

A novel approach for monitoring extracellular acidification rates: based on bead injection spectrophotometry and the lab-on-valve system†

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Monitoring extracellular acidification rates (ECARs) is important for the study of cellular activities, since it allows for the evaluation of factors that alter metabolic function, such as stimulants, inhibitors, toxins as well as receptor and non-receptor mediated events. While the light addressable potentiometric sensor (Cytosensor® Microphysiometer) has been the principal tool for ECARs measurement in the past, this work introduces a novel method that exploits an immobilized pH indicator on the surface of microcarrier beads (Sephadex®) and is probed with a fiber optic coupled spectrophotometer. Likewise, live cells under investigation were also immobilized on microcarrier beads (Cytopore®). These beads are metered, transported and monitored within a microfluidic system, termed as the Lab-on-Valve (LOV). Use of carrier beads in conjunction with Bead Injection Spectrophotometry and a Lab-on-Valve module (BIS-LOV), makes ECAR measurements reliable and automated. The feasibility of the BIS-LOV approach is demonstrated measuring ECARs of the mouse hepatocyte cell line, TABX.2S, grown on Cytopore® beads packed within the central channel of the LOV system. These immobilized cells were perfused in a phosphate buffer carrier solution (capacity: 1 mmol L⁻¹, pH 7.4). Protons extruded from 10⁵ to 10⁶ cells were accumulated during a stopped flow period of 220 s followed by a pH measurement, detected by changes in absorbance of the pH indicator bonded to the microcarrier beads. Addition of metabolic inhibitors (sodium azide, oxamic acid) to the carrier buffer solution can induced an increase or decrease of the basal proton extrusion rate in a very reproducible manner. Comparison of the BIS-LOV technique to the Cytosensor® microphysiometer and literature confirms the validity of this novel approach, highlighting its advantages and suggesting future improvements that will make the BIS-LOV a practical tool for routine ECARs measurement.

Introduction

Metabolic pathways in living cells are comprised of numerous biological interactions and subsequent chemical reactions. The processes that yield cellular energy start with the uptake of nutrients and oxygen, with the excretion of waste products, such as lactic acid and carbon dioxide as by-products of catabolism. While monitoring glucose consumption and lactate extrusion provides some information on the energy balance of a cell by measuring glycolysis, measurement of acidification rates in the cell culture milieu is often preferable, since the extracellular acidification rate (ECAR) integrates the influence of a wider scope of metabolism than glycolysis alone. Consequently, the ECAR technique has been widely applicable to many areas including studies of functional response of membrane bound receptors,^{1–6} evaluation of drug candidates (inhibition of ATPase of P-glycoprotein⁷), chemosensitivity of tumor cells to therapeutic agents,⁸ detection of deficient cellular signaling pathways⁹ and cytotoxicity studies.¹⁰

Many of these studies within the last ten years were carried out using a Cytosensor® microphysiometer,¹ a potentiometric based monitor of pH change. Proton extrusion from living cells is measured in a custom sensor chamber that permits a low capacity buffer to be perfused. The bottom surface of this chamber is a silicon chip that functions as a light addressable potentiometric sensor (LAPS). Since a treated silicon nitride surface can respond to pH changes in a Nernstian fashion, the transducer yields a response of 61 mV pH⁻¹ at 37 °C when connected to current compensating circuitry.¹ The light addressable mode allows one pH

