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Real-Time Cell Dynamics With a Multianalyte Physiometer

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Summary

A technique for simultaneously measuring changes in extracellular glucose, lactate, and oxygen concentrations in conjunction with acidification rates on a Cytosensor™ Microphysiometer is described. Platinum electrodes are inserted into the standard Cytosensor plunger head and modified with enzymes and biocompatible polymeric films. The lactate and glucose oxidase enzymes catalyze the reaction of lactate and glucose. An end product of these catalyses, H_2O_2, is measured amperometrically. Extracellular oxygen is also measured amperometrically, while the acidification rate is measured potentiometrically by the Cytosensor. Useful information is obtained during the Cytosensor stop-flow cycles, which produce increasing or decreasing peaks, owing to the production of lactic and carbonic acid and consumption of glucose and oxygen by the cells. Fabrication of the modified sensor head and deposition of the electrode films is detailed, and the operation of the technique is described and illustrated by the simultaneous measurement of all four analytes during the addition of 20 m*M* fluoride to mouse fibroblast cells.

Key Words

Cellular physiology; Cytosensor™; Microphysiometer; enzyme electrodes; oxygen; acidification; multianalyte; Nafion.

1. Introduction

Living cells represent the ultimate nanoscale analytical detectors based on their nanostructured protein receptors coupled directly to cell metabolism and function. Significant advances in nanobiotechnology research at this point have been made in artificially re-creating the sensing strategies found in cell membranes. Despite this progress, the components of the living cell remain the most complex of all nanostructured devices. One approach is to use cell physiology

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as an analytical platform by coupling the quick response of cell receptors to the cell's energy metabolism.

New classes of biosensors have been used to determine the physiological response of cells to various chemical environments and toxic agents by detecting changes in extracellular analyte concentrations ***(1–3)***. Since the responses are related to the metabolic activity of the cells, they can be used to screen the cellular responses to various agents such as drugs and toxins. The simplest case would be the determination of a single analyte indicative of metabolic activity. However, if responses are obtained simultaneously from multiple analytes, these biosensors offer a multidimensional approach to the elucidation of cellular metabolic pathways and responses to chemical and biological agents.

Although there are many analytes in metabolism to choose from, the choice is normally limited to those that are directly involved in the energy-producing pathways within the cell and that can be measured in the extracellular environment: oxygen consumption, glucose consumption, lactic acid production, and acidification rate (which is a combination of lactic acid and carbonic acid release). Using glucose and oxygen as inputs and lactate and acidification as outputs, real-time cell dynamics can be described by this simple model of metabolic physiology. The two major pathways in this model can be described as anaerobic, in which lactic acid is produced by the cells, and aerobic, in which extracellular glucose and oxygen are eventually converted into carbon dioxide (carbonic acid) and water. Acidification, measured potentiometrically, and oxygen concentration, measured amperometrically, can be determined directly. Glucose and lactate concentrations can be determined indirectly by measuring the H_2O_2 produced by selective catalysis of these analytes with glucose oxidase (GOX) and lactate oxidase (LOX), respectively ***(4)***. Cellular physiological responses to a particular agent can then be inferred from the direction and magnitude of the change in these analytes. In addition, constructing dose–response curves for specific agents would be analogous to analytical calibration curves.

Karube and colleagues ***(5,6)*** first described indirect detection of an agent by using the change in the oxygen consumption rates of yeast cells exposed to a wide variety of physiological agents. This technique, which measured oxygen concentration amperometrically by a Clark electrode, was never commercialized as an analytical instrument. The Cytosensor™ Microphysiometer, however, has been commercialized and used for a large number of cellular studies ***(7–11)*** in which the change in pH in the extracellular environment is related to metabolic signals from the cell. However, this does not provide multidimensional extracellular measurements, which are more useful to characterize cellular response to chemical stimulations such as drugs and toxins, nor can the internal dynamics of the cell metabolic pathways be investigated.

Measurements detecting species such as oxygen ***(12,13)***, glucose ***(4,14)***, and lactate ***(4)*** have been made at modified platinum electrodes. By incorporating similarly modified electrodes into the Cytosensor Microphysiometer, producing a multianalyte sensor head, measurements can be made simultaneously in conjunction with acidification rates, and a broader picture of cellular physiology can be realized. This technique presents the methodology to simultaneously measure the change in extracellular glucose, lactate, and oxygen concentrations in conjunction with acidification rates in response to metabolic agents. These measurements help to elucidate cellular activity in response to drugs and toxins and to help distinguish between aerobic and anaerobic metabolic pathways.

