf) < atmospheric pressure is applied to well 34 to draw the front-side solution through the front-side channel 20 and over the aperture until it reaches well 34. The impedance measuring means 112 then checks that continuity has been achieved through the aperture.
3. Cell suspension is then dispensed into the inlet well 30 and drawn through the front channel 20. The detection amplifier 110 monitors the impedance between the front-side inlet and outlet electrodes 36 and 38. In figures 1a and 1b the detection region is the narrow part 50 of the front-side channel 20; the detection amplifier will register an increase in impedance when a cell enters this region. The detection amplifier then optionally compares the impedance rise with that expected for a single healthy cell in order to determine whether this is the cell type present.

4. The detection amplifier then instructs the location control means 114 to apply negative pressures (Pr1) and (Pr2) to the rear-side to draw the cells towards the aperture. Optionally the impedance measuring means 112 then monitors the impedance through the aperture, and when this is increased by the approach of a cell, regulates the rear-side pressure to achieve a gigaseal without disrupting the cell membrane.

5. Once a gigaseal is established, the whole-cell configuration is achieved by means of a sudden negative pressure pulse to the rear-side and/or a pulsed potential across the aperture between electrodes 40 and either/both of 36 and 38.

6. Once the measurement configuration has been checked by the measurement means 112, the compound to be tested is dispensed into the inlet well 30, and drawn through a channel 20 over the test cell by negative pressure (Pf). The resulting changes in ion channel activity are determined by the measurement means.
Figure 4 shows further embodiments, which enhance the operation of the apparatus of the invention. Figures 4a, 4b and 4c are partial plan diagrams of the front-side channel, analogous to figure 1b.

In each case, the embodiments can be fabricated by the same means as described for that in figures 1a and 1b.

In Figure 4a, the measurement site is located at the downstream end of the constriction 50. The detection region is therefore entirely upstream of the aperture, enhancing the sensitivity of the system and avoiding the possibility of a cell in a constriction downstream of the measurement site adding to or masking the signal from a second upstream cell. It is advantageous to keep a constriction short in order to increase the sensitivity of the detection method and this embodiment assists this.

In figure 4b, the measurement site is located further downstream of and outside a constriction. This confers a more effective capture of a target cell as its velocity will be smaller in this region of greater cross-sectional area, and hence slower liquid flow.

Where a cell is trapped from a flowing stream intentionally, detection means some way upstream of the measurement site offers the advantage that the location means has sufficient time to operate. For example, in suction through an aperture, a certain amount of liquid has to be drawn through the aperture in order to entrain a cell towards it, the amount depending on the position of the cell in the front channel. For relatively high flow rates in the front flow channel relative to those through the aperture, a cell detected far upstream will have a chance of being trapped whereas one detected only as it passes the aperture will have a much reduced chance of being captured at the aperture.
In certain applications it is advantageous to select the cell to be trapped on the basis of its velocity. A measurement of the cell velocity allowing location means to be actuated only for cells having a velocity above a predetermined value can be achieved by measuring the transit time of the cell through a constriction: conductivity falls as the cell enters a constriction and rises again as the cell leaves.

Figure 4c demonstrates a further embodiment in which two contractions 50 and 70 are provided. The velocity of the cell is given by the time between successive falls in conductivity. The measurement site can be located either in a wider region as shown in Figure 4c, or within the downstream constriction 70.

A constriction of the type shown in figures 4a, 4b and 4c, can be formed as a narrowing of the front-side channel as demonstrated, a change in its height, or both. Such a constriction can be an integral part of the front-side channel passing adjacent to the measurement site or might be formed upon or as part of a separate component in the liquid flow path leading to the measurement site.

Figure 5 illustrates the resistance of a typical front-side channel of the design shown in figure 1b, when filled with standard front-side buffer of conductivity 0.014 Scm⁻¹, corresponding to a resistivity of 71.4 Ωcm. Two choices of channel dimension have been tested in apparatus in figure 1a and shown in figures 2a and 2b: 50 μm x 50 μm x 400 μm and 200 μm wide x 50 μm high x 650 μm.