readout to be made each second. The cells are kept in contact with the transducer surface by means of a membrane that prevents cell loss into the flowing carrier stream. A typical measurement cycle^{2,20} is comprised of a 60 s flow period, followed by 120 s of stopped flow, during which extruded protons are accumulated around a cellular mass (typically 10⁵ cells in a 10.5 µL capsule) monitored by the LAPS transducer. Since a typical mammalian cell extrudes ~ 10⁸ H⁺ s⁻¹ basally, a change of 0.10 to 0.15 pH units can only be observed in a low capacity buffer (e.g. 1 mmol L⁻¹ NaH₂PO₄, pH 7.4) during this stopped flow period. Changes in acidification rates on the order of ~120% to ~170% are measurable upon external stimulation. The Cytosensor® microphysiometer, operating in a flow injection mode, delivers an injected stimulant bolus into a flowing stream and into the sensor chamber using a peristaltic pump. This method employs a *go/stop/go* routine¹¹ that introduces the stimulant, stops for acid accumulation and followed by re-equilibration through renewed perfusion. The microphysiometer has been widely accepted by the research community, and has been used to study many cell types, including adherent and nonadherent, mammalian as well as insect cell lines. Stimulants included antibodies, hormones, growth factors, cytokines, while inhibitors included drugs, toxins, disinfectants and channel blockers (Table 1). Unfortunately this instrument is no longer commercially available, and within the next two years Molecular Devices will also cease its technical support.¹²

Previously we have demonstrated the suitability of the BIS-LOV technique for cellular metabolic studies measuring glucose consumption and lactate extrusion.^{13,14} We have extended this to develop a novel approach to ECAR sensing, based on spectrophotometric measurement of a pH indicator that is immobilized onto microcarrier beads. The principle of this technique (Fig. 1A) differs

† Electronic supplementary information (ESI) available: Derivation of eqn. (4). See <http://www.rsc.org/suppdata/an/b315007k/>

from LAPS measurement in that the pH transducer (sensor beads) is not in direct contact with the cell culture. Rather the cellular matrix is held separate and upstream, forming a micro-column that can be perfused by a low capacity buffer stream. During a stopped flow period, proton extrusion by the cellular matrix is accumulated within the micro-column, and when the flow is resumed, the acidified buffer is then carried into a separate sensing column, composed of indicator beads that change color with pH, generating a flow injection peak. The area of the peak is proportional to the total amount of extruded protons during the stopped flow period as discussed in theory and confirmed by calibration. By keeping the stopped flow period constant throughout a series of measurements, changes in the metabolic rate are reflected by changes in peak area and hence the rate of proton extrusion by a cell can be derived. Our work introduces this novel approach and verifies its feasibility, highlighting its advantages as well as its limitations.

Theory

Calculation of the proton extrusion rate by living cells is based on the measurement of pH change ($-\text{dpH}/\text{dt}$) within a known volume V of buffered solution that has an overall buffering capacity β :

$$\beta = -\text{d}(n_{\text{H}^+}/V)/\text{d}(\text{pH}) \quad (1)$$

where n_{H^+} is the number of protons excreted into a fixed volume (V), within which the change of pH takes place. The buffer capacity β depends on the weak acid concentration of the buffer. For a weak phosphate buffer:

$$[\text{A}]_{\text{tot}} = [\text{H}_2\text{PO}_4^-] + [\text{HPO}_4^{2-}] \quad (2)$$

The pK_2 value of phosphoric acid,^{15,16} and the predominant pH value of the interrogated solution becomes:

$$\beta = -\Delta(n_{\text{H}^+}/V)/\Delta\text{pH} = \ln(10)[\text{A}]_{\text{tot}}(10^{\text{pK} - \text{pH}}/(1 + 10^{\text{pK} - \text{pH}})^2). \quad (3)$$

The equation is obtained by the first derivative with respect to pH using the Henderson–Hasselbalch equation and solving the outcome to $-(n_{\text{H}^+}/V)$:

$$-(n_{\text{H}^+}/V) = [\text{A}]_{\text{tot}}(10^{\text{pK} - \text{pH}}/(1 + 10^{\text{pK} - \text{pH}})) - [\text{A}^-]. \quad (4)$$

See Electronic supplementary information (ESI)† for derivation of eqn (4). This calculation assumes that the buffering capacity is constant within the interrogated pH range and that the transducer response to pH is linear within the range of the interrogated pH change. Also, the amount of indicator must be small compared to the buffer capacity of the interrogated sample. Under these conditions, the transducer will: (1) provide a *dynamic response* that is directly related to ECAR, provided that it is in contact with the buffer solution surrounding the cell culture, as is the case in the Cytosensor® microphysiometer, and (2) provide an *integral response* as the interstitial buffer (volume V) is transported through the column of indicator beads (Fig. 1).