The Cytosensor Microphysiometer operates via a program in which a cell medium (usually RPMI-1640) is pumped through a cell chamber (containing approx 3×10^5 cells) in a series of flow and stop-flow cycles. During the stop-flow cycles, the solution in the chamber is stationary and the acidification in the cell chamber changes at a rate comparable to the number and activity of the cells. This rate is measured by the instrument using a light-addressable potentiometric sensor ***(9)***. Once a baseline cellular activity is achieved, a metabolic agent is introduced and the change in acidification rate is recorded.

The technique of multianalyte determination takes advantage of the Cytosensor Microphysiometer technology by measuring the change in glucose, oxygen, and lactate concentrations in conjunction with the Cytosensor Microphysiometer acidification rates. Amperometric curves for glucose, lactate, and oxygen are obtained by applying appropriate potentials at the modified platinum electrodes while running the acidification rate program. When the stop-flow cycle begins, the current begins to decrease or increase, depending on whether the analyte is being consumed or produced by the cells. The raw data (**Fig. 1**) appear similar to the Cytosensor acidification raw data but generally have curved slopes, and instead of measuring the slope of the change in pH in negative microvolts per second, the area of the resultant peaks during the stop-flow cycle (in Coulombs of charge), or the difference in peak heights, is plotted vs time and is proportional to the analyte concentration. The Coulombs of charge for the glucose and lactate can be converted into concentrations by an *in situ* addition of known quantities of analyte, usually at the end of the experiment, thereby internally calibrating the sensors. The oxygen consumption can be estimated by assuming the baseline current as the ambient oxygen concentration and extrapolating to zero.

The GOX- and LOX-modified platinum electrodes themselves are coated with a solution containing the enzyme, bovine serum albumin (BSA), and the crosslinking agent glutaraldehyde. The chemical reactions catalyzed at the respective enzyme electrodes can be simply described as

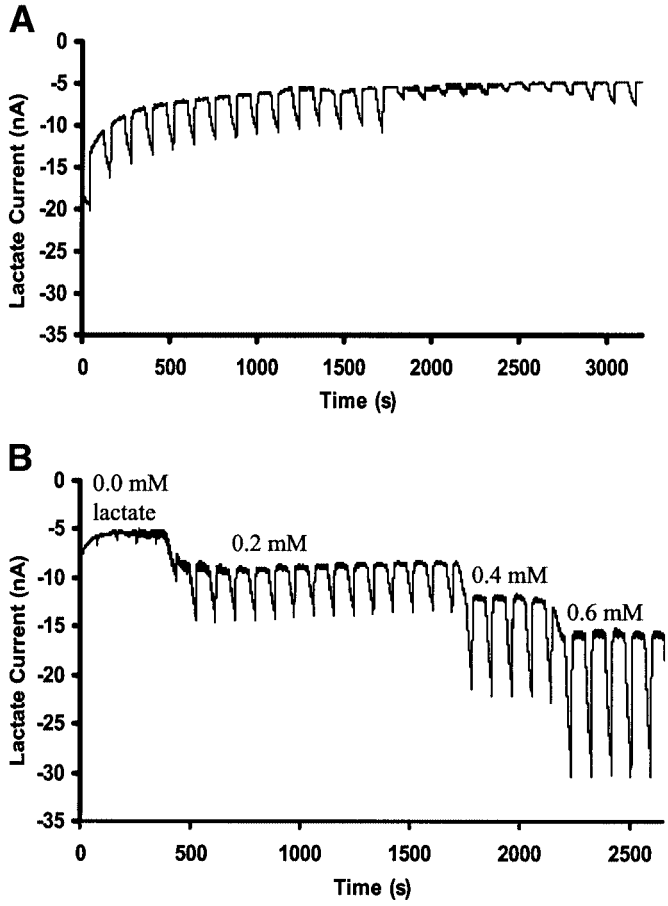


Fig. 1. Modified lactate electrode response in Cytosensor Microphysiometer with **(A)** approx 3×10^5 CHO cells (with 20 m*M* NaF added at 1800 s and stopped at 2700 s) and **(B)** additions of 0.2 m*M* lactate. The medium used in both cases was RPMI-1640.