The table relates to the channels shown in Figures 2a and 2b.
### Front channel SU8 central part

<table>
<thead>
<tr>
<th></th>
<th>(Fig 2a)</th>
<th>(Fig 2b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Width</td>
<td>50μm = 0.005 cm</td>
<td>200μm = 0.02 cm</td>
</tr>
<tr>
<td>Height</td>
<td>50μm = 0.005 cm</td>
<td>50μm = 0.005 cm</td>
</tr>
<tr>
<td>Length</td>
<td>400μm = 0.04 cm</td>
<td>650μm = 0.065 cm</td>
</tr>
<tr>
<td>Cross section</td>
<td>25×10⁻⁶ cm²</td>
<td>100×10⁻⁶ cm²</td>
</tr>
<tr>
<td>Volume</td>
<td>1 nl</td>
<td>6.5 nl</td>
</tr>
<tr>
<td>Channel resistance</td>
<td>114 kΩ</td>
<td>46.5 kΩ</td>
</tr>
</tbody>
</table>

For the 50 μm (200 μm) channel, the SU8 channel widens out to 300 μm over a distance of 200 μm (100 μm) in each end. This is then followed by roughly 300 μm of channel (to the centre of the vertical connection 52 to the inlet channel 28 in the polymer housing component 14 with a constant width of 300 μm.

The part of the channel widening out has a resistance of approximately 14 kΩ for the 50 μm channel and approximately 5.7 kΩ for the 200 μm. The constant part is roughly 300 μm long in both cases, providing a resistance of 14 kΩ.

The total resistance of the SU8 channels on the substrate is thus:

<table>
<thead>
<tr>
<th></th>
<th>R\text{ch}</th>
<th>R\text{end}</th>
<th>R\text{tot} = R\text{ch} + 2× R\text{end}</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 μm</td>
<td>114 kΩ</td>
<td>14+14=28 kΩ</td>
<td>170 kΩ</td>
</tr>
<tr>
<td>200 μm</td>
<td>46.5 kΩ</td>
<td>5.7+14=19.7 kΩ</td>
<td>85.9 kΩ</td>
</tr>
</tbody>
</table>

A series resistance from the larger flow channels in the housing component 14 has to be added to the channel resistance in order to make up the total resistance between electrodes 36 and 38. These channels have a width of 0.5
33 mm and a depth of 0.25 mm. The total length is estimated to be 11 mm. This results in a resistance of 63 kΩ.

Figure 5 shows a schematic model of the resistances.

The total resistance for the two types of channels shown in Figures 2a and 2b respectively when mounted on the housing component is:

| 50 Microns | 233 kΩ |
| 200 Microns | 149 kΩ |

Assuming a cell diameter of 12 µm gives a cell volume of 9.0×10⁻⁴ nl.

The resistance change when the cell enters the channel is given as:

$$\frac{\Delta R}{R_{chan}} = \frac{V_{cell}}{V_{chan}}$$

Using this one gets:

<table>
<thead>
<tr>
<th>渠道宽度</th>
<th>R_{chan}</th>
<th>V_{cell}</th>
<th>V_{chan}</th>
<th>ΔR</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µm</td>
<td>114 kΩ</td>
<td>9.0×10⁻⁴ nl</td>
<td>1 nl</td>
<td>103 Ω</td>
</tr>
<tr>
<td>200 µm</td>
<td>46.5 kΩ</td>
<td>9.0×10⁻⁴ nl</td>
<td>6.5 nl</td>
<td>6.4 Ω</td>
</tr>
</tbody>
</table>

The relative change of the total resistance: R_{ch}=233 kΩ or 149 kΩ, is 440 ppm for the 50 µm channel and 40 ppm for the 200 µm channel.