The first condition requires a constant buffering capacity that cannot be entirely satisfied given a pK_a value^{15,16} of 7.21 for phosphate buffer. However, since other buffers are less suitable for use on cell cultures, a compromise has been adopted in Cytosensor®

Table 1 ECAR measurements carried out by Cytosensor®.

Type of study	Type of cells or cell line	Ref.
Receptor pharmacology	Extracellular acidification rates (ECARs) for two classes of receptors	CHO C, human bone marrow CL (TF-1), PC-12 neuroblastoma C, mouse 929L cells
	ECARs in response to different transmembrane-domain receptors	Human osteoblast-like SaOS-2 cells
	Two-phase response of acid extrusion triggered by purinoceptor	CHO cells
	Identification and characterization of small molecule functional antagonists of the CCR1 chemokine receptor	Human monocytic (THP1) and human embryonic kidney 293 cell line
	Real-time evaluation of human secretin receptor activity	CHO cells
	ECARs associated with T lymphocyte responses to peptide ligands interacting with T-cell receptor	Ag-specific T-cell clones, designated E11, generated from MSF132 Patient blood (HLA-typed)
	ECARs in response to the gonadotropin-releasing hormone (GnRH), acting via the GnRH receptor	Mouse gonadotrope-derived α T3-1 cells
	ECAR response triggered by α_1 adrenoceptor	CHO cells
	ECARs in response to drugs activating or inhibiting the ATPase of P-glycoprotein (Amitriptyline, calcein – AM, cyclosporin A, diltiazem, progesterone, trifluoperazine, R/S – verapamil, vinblastine) activating or inhibiting the ATPase of P-glycoprotein	Pig kidney cells (LLC-MDR1, LLC-PK1), mouse embryo fibroblasts (NIH-MDR-G185 and NIH3t3)
	ECARs in response to drugs (Fluorouracil, Insulin, Chlorpromazine, Thyroxine, Dilantin)	Nephridium and cardiac muscle cells of the suckling rat
Drug discovery	ECARs in response to drugs (verapamil, glybenclamide, KCl) to track insulin secretion	Rat β insulinoma cells (INS-1, HIT-T15)
	ECARs in response to drugs (quinpirole, amiloride)	C ₆ glioma cells, L fibroblasts
	Biological implication of conformational flexibility in Ouabain	Dog kidney cell line (MDCK)
	Monitor the basal and cholinergic-simulated activity of the Na^+/K^+ ATPase	Human rhabdomyosarcoma cell line TE671
	ECARs in response to vasoactive amine	Cerebovascular endothelial cells (CVE) derived from SJL/J mice and BALB/c-derived CVE cells
	ECARs in response to different drugs (dicumarol, epiandrosterone, iodoacetate, fluoride, oxamate, rotenone, antimycin, azide)	Chinese hamster ovary (CHO) cells
	Toxicity studies, ECARs in response to drugs (paracetamol, acetylsalicylic acid, ferrous sulfate, diazepam, amitriptyline, digoxin, ethanol, ethylene glycol, methanol, isopropanol)	Human liver cell line (ATCC-CCl-13)
	Signal transduction	PC12 or TrkA-transfected CHO cells
	Others	Mouse fibroblast line 3T6
	Limits of acidification	Fibroblast cells (ATCC CRL-10225)
Others	Simultaneously measurements of acidification and oxygen consumption	
	Microphysiological testing for chemosensitivity	Tumor cells: human adherent and murine sarcoma S180 cell line

methodology¹⁹ as well as the BIS-LOV technique. That is to accept a small decrease in buffering capacity as the buffer becomes acidified within a limited working range of pH 7.4–7.2 and to use an average value of $\beta/A_{\text{tot}} = 0.35$, since this same change will occur in every consecutive measurement. Keeping to the same conventions used in the Cytosensor[®] studies was justified as documented by the linearity of dpH/dA, as well as in the $\Delta A/\Delta H^+$ calibration curves.