$$\text{glucose} + O_2 + H_2O \rightarrow \text{gluconic acid} + H_2O_2 \quad (1)$$

and

$$\text{lactate} + O_2 + H_2O \rightarrow \text{pyruvate} + H_2O_2 \quad (2)$$

The platinum electrodes are set at a potential that will oxidize the H_2O_2 product back to water and oxygen (usually +0.6 V vs the Ag/AgCl reference electrode), and so any change in glucose or lactate concentration will be seen as a change in H_2O_2 concentration. These films give rapid time response and very high currents in the concentration ranges of the target analytes: glucose (10 m*M* in

RPMI-1640) and lactate (roughly 0.3 m*M* scale with approx 3×10^5 fibroblast cells at an acidification rate of –200 to –250 μV/s). These currents approach the region where Michaelis-Menten kinetics giving rise to nonlinear sensors would need to be considered. Fortunately, it is easy to modify the enzyme loading on the electrode so that the concentrations of interest are in a linear response range of the modified electrode, i.e., in the more linear portion of the Michaelis-Menten curve. This modification is achieved by adding a diffusion barrier to the electrodes in the form of a Nafion film. Nafion also has the benefit of adding resistance to biofouling, because the Nafion is negatively charged and very unreactive. The Nafion is diluted before coating the LOX membrane so that it does not completely block the negatively charged lactate ions from diffusing through the membrane.

The lactate response raw data for Chinese hamster ovary (CHO) cells exposed to 20 m*M* NaF is shown in **Fig. 1A**, where the peaks represent the increase in current owing to cellular lactate production during the stop-flow cycle of the Cytosensor Microphysiometer program. When NaF was added at 1800 s, cellular lactate production was suppressed, shown as a decrease in the peak area (or height). The cellular lactate production resumed when NaF was stopped. The effect of 0.2 m*M* lactate additions as a calibration are shown in **Fig. 1B**, although lactate (and glucose) additions are usually added at the end of the experiment. Since the lactate and glucose current responses are fairly linear, simple two-point calibration curves are generally sufficient to convert the Coulombs of charge (or peak height current differences) to concentrations. The oxygen concentration can be estimated by extrapolating from the baseline current, assumed to be ambient oxygen at 0.24 m*M*. The acidification rate data can be directly imported from the Cytosensor Microphysiometer program. Note that in determining concentrations of analytes, any analyte additions to the flow medium, such as the lactate additions shown in **Fig. 1B**, produce an increasing baseline during the flow cycle on each successive addition. However, when analyte concentration changes are owing to cellular activity, such as the lactate in **Fig. 1A**, the baseline during the flow cycle generally does not change when the analyte concentration increases or decreases, because the baseline concentration in solution does not change in the vicinity of the electrode until the analyte concentration gradient from the cells during the stop-flow cycle reaches the electrode. Peak currents during stop-flow may therefore be more representative of the actual analyte concentration because they are not affected by the time for the concentration gradient from the cells, whether decreasing or increasing, to reach the electrode. This has been found to be especially true for the lactate determination, in which the difference in lactate concentration calculated from peak heights was two to three times larger than that calculated from areas. However, there

may be times when it is difficult to analyze the peak height owing to a non-standard peak shape, especially for glucose consumption.

The effect of 20 m*M* NaF on fibroblast cells is illustrated in **Fig. 2**, which shows the changes in the extracellular concentrations of glucose, lactate, and oxygen and the acidification rate. Fluoride shut down the metabolic activity of the cells, shown as an increase in the glucose and oxygen concentrations (the cells are using less) and as a decrease in the lactate concentration and acidification rate (the cells are producing less). Once the fluoride was removed, the cells appeared to recover nearly to their previous metabolic state.

2. Materials

2.1. Equipment

1. Cytosensor Microphysiometer instrument and all corresponding materials necessary for measuring acidification rates (Molecular Devices, Sunnyvale, CA).
2. CHI 1030 Multipotentiostat (CH Instruments, Austin, TX) with a PC to run the instrument and accompanying software.
3. Aqueous reference electrode, Ag/AgCl (can be obtained from CH Instruments).