Referring to Figure 6, a circuit is shown which enables the output from a Wheatstone bridge to be amplified by differential amplifier 700.
Referring to Figure 7 a circuit for use in conjunction with the Coulter counter forming part of the present invention is shown as a block diagram. The circuit comprises a Wheatstone bridge 900. By means of the bridge circuit it is possible to balance the bridge so that the output from the circuit is zero when there is no cell in the channel. The signal can thus be amplified with a very high gain to obtain a large signal when a cell enters the channel. For maximum signal the fixed resistor R₁ should be matched to the channel resistance. For a channel resistance of 100kΩ and a bridge supply of 1 V DC the maximum output from the bridge would be 2.5 μV /Ω. By using a DC measurement system instead of an AC system, problems relating to cross talk are prevented when the system is scaled to a 96 channel parallel system. Such a parallel system would be required for example when the apparatus comprises a plurality of test structures. An alternating current would otherwise be advantageous in terms of drift and noise reduction as it would be possible to use a lock-in technique.

The signal is amplified by a high quality instrumentation amplifier 910 and filtered by an anti-aliasing filter 920 before it is sampled by an A/D converter 930. The anti-aliasing filter is a low-pass filter which ensures that the Nyquist criterion is not violated (i.e. at the filter cutoff, frequency must be below half the sample frequency).

The sampled signal is then processed by the digital signal processor 940, which closes the feedback loop by controlling the bridge supply and the position of the digital potentiometer 950.

The digital signal processor (DSP) also performs the bandpass filtering and checks to see if the signals (amplitude and speed) from the passing cells fall within the allowed window.
The DSP can thus be used to trigger a pump, or other location means, for cell capture.

After power up, the DSP 940 sets the digital potentiometer 950 to mid position. It then samples the signal and adjusts the potentiometer to achieve the minimum DC-offset depending on the channel resistance and electrode offsets. The adjustment could be done by recursive bisection.

During the measurement of Coulter signals, the DSP monitors that the DC-offset does not drift outside the dynamic range of the amplifier. If such drift occurs, the DSP acts to adjust the potentiometer again to minimise the offset, so that amplifier does not saturate.

Typical causes for the drift could be changing electrode potentials, streaming potentials and temperature drift in the electronics.

Due to the high gain in the instrumental amplifier, even small changes in the electrode potentials will result in large offset errors.

The maximum achievable gain is also influenced by the smallest step that the digital potentiometer has. For example an AD5250 50K Ω potentiometer has 256 steps giving a step size of 195 Ω, which results in an output voltage change of 0.488 volts with a gain of x1000.

The DSP further provides a convenient way of processing the Coulter signal to remove problems relating to drift and noise, by either differentiating the signal or applying a digital bandpass filter to it.

Referring to Figure 8a it can be seen that the output voltage of the Coulter detector increases linearly as the size of the cell detected increases.
An example of the output from the circuit described above and shown in figure 7 is given in figure 8b. The channel dimensions were as described above, and the apparatus was operated using Human Embryonic Kidney (HEK) cells and data sampling conducted at 20 kHz averaged in a 3 ms window.

From the results, it is apparent that significant drift is still present in the output from the bridge circuit (upper trace) so that the output is preferably band-pass-filtered (1st order Butterworth, 300 Hz < f < 2 kHz) which gives a stable output as in the lower trace.

Figure 8c shows typical Coulter traces from individual cells, again with raw data in the upper trace and filtered in the lower trace. The height of the pulses represents the size of the cells and the length of the pulse the time taken to pass through the channel.

In a preferred embodiment the pulse height is therefore used to discriminate between single cells desirable for testing, undesirable agglomerations of cells, and debris, which in general will be smaller than a single cell.

The apparatus of the invention is able to distinguish between dead, non-viable and healthy cells, as in many cases the cell membrane properties change so that the Coulter signal from these cells will fall outside the range of those from healthy cells.