Experimental

BIS-LOV technique

System components. The experiments were conducted using a FIALab 3000 Sequential injection analyzer (FIALab Instruments, Medina, WA) configured with Lab-on-Valve manifold (Fig. 1B). It is comprised of a high precision bi-directional 1000 μL syringe pump, a six-position selector valve with a LOV module and a second auxiliary six position valve (MPV). PEEK tubing with an id of 0.5 mm (Upchurch Scientific, Murrieta, CA) is used as the conduit for all solutions. A 7 W tungsten halogen lamp (fabricated in-house) was used as the light source for a UV/VIS S2000 spectrophotometer (Ocean Optics Inc., El Dorado Hills, CA). Two 400 μm fiber optic cables ZP400-1-UV/VIS (Ocean Optics Inc., El Dorado Hills, CA) furnished with stainless steel probes (1.6 mm od) were inserted into the LOV module and connected to a light source and UV/VIS spectrophotometer. Inside the LOV module, the fiber optic tips were separated to form a 1.7 mm gap. This gap serves as a flow-through cell filled with the pH indicator beads to a bead volume of $\sim 2 \mu\text{L}$. At the bottom of this flow-through cell

and atop of the central port channel, bead retention plugs (Fig. 1C and D) were inserted and are made from 1.6 mm PEEK tubing (0.13 mm id and 3 mm long). The entire instrument was placed inside an incubator Imperial III (Barnstead Int., Dubuque, IA) for temperature control. Data collection and fluidic control was accomplished by using FIALab for Windows software (version 5.9.87).

A digital pH meter, Model 4500 (Beckman Coulter, Fullerton, CA) was used to adjust the pH of all prepared buffer solutions. The absorption spectrum of the nonbleeding indicator was recorded using a standard 1 cm² cuvette equipped spectrophotometer, Model 8452A (Hewlett Packard, Palo Alto, CA).

Materials and reagents. All standards and reagents were prepared fresh daily, using boiled DI water. When in use, carrier solutions were continuously aerated and carbon dioxide was removed at the inlet by using a scrubber tube filled with potassium hydroxide pellets (Fisher Scientific, Fairlawn, NJ).

Carrier buffer solution. For the pH measurements, a phosphate buffered balanced salt solution (PBS) with 1 g L⁻¹ bovine serum albumin, BSA, (Calbiochem-Novabiochem corporation, San Diego, CA) and 10 mmol L⁻¹ glucose (Sigma, St. Louis, MO) was prepared. The PBS solution contained 0.5 mmol L⁻¹ potassium phosphate (KH₂PO₄), 0.5 mmol L⁻¹ sodium phosphate (Na₂HPO₄), 0.3 mmol L⁻¹ calcium chloride (CaCl₂), 0.6 mmol L⁻¹ magnesium chloride (MgCl₂), 3.0 mmol L⁻¹ potassium chloride (KCl) and 130.0 mmol L⁻¹ sodium chloride (NaCl) (all J. T. Baker Chemical Co., Phillipsburg, NJ). During the cell experiments the pH was adjusted to 7.4 followed by filtration through a 0.2 μm sterile filter (Pall corporation, Ann Arbor, MI). The calibration was performed through the spiked addition of 1 mol L⁻¹ hydrochloric acid (VWR International, West Chester, PA) into a sample of phosphate buffer.

Indicator Beads. Fig. 2A illustrates the absorption spectra of aqueous solutions of indicator (pH indicator, Cat. no. 2.79602.000, Merck KgaA, Darmstadt, HE, Germany), recorded at various pH values, showing two absorption maximums, the acid form at 457 nm and the base form at 556 nm.