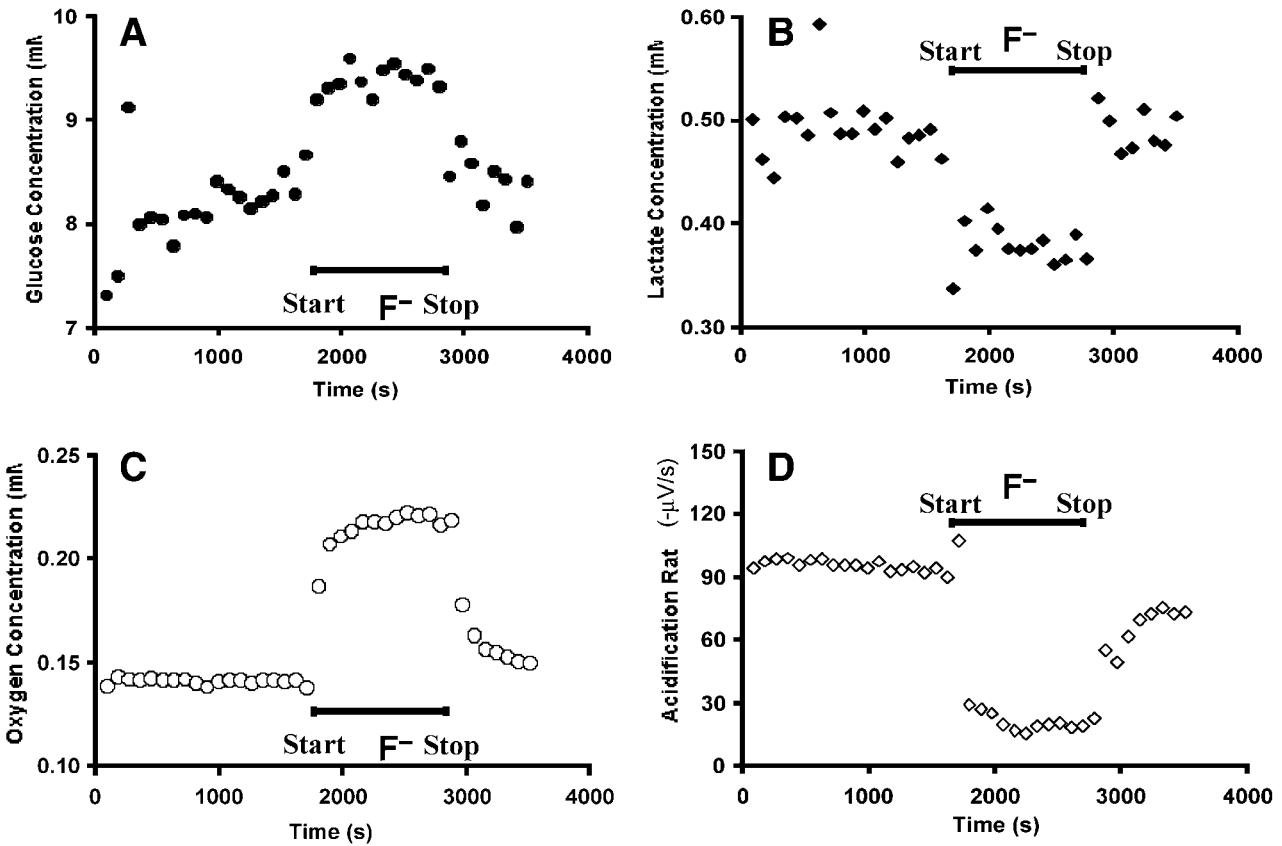
2.2. Membrane Solution Reagents

1. GOX (*Aspergillus niger*; Sigma, St. Louis, MO).
2. LOX (from *Pediococcus* species; Sigma).
3. BSA (Fraction V, 96%; Sigma).
4. Glutaraldehyde (glutaric dialdehyde), 25% solution in water.
5. 1 m*M* Phosphate buffer, pH 7.0.
6. Phosphate-buffered saline (PBS): 1 m*M* Phosphate; 100 m*M* NaCl, pH 7.0.
7. Triton X-100.
8. Nafion solution: perfluorsulfonic acid–PTFE copolymer, 5% (w/w) solution from Alfa Aesar.