Necrotic cells often lose the ability to osmotically regulate and therefore tend to swell up to around 1.5 times their previous diameter. Apoptotic cells however, tend to shrink, and at the same time the membrane permeability increases, so the Coulter signal will be smaller than would be
expected for a cell of that size. Therefore the pulse height can be used to increase the chance of selecting a healthy cell from a mixed population.

AC or pulse techniques can be used together with further signal processing and interpretation steps to give more detailed interrogation of the cell population.

The present apparatus allows detailed discrimination between different cell types, and live and dead cells, in flowing streams with reduced incidence of failed tests.

Figure 9a shows a partial cross section diagram and figure 9b a partial plan view of a constriction in the inlet channel of an apparatus equivalent to figure 1a.

Here a constriction 50 is formed by a reduction of the height of the inlet channel between the electrodes 36 and 38. The measurement site is shown located at the downstream end of a constriction, but, as in previous embodiments, may be located at any point within or downstream of a constriction.

The embodiment in Figure 10a comprises a single pair of electrodes 60 and 62 on opposite sides of the channel such that they contact the liquid contained within the channel. The detection region analogous to a constriction in previous embodiments is therefore the region between the electrodes.

The electrodes are connected to tracking and contact means provided on the substrate 12, which is part of an assembly as shown in figure 1a. These are arranged in such a way that they contact further conductor means included
within the housing component 14, so allowing contact to the detection amplifier outside the measurement device.

The single pair of detection electrodes gives a fall in conductivity while the cell is between them, so allowing the speed of the cell to be determined from the measured pulse length and the known length of the electrodes. If further information is required, or smaller individual electrode areas are preferable, then further electrodes 64 and 66 are provided (figure 10b), where the transit time between the first and second electrode pairs can be used to calculate the speed of the cell.

The further electrode pair can be provided in the same orientation as the first pair, or can be located on different faces from the first so as to interrogate where in the channel the cell is located by combination measurements of impedance between combinations of the four electrodes.

In a further embodiment of the apparatus of figure 10a, and where only cells moving close to the face of the front channel at which the aperture is located are capable of being trapped, the electrode arrangement is made to be sensitive preferentially to these cells.

The electrode arrangement shown in plan in figure 11a and in cross-section X-X of figure 11b achieves this. Here the electrodes 60 and 62 are formed such that they occupy only part of the height of the inlet channel 20, the region between the electrodes is adjacent the wall of the inlet channel at which is located the measurement site. The inlet channel is formed from photo-patterned material 68 in a layer on the component 12, and closed by a cover layer 69, which may be part of the housing component.
The cell at position A causes a greater change in the resistivity between the electrodes than that at position B; only the cell at position A (close to the wall of the channel where the measurement site is located) is therefore able to trigger the location means.

The embodiments in figure 11 are fabricated using standard micro-fabrication techniques, for example using photo-patterned SU8, coated on a silicon substrate, with the electrodes formed in recesses in the SU8 by electroplating. Contact means on the substrate and on the housing component are those used in semiconductors, with joining techniques as used in electronic assembly processes.

The foregoing embodiments all have as a common feature a front channel running parallel to the surface of the substrate on which the aperture is located.

Figure 12 shows a partial cross-section diagram of an alternative embodiment of a test structure according to the invention in which the front channel runs normal to the surface of the substrate in the region of the aperture and in the region of a constriction.

In this embodiment the substrate 152 comprising a measurement site 154 is mounted in a housing formed from two components 156 and 158.