The amount of indicator covalently bound to the Sephadex[®] beads was adjusted in order to yield an absorbance < 1 at all VIS wavelengths, when the indicator beads filled the gap between the optical fibers and are equilibrated to pH 7.4 (Fig. 2B). After testing several different concentrations of immobilized indicator, the optimal formulation for the synthesis of pH sensor beads is described as follows.

A 2 mL solution containing 423 $\mu\text{g mL}^{-1}$ of indicator in water was doped with 10 μL of sodium hydroxide (32%), 0.1 g sodium carbonate (Na₂CO₃) and 0.05 g sodium chloride (all J. T. Baker Chemical Co., Phillipsburg, NJ). After all components are dissolved, 40 mg of Sephadex[®] G-50 microcarrier beads (Sigma, St. Louis, MO) was added and allowed to mix for 5 min in a tumbling mode mixer. The resulting pH beads are placed onto filter

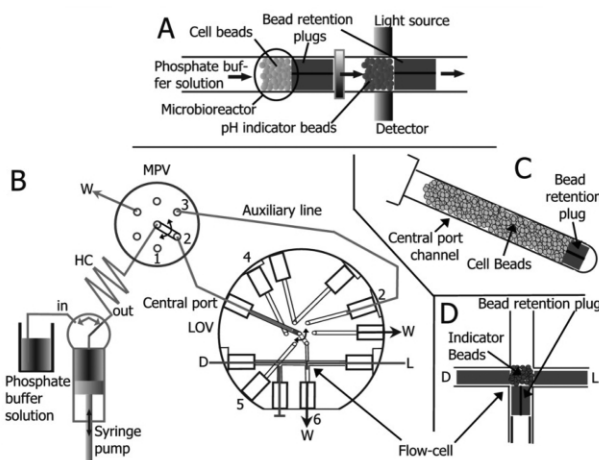


Fig. 1 Schematic diagram of the micro Sequential Injection (μ -SI) system for the measurement of proton extrusion from live cells. This entire Lab-on-Valve (LOV) system is placed in a small conventional incubator. The auxiliary 6-port multiposition valve was used during (a) the calibration for standard solutions and (b) the measurement of the cellular response to avoid the diffusion of the produced protons by positioning it during the stop flow time to port #1. Abbreviations: HC→holding coil, W→waste, D→detector, L→light source, MPV→multiposition valve, LOV→Lab-on-Valve.

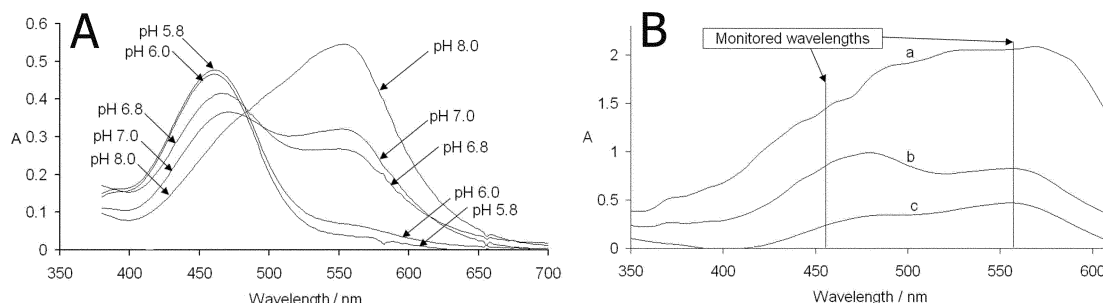


Fig. 2 A: Absorption spectra of the pH indicator in a solution at various pH values. B: Absorption spectrum of the Sephadex[®] pH indicator beads recorded in LOV module and corrected by subtracting spectra of beads without indicator. Indicator concentrations: a: 10.8 mg mL⁻¹, b: 423 $\mu\text{g mL}^{-1}$ and c: 267 $\mu\text{g mL}^{-1}$.

paper (VWR Scientific Inc., San Francisco, CA) and washed with DI water to thoroughly remove excess dye and reactants.