2.3. Sensor Head Materials

1. Cytosensor Microphysiometer plunger heads with four holes drilled lengthwise through the body to accommodate the platinum wires.
2. Platinum wire, 24 gage (0.51-mm diameter) and 36 gage (0.127-mm diameter).
3. Hysol® Epoxy-Patch® structural adhesive (1c; Dexter).

Fig. 2. *(opposite page)* Modified sensor head response to changes in extracellular **(A)** glucose, **(B)** lactate, **(C)** oxygen concentrations and the **(D)** Cytosensor Microphysiometer-measured acidification rate on addition of 20 m*M* NaF to approx 3×10^5 fibroblast cells. NaF addition began at 1700 s and was stopped at 2800 s. The medium was RPMI-1640 with a 60-s flow cycle and a 30-s stop-flow cycle. The curves in (A–C) have been baseline corrected. Details of the effects of NaF on fibroblasts have been published ***(15)***.


A
Glucose Concentration (mM
10
9
8
7
Start F⁻ Stop
0
1000
2000
3000
4000
Time (s)
B
Lactate Concentration (mM
0.60
0.50
0.40
0.30
Start F⁻ Stop
0
1000
2000
3000
4000
Time (s)
C
Oxygen Concentration (mM
0.25
0.20
0.15
0.10
Start F⁻ Stop
0
1000
2000
3000
4000
Time (s)
D
Acidification Rat (-μV/s)
150
120
90
60
30
0
Start F⁻ Stop
0
1000
2000
3000
4000
Time (s)

4. Brass crimp connectors (CCD-202-1-SP; SPC Technology).
5. Polyurethane (Crystal Clear 200 or harder version, from Smooth-On).
6. Silver epoxy (Epo-Tek®, Epoxy Technology®, Billerica, MA).
7. Diamond-polishing compounds (1, 6, and 15 µ, Metadi II, from Buehler).
8. Cotton-tipped swabs.

2.4. Cell Lines

1. CHO cells M3WT4 (American Type Culture Collection [ATCC] no. CRL-1981).
2. Mouse sc fibroblast cells A9 L HD2 S.C.18 (ATCC no. CRL-10255).

3. Methods

3.1. Construction and Preparation of Sensor Heads

3.1.1. Physical Modifications to Sensor Head

1. Insert four platinum wires (24 gage; *see* **step 2** for the smaller oxygen electrode) in holes drilled lengthwise through a Cytosensor Microphysiometer plunger head (*see* **Note 1**). Then seal them at the surface with Hysol Epoxy-Patch and allow to harden for the recommended time **(Fig. 3) (*see* Note 2**).
2. For the oxygen electrode, the smaller platinum wire (36 gage) can be attached to the larger platinum wire with silver epoxy before sealing in the electrode **(Fig. 3)**. To do this, leave the 24-gage platinum wire approx 3 mm below the surface of the sensor face, fill with a small amount of silver epoxy, then insert a short piece of the smaller platinum wire. The wire can be sealed with Hysol Epoxy-Patch after the silver epoxy hardens (*see* **Note 3**).
3. Solder crimp connectors to the platinum wires at the back.
4. Attach a rubber collar, and then pour freshly mixed polyurethane into the back of the sensor head. Before the polyurethane hardens, insert a wooden stick in the center of the back of the sensor head to form a hole for the Cytosensor plunger arm to fit. The wooden stick can easily be removed before complete hardening of the polyurethane (*see* **Note 5**). Allow the polyurethane to harden for the recommended time. This provides electrical insulation and mechanical strength for the crimp connectors (*see* **Note 5**).
5. Polish the electrodes with sandpaper glued to a machined brass jig that fits over the electrode area alone, minimizing damage to the O-ring seat (**Fig. 2**). Then rub diamond-polishing compound over the electrodes with cotton-tipped applicators to achieve a fine polish (*see* **Note 6**).

3.1.2. Preparation of Oxidase-BSA Coating Solution

1. In a small vial, weigh out 5 mg of GOX.
2. Add 500 µL of 1 m*M* phosphate buffer containing 0.02% (v/v) Triton X-100 and dissolve the GOX.
3. Thoroughly dissolve 50 mg of BSA in the mixture (*see* **Note 7**).
4. Add 5 µL of the 25 wt% glutaraldehyde solution and mix well (*see* **Note 8**).
5. Repeat this process for the LOX solution, except use approx 2.5 mg of LOX (*see* **Note 9**).