A front side inlet well 162 is in communication with a liquid path leading over the measurement site. The plastic housing further comprises the path formed from an inlet channel 164 leading to the measurement site, a front-side channel 166 leading away from the measurement site and an outlet channel 168.
Electrodes 170 and 172 are provided which contact the solution at each end of the liquid path. Inlet channel 164 comprises a constriction which forms a Coulter detection region to detect cells moving from the well 162 towards the measurement site. The electrode 170 is shown as located on the sidewall of the well 162, but may equally be located within the inlet channel 164, on the opposite side of a constriction from the measurement site. The electrode and the conductor track making contact with it from the outside may then be provided, associated with the upper housing component 156, or may be in the form of an electrode external to the test structure, dipping into the well from above.

Figure 13 shows a partial cross section diagram of a yet further embodiment of the test structure, in which the Coulter orifice is formed within the substrate itself. A substrate component 202 having a measuring site 204 comprising an aperture (not shown) formed within the surface of the component, is mounted on a housing component 206 by means of a seal layer 208. The housing component is in turn mounted on second housing component 210 using a further sealing layer (not shown).

The substrate comprises a front channel 220 formed on the surface of the component adjacent the measuring site. Housing components 206, 210 together form a housing inlet channel 228 which communicates with the front channel through an inlet via 224 in the substrate, and also forms a housing outlet channel 232 which communicates similarly with the front channel 220 through an outlet via 226. The housing inlet and outlet channels communicate with input and output wells in the manner of the embodiment shown in figure 1.
The aperture at the measuring site 204 opens to the rear-side compartment 222, which in turn communicates with further channels and wells open to the exterior, and is contacted by a rear-side electrode 40.

Electrode 236 is provided mounted on the base-plate which in use contacts liquid in the housing inlet channel 228, and electrode 238 is provided which in use contacts liquid in the housing outlet channel 232. The front channel 220 is formed in a preferred embodiment from a photo-patterned polymer layer 246, for example formed as in previous embodiments from the photo-patternable epoxy SU8, and closed by a cover 248.

In this embodiment the inlet via 224 forms the Coulter constriction, and so is made with smaller cross-sectional area than the outlet via, or any other part of the liquid path formed within the test structure which lies between the electrodes 236 and 238.

The apparatus of the invention comprising the embodiments of test structures in figures 12 and 13 is operated in essentially the same manner to that comprising the test structures shown in figures 1a and 1b.
CLAIMS

1. An apparatus for determining and/or monitoring of electrophysiological properties of ion channels in ion channel containing objects the apparatus comprising:

(i) a substrate comprising a first measuring site for holding ion channel containing objects, the site comprising a passage in the substrate, a first end of the passage defining a first aperture in a first upper surface of the substrate and being in contact with a first domain, the first upper surface of the substrate in the vicinity of the aperture being adapted to form a seal with an ion channel containing object held at the site, and a second end of the passage being in contact with a second domain;

(ii) an inlet channel in liquid communication with the first domain and the first aperture;

(iii) a first measurement electrode in electrical contact with the first domain;

(iv) a second measurement electrode in electrical contact with the second domain;

(v) measuring means electrically connected to the first and second measurement electrodes and adapted to make electrical measurements on an object sealed to the sealing region at the measurement site;
(vi) location means for causing movement of the objects towards the measuring site to cause an object to form a seal with the first aperture;

wherein the apparatus further comprises a Coulter detector for detecting the presence of an object in the inlet channel, the detector controlling operation of the location means, such that movement of an object towards the measuring site is caused by the location means in response to signals emitted from the detector.

2. An apparatus for determining and/or monitoring of electrophysiological properties of ion channels, in ion channel containing objects, the apparatus comprising:

i) a substrate comprising a first measuring site for holding ion channel containing objects, the substrate having a first surface in contact with a first domain, the measuring site comprising a first measurement electrode; a sealing region surrounding the first measurement electrode; and a conductive track for connecting the first measurement electrode to a measuring instrument;

ii) an inlet channel in liquid communication with the first measuring site and the first domain;

iii) a second measurement electrode which, in use is in electrical contact with the first domain;

iv) measuring means electrically connected to the first and second measurement electrodes and adapted to make electrical
measurements on an object sealed to the sealing region at the measurement site;

v) location means for causing movement of an object towards and onto the measurement site;

vi) wherein the apparatus further comprises Coulter detection means which determines the presence of the object in the inlet channel which detection means outputs a signal which controls the location means whereby the flow of an object towards the measuring site is caused by the location means in response to signals emitted from the detector.