Loading the pH indicator beads into the flow cell was carried out using a syringe filled with the bead suspension and connected to port #4 of the LOV (Fig. 1B). A 15 μL volume of suspension was aspirated into the central channel of the LOV module at a flow rate of 100 $\mu\text{L s}^{-1}$. The valve was then switched to port #6 and the bead suspension was propelled into the flow-cell. Excess pH beads were discarded to waste through port #1. In order to pack these pH sensor beads into a well-defined geometry, a flow pulse of 10 $\mu\text{L s}^{-1}$ and 700 μL of carrier solution was used. In this way, $\sim 2 \mu\text{L}$ of beads filled the gap between the optical fibers, forming a colorimetric pH sensor. Because of the compressibility of these indicator beads, they can be removed by accelerating the forward flow to 200 $\mu\text{L s}^{-1}$, that forces the beads through the central gap of the bead retention plug.

Cell culture. All reagents were purchased from Sigma, St. Louis, MO, unless specified from another source. Cells were cultured in an incubator at 37 °C with 5% CO_2 . The cells of the mouse hepatocyte cell line, TABX.2S,¹⁷ were grown to $\sim 80\%$ confluence. A polystyrene cell culture flask containing the culture medium, consisting of Dulbecco's modified Eagle's medium/Ham's F12 medium with 100 $\mu\text{g mL}^{-1}$ penicillin-streptomycin (both Gibco BRL, Bioscience, Inc., Gaithersburg, MD), 5 mg mL^{-1} insulin, 5 mg mL^{-1} transferrin, 5 ng mL^{-1} selenium, 100 nmol mL^{-1} dexamethasone, 10 mmol mL^{-1} nicotinamide, and 225 $\mu\text{g mL}^{-1}$ G418 (Geneticin). The cells were treated with trypsin to release them from the surface, washed in fresh medium and added a spinner culture flask containing 20 mg of dry weight Cytopore® microcarrier beads (Amersham Pharmacia Biotech, Arlington Heights, IL) in 20 mL cell culture medium. The beads had been previously prepared by hydration in Hank's balanced salt solution (Gibco BRL), sterilized by autoclaving (121 °C, 20 min), followed by overnight incubation in cell culture medium containing 10% fetal calf serum (HyClone, Logan, UT). After the inoculation, the cell bead mixture were gently stirred and incubated for several days in a spinner culture flask at 37 °C with 5% CO_2 until there were approximately 200–500 cells per bead.

Loading the cell beads into the LOV module. Cells grown on Cytopore® beads were loaded manually into the LOV module in the following way. The tubing leading from the MPV into the central channel was temporarily disconnected and a syringe containing the Cytopore® beads was mounted into the LOV port. The lower end of the central channel was then connected to port #2 so that the auxiliary line communicated with the syringe pump *via* the MPV. This arrangement allows the bead suspension to be aspirated stepwise, until an 18 mm long bead column is formed in the central port. Next, the central port was reconnected to MPV in the manner shown in Fig. 1B and the cell column was perfused with phosphate

carrier buffer for approximately 15 min in order to establish a stable baseline metabolic rate.

Cell number determination. The number of living cells per bead was measured by sampling an aliquot of the cell bead culture and reacting this subset to a solution containing 0.1 mol L^{-1} citric acid and 0.1% crystal violet, at a ratio of 1:5 (v/v). Next, the cell bead solution was gently mixed for 1 h to allow complete cell lysis, releasing the stained nuclei from the beads. These nuclei were counted on a hemacytometer. Typically a single bead accommodates between 200 to 500 cells.