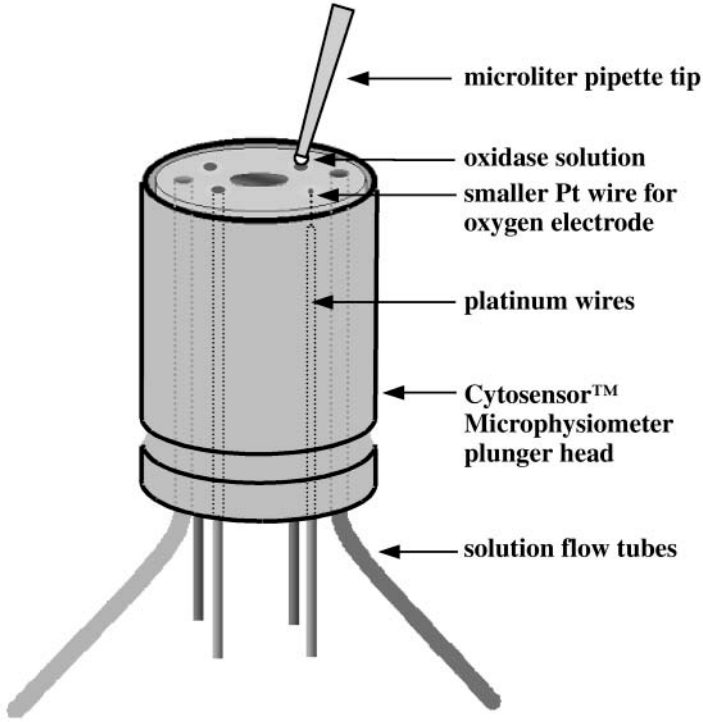


Fig. 3. Modified sensor head for use in Cytosensor Microphysiometer. The four platinum electrodes are shown placed around a center electrode. The center iron electrode comes as part of the plunger head before the modifications and is the counter-electrode for the acidification measurements. The smaller of the four platinum electrodes is used for oxygen measurements. Deposition of the oxidase solution, whether GOX/BSA, LOX/BSA, or Nafion, is shown by the droplet from the microliter pipet tip.

3.1.3. Coating of Modified Sensor Head Electrodes

1. Draw a small amount of the coating solution into a 10 µL plastic pipet tip.
2. Gently force the solution out of the tip by pressing a finger (moistened for good seal) at the back of the pipet tip, and allowing a partial droplet to form at the tip. Touch the droplet to the electrode area (**Fig. 3**) and allow it to cover the electrode but minimize droplet spread outside of the electrode area as much as possible (*see* **Note 10**).
3. Let dry for approx 30 min in room air.
4. After drying, coat the GOX-BSA membrane with a droplet (using the pipet tip as in **step 2**) of 5% Nafion solution, and allow it to dry for 10–15 min before use.

5. Coat the LOX-BSA membrane with three or four droplets of a Nafion solution consisting of 1 part (v/v) 5% Nafion solution to 9 parts ethanol.
6. Coat the smaller of the four electrodes (oxygen electrode) with a droplet of 5% Nafion solution.
7. Store the sensor heads at 4°C with the sensor ends immersed in 50 m*M* phosphate buffer (pH 7.0).

3.1.4. Testing of Sensor Quality

1. Insert the sensor end only of the sensor head into a small glass bottle containing 10 mL of a stirred, 1 m*M* PBS solution.
2. Connect the leads of the CHI 1030 to the electrodes as described in **Subheading 3.2.**, except using a separate Ag/AgCl aqueous reference electrode (this can be obtained from CH Instruments).
3. Run the same amperometric program as described in **Subheading 3.3.1.** for the CHI 1030.
4. When the current approaches a steady state, add successive aliquots of solutions of glucose (200 µL of a 0.1 *M* solution = 2 m*M*) or lactate (50 µL of a 0.1 *M* solution = 0.5 m*M*) and observe the increase in current. The currents should be roughly 5–10 nA/m*M* for glucose (up to 10 m*M* glucose) and 50–100 nA/m*M* for lactate (up to 2 m*M* lactate), depending on the quality of the coatings.