3. An apparatus according to Claim 1 or Claim 2 wherein the seal comprises a gigaseal.

4. An apparatus according to Claim 1 or Claim 3 wherein the first and second domains comprise first and second ionic solutions respectively.

5. An apparatus according to any one of the preceding claims wherein the detector comprises first detection means comprising a pair of detection electrodes comprising between them a detection region within the inlet channel, the volume of the detection region being sufficiently small that the resistance of the solution increases measurably when a cell is within the solution.

6. An apparatus according to any one of the preceding claims wherein the cross sectional dimension of the detection means is less than 20 times the dimension of a cell to be measured.
7. An apparatus according to Claim 2 and any claims dependent thereon, wherein the first measurement electrode has a diameter of less than 5 microns.

8. An apparatus according to Claim 2 and any claim dependent thereon wherein the first and second measurement electrodes are formed on or proximal to the first surface of the substrate.

9. An apparatus according to any one of Claim 1 any any claim dependent thereon wherein the second measurement electrode is located on a second surface of the substrate and is separated from the first measurement electrode by the passage.

10. An apparatus according to any one of the preceding claims wherein the detection region is defined between electrodes located in the inlet channel, either transversely across the channel or adjacent to one another.

11. An apparatus according to any one of the preceding claims wherein the inlet channel comprises a constriction forming a narrowest region within the channel, and the detection region is located in the narrowest region of the channel.

12. An apparatus according to Claim 11 wherein the constriction has dimensions comprising a height and/or width of less than 200 microns and/or a length less than 1 millimetre.

13. An apparatus according to any one of the preceding claims wherein the measurement site is positioned downstream of the detection region.
14. An apparatus according to any one of the preceding claims wherein the detector comprises a plurality of detection regions.

15. An apparatus according to any one of the preceding claims wherein the detector comprises one or more further detection means, each detection means comprising an electrode pair provided downstream of the first pair of electrodes.

16. An apparatus according to any one of the preceding claims comprising a plurality of measuring sites.

17. An apparatus according to any one of the preceding claims comprising a plurality of location means.

18. An apparatus according to any one of the preceding claims wherein a DC current is applied across the detector.

19. An apparatus according to Claim 18 comprising a self-balancing DC bridge with the resistance of the inlet channel in one arm.

20. An apparatus according to Claim 19 wherein the self-balancing DC bridge is a Wheatstone bridge, and the apparatus further comprises an instrumental amplifier for amplifying the output from the Wheatstone bridge, an A/D converter for converting the signal into digital signal, a digital signal processor for receiving the digitised signal and for controlling the output of the circuit, a digital to analogue converter for receiving a signal from the digital signal processor and converting the signal into an analogue signal for feeding back into the Wheatstone bridge in accordance with signals from the digital signal processor.
21. An apparatus according to Claim 20 wherein an output from the digital signal processor causes the location means to be activated.

22. A test structure forming part of an apparatus according to any one of the preceding claims.

23. A measurement system comprising the apparatus according to any one of Claims 1-21.

24. A method of making electrical measurements on an ion channel containing object, comprising the steps of:

(i) supplying a first conductive solution containing at least one object through an inlet channel to a first surface of a substrate comprising a measuring site having an aperture, such that the first solution contacts the first surface, the first surface being in fluid communication with a second surface of the substrate via the aperture;

(ii) supplying a second conductive solution to the second surface so as to establish liquid contact between the first and second surfaces;

(iii) measuring electrical continuity to test that liquid contact has been achieved between first and second electrodes respectively located in contact with the first and second conductive solutions;

(iv) detecting the presence of an object using a Coulter detection means provided in the inlet channel;
(v) using the output of the detector to control a location means to drive an object in the first solution towards a measurement site having a sealing region surrounding the aperture;

(vi) performing electrical measurements on the object.

25. A method of making electrical measurements on an ion channel containing object, comprising the steps of:

(i) supplying a first conductive solution comprising at least one ion channel containing object through an inlet channel, to a first surface of a substrate comprising a measurement site having a first measurement electrode located on the first surface, and a sealing region surrounding the first electrode;

(ii) providing a second measurement electrode in electrical connection with the first solution;

(iii) detecting the presence of an object using a Coulter detector provided in the inlet channel;

(iv) using an output of the detector to control a location means to drive an object to the measurement site to form a seal around the first measurement electrode; and

(v) performing electrical measurements on the object.
Figure 2a
50 μm channel

Figure 2b
200 μm channel
Figure 5

Schematic model of resistances for the 50 and 200 μm chips

The total resistance for the two types of channels mounted into a chip is:

<table>
<thead>
<tr>
<th>Size</th>
<th>Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 μm</td>
<td>233 kΩ</td>
</tr>
<tr>
<td>200 μm</td>
<td>149 kΩ</td>
</tr>
</tbody>
</table>
Figure 6

[Diagram showing a circuit with labels for components such as $V_b$, $R_{ph}$, $R_1$, $R_{th}$, $R_{ph} = 100K$, $R_{th} = 1.1M$, and various resistors and operational amplifiers.]

Digital Potentiometer
Figure 7

[Diagram of a circuit with labeled components: Bridge, Digital Potentiometer, Instrumental Amplifier, Active Filter, A/D, DSP, EOF pump, Autozero ctrl signal, R_{PH}, R_{PL}, R_{ch}, D/A, and other circuit elements.]
Figure 8b

Sampled data after 3 msec averaging

Data after bandpass filtering and subtraction of DC-value

300 Hz < $f_{\text{pass}}$ < 2000 Hz

Amp output [V]

Time [sec]
Figure 8

[Graph showing data over time]
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N33/487 C12M1/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, INSPEC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>WO 02 29402 A (SOPHION BIOSCIENCE AS)</td>
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<td></td>
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<td></td>
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<td></td>
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<td>the whole document</td>
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<tr>
<td>Y</td>
<td>WO 02 03058 A (HAEMMERLE HUGO ;KNOTT THOMAS (DE); NISCH WILFRIED (DE); STETT ALFR) 10 January 2002 (2002-01-10) page 16, line 5 - line 16</td>
<td>1, 2, 24, 25</td>
</tr>
<tr>
<td>A</td>
<td>WO 01 48474 A (DODGSON JOHN :ASTRAZENECA UK LTD (GB); ASTRazeneca AB (SE))</td>
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</tr>
<tr>
<td></td>
<td>5 July 2001 (2001-07-05)</td>
<td></td>
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<tr>
<td></td>
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Date of the actual completion of the international search: 24 February 2004

Date of mailing of the international search report: 04/03/2004

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<tr>
<td></td>
<td></td>
<td>CA 2424498 A1</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>WO 0229402 A2</td>
<td>11-04-2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 1322955 A2</td>
<td>02-07-2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2002063067 A1</td>
<td>30-05-2002</td>
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<td></td>
<td></td>
<td>WO 0203058 A2</td>
<td>10-01-2002</td>
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<td>JP 2004502936 T</td>
<td>29-01-2004</td>
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<td>US 2003153067 A1</td>
<td>14-08-2003</td>
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<tr>
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<td></td>
<td>EP 1244910 A1</td>
<td>02-10-2002</td>
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<tr>
<td></td>
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<td>WO 0148474 A1</td>
<td>05-07-2001</td>
</tr>
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<td></td>
<td>JP 2003527581 T</td>
<td>16-09-2003</td>
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