3.2. Attachment of Modified Sensor Head to Cytosensor Microphysiometer

The Cytosensor Microphysiometer program is used in the usual manner for obtaining acidification rates and controls the flow/stop-flow cycles. The CHI 1030 is run in conjunction with this to obtain the glucose, lactate, and oxygen concentration data. However, the only shared electrode between the instruments is the reference electrode. The CHI 1030 connects to the other electrodes separately from the Cytosensor Microphysiometer.

1. Insert the seated cells into the Cytosensor Microphysiometer in the usual manner for obtaining acidification rates, except replace the usual plunger with the modified sensor head.
2. Attach the CHI 1030 leads 1 (green), 2 (yellow), and 3 (yellow) directly to the GOX, LOX, and oxygen electrodes, respectively, on the modified sensor head.
3. Attach the counterelectrode lead (red) to the remaining platinum electrode on the modified sensor head.
4. Attach the reference electrode lead (white) to the reference electrode on the Cytosensor Microphysiometer instrument.

3.3. Standard Operating Procedure

3.3.1. CHI 1030 Program Parameters

1. Select the amperometric *i-t* curve program from the technique menu.
2. Select the program setup and insert the following parameters:

a. Electrode 1, GOX: Init E = +0.6 V, Sample Interval = 0.2 s, Sampling Time = length of experiment (recommend at least 10,000 s), Quiet Time = 0 s, Scales During Run = 1, Sensitivity = 1.e-007.
b. Electrode 2, LOX: Init E = +0.6 V, Sensitivity = 1.e-007; check "on" box.
c. Electrode 3, Oxygen: Init E = –0.45 V, Sensitivity = 1.e-007; check "on" box.

3.3.2. Initial Preparations

Once the cells are inserted into the modified sensor head and the electrodes are attached the Cytosensor program can be started to determine the quality of the connections and activity of the cells. *See* **Note 11** for the Cytosensor Microphysiometer program parameters.

The Cytosensor Microphysiometer should return a "no problem" signal to ensure good electrical connections. The cells should be fairly active—the larger the acidification rate, the more active the cells, and the larger the signal change that will be generated for the sensors. However, this may vary between cell types. Best results have been obtained with acidification rates approx –200 μV/s or greater.

The modified sensor head should also be tested briefly with the amperometric *i-t* curve program described in **Subheading 3.3.1.** to ensure that the CHI 1030 leads have good connections and that the sensors are working properly. Be sure that the sensor head plunger hole is dry. Then with the Cytosensor Microphysiometer pumps running, select "run" on the CHI 1030 and observe the currents. The GOX and LOX electrodes give negative currents and the oxygen electrode gives positive, all of which begin to decrease to a steady state (ideally in the 10- to 40-nA range).

Once both programs are functioning properly, they can then be started simultaneously. Mark the difference in the starting time or some common point during the run, and let them run until the cells have adjusted to the environment, i.e., achieve a steady baseline with respect to the acidification rate (*see* **Note 12**).

3.3.3. Addition of Metabolic Agent and Calibration Standards

1. Add metabolic agents either during a stop-flow cycle or near the start of a flow cycle. This allows the agent to begin taking effect before the next measurement (i.e., stop-flow cycle) (*see* **Note 13**).
2. After the desired number of cycles under metabolic agent flow, switch back to regular RPMI flow without the metabolic agent, and allow the cells to recover to a steady state for a desired number of cycles.
3. For construction of the calibration curve to convert the peak areas to concentrations, at the end of the experiment, add to the flow solution standard additions of lactate to change the concentration by 0.2 m*M* and glucose to change the concentration by 0.5 m*M* for each aliquot.

4. Remove the cells and repeat the experiment. This serves as a background check on the effect of the metabolic agent on the electrodes themselves in the absence of cells.

3.4. Data Accumulation and Calibration

1. Model the area or height of the peaks during stop-flow in Coulombs as measured against the flow baseline as directly proportional to the concentration.
2. Use the standard additions of glucose and lactate at the end of the experiment to construct a calibration curve of peak current (measured from the current baseline) or Coulombs of charge (also measured from the current baseline) vs concentration.
3. Estimate the oxygen concentration by extrapolating from ambient as a baseline. This may require measurement of the dissolved oxygen in the bulk solution if it is not known or it may be assumed. Then take the oxygen response (in Coulombs during stop-flow) of the sensor head in the absence of cells as the ambient oxygen concentration, and use a two-point calibration.
4. Convert all files to .TXT files and import into a spreadsheet program such as Excel for data analysis. This includes the acidification rate data from the Cytosensor Microphysiometer.

4. Notes

1. The wires should not touch the center electrode that comes with the plunger, should run the length of the sensor body, and should be electrically insulated. It may be helpful to bevel the holes at the surface before inserting the wires and filling with epoxy. When depositing epoxy around the electrode, it is useful to push the wire a few millimeters above the surface, depositing epoxy, then pulling the wire back down. This helps to ensure good adhesion of the epoxy to the platinum wire.
2. The silver epoxy can be deposited into the hole by filling a small, disposable syringe needle with some of the silver epoxy and then injecting it into the hole. To prevent the silver epoxy from making a silver electrode by being exposed at the surface, the hole with the silver epoxy can be beveled slightly with a larger drill bit after the silver epoxy has been injected. This will remove excess silver epoxy around and near the surface before the smaller platinum wire is inserted.
3. Polyurethane can also be used as the sealant. However, a version that is harder than Crystal Clear 200 is recommended. Make sure that the sensor head crimp connectors will not touch the plunger arm. They should be angled out similar to the flow tubing when the polyurethane sealant is used and before it hardens.
4. A Teflon rod has been used as well, which can be left in for the duration of hardening, since the material does not stick to it, and the Teflon is easily removed later. The wooden stick can also be drilled out. In addition, it is possible that the material could just be drilled out of the back after the hardening process, but having the wooden stick in provides a soft channel for the drill bit to follow and thus minimizes any possible damage to the tubing or crimp connectors from the drill bit.

5. Hysol Epoxy-Patch or some other insulating and hardening epoxy can be used in place of the polyurethane.
6. Alumina-polishing compound (Buehler) could be used in place of the diamond. Be careful not to grind the electrode surface area too far below the plunger lip/O-ring seat. This causes the electrodes to be too far from the cells when placed in the cup and, hence, an increase in the diffusion distance. In this case, the solution stop-flow cycle would have to be increased in proportion to the increased diffusion distance, i.e., beyond a practical time in order to measure a signal. The closer the electrodes to the cell membrane, the better and more rapid the signal response.
7. The more powdery versions of BSA go more easily and quickly into solution. The flakier BSA works but takes much longer to dissolve fully.
8. Be sure to mix thoroughly the glutaraldehyde into the solution. This prevents concentrated regions of the glutaraldehyde, which can cause portions of the mixture to gel early.
9. Because LOX is expensive and the solution has a tendency to gel more quickly, depending on how accurately the amount of glutaraldehyde was added, as many heads as possible should be prepared at one time for coating, because the solutions do not last more than 1 d. We found that storage of the solutions in a freezer or refrigerator overnight does not preserve them. Because the GOX is much cheaper, it can be used to practice making the solutions and applying the coatings before the LOX is used. Once coated on the electrodes, however, the coatings can last for 1 mo or longer.
10. If the coating spreads too far outside the sensor head area, the current is too high and it will be difficult to control the linear response even with the Nafion layers. In addition, excess H_2O_2 will be produced outside the platinum electrode area and may adversely affect the cells.
11. The Cytosensor Microphysiometer program can be set up to run 90 s flow, and 30 s stop-flow. This program may vary widely, but note that the shorter the stop-flow cycle, the less time for the analyte concentration gradient from the cells to reach the electrodes, so at least a 30-s stop-flow cycle is recommended as a minimum. In attempting to shorten the overall experiment time, we found that no adverse effects could be detected on reducing the 90-s flow cycle to 60 s.
12. There will be a slight amount of noise visible on the Cytosensor display, but it has no adverse effect on the determination of acidification rate. However, care should be taken not to allow any solution to get into the plunger hole of the sensor head, because this can cause unacceptable electrical noise between the two instruments. Water in the plunger hole is the main cause of bad signals and can easily get in when the tubing running to the reference electrode is disconnected while the solution is still flowing or during insertion of the sensor head into the cell holder. Drying with absorbent tissue usually removes the water.
13. The time for a metabolic agent to begin to affect the cells and to what extent will vary widely depending on the particular agent itself and the type of cells used. This includes the recovery period, if any, as well.

Acknowledgments

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