Total number of living cells per assay was calculated from the number of cell beads held within the 40 μL volume of the central port channel. All spent cell beads were extracted after the experiment with a 1000 μL aspiration of solution into a standard 1.5 mL microtube. The number of beads from a 10 μL sample were counted using a hemacytometer. It has been estimated that 3500 cell beads occupy the 40 μL cavity within the central port channel of the LOV module. The determination of the interstitial volume around the cell bead column was volumetrically measured. The fitting to port #2 of the MPV, was disconnected to create an air/liquid junction just above the central port channel. The solution around the cell beads was gradually aspirated with the syringe pump until the air/liquid meniscus was spotted at the rotating selector slot of the LOV, marking the bottom of the bead column. The extracellular (interstitial) volume within the bead column was found to be 15 μL . Thus the ratio of the free liquid volume to the total volume of the column was found to be 37.5%.

ECAR calculations were based on the number of cells and cell beads measured. Thus, a typical experiment uses 10^5 to 10^6 living cells packed into the central port channel and the extruded protons were measured from the interstitial volume of 15 μL .

Inhibitors. A 5 mmol L^{-1} solution of sodium azide and a 5 mmol L^{-1} oxamic acid solution (both Sigma, St. Louis, MO) were made in the phosphate buffer carrier solution. Sodium azide is a reversible inhibitor of oxidative metabolism and promotes anaerobic glycolysis, resulting in an increase of lactic acid extrusion. Oxamic acid blocks conversion of pyruvate to lactate resulting in a decrease of proton extrusion. These inhibitors were used to test the sensitivity of the pH indicators to metabolic changes occurring in the cells.

Experimental Protocol. The pH measurement for the determination of proton extrusion by living cells was carried out inside an incubator at 37 °C. Information on metered volumes and flow rates during the individual steps of the experimental protocol is given in Table 2. At the beginning of an experimental run, indicator beads and cell beads are manually loaded into the LOV module as described earlier. All subsequent steps were automated by software control.

Table 2 Experimental protocol for the ECAR in BIS-LOV system^a

Step	Operation	MPV port	LOV port	Time/s	Flow rate/ $\mu\text{L s}^{-1}$	Vol./ μL
1.	S.p. (out) disp. buffer sol. through cell beads	#2	#1	900	2	1800
2.1	S.p. (in) asp. buffer sol. from reservoir			0.5	100	50
→ 2.2	S.p. (out) disp. buffer sol. through cell beads	#2	#6	25.5	2	50
↑ 3.1	S.p. stop	#1	#5	0		
↑ 3.2	Stop flow period	#1	#5	103		
↑ 3.3	S.p. (in) asp. buffer sol. from reservoir	#1	#5	14	50	700
↑ 3.4	Stop flow period	#1	#5	103		
↑ 3.5	Reference Scan	#1	#5	0		
↑ 3.6	Start absorption scanning	#1	#5	0		
↑ 4.1	S.p. (out) disp. to flow cell	#2	#6	325	2	650
↑ ← 4.2	Stop absorption scanning	#2	#6	0		

^a Abbreviations: s.p. – syringe pump, sol. – solution, LOV – Lab-on-Valve, MPV – Multiposition valve, Vol. – volume, asp. – aspirate, disp. – dispense, → – loop

In the first step, the cell beads were perfused with the phosphate buffer solution for about 15 min at a flow rate of $2 \mu\text{L s}^{-1}$ in order to stabilize the cells to their basal metabolic rate. In the second step, an initial baseline measurement is determined by pumping $50 \mu\text{L}$ of phosphate buffer solution from the MPV over the column of cell beads and into indicator beads chamber. In the third step, a stop flow period is initiated by isolating the cell bead chamber through repositioning the selector valves (LOV module and MPV) to alternative ports (#5 and #1). This physically isolates the cell beads and proton diffusion is thus contained within the interstitial volume of the beads. In the fourth step, the end of stopped flow period, $650 \mu\text{L}$ of the phosphate buffer solution is pumped at $2 \mu\text{L s}^{-1}$ through the cell culture and into the indicator beads. Spectrophotometric measurement of the absorption at 457 and 556 nm is recorded during this step.

Cytosensor®

System components. A Cytosensor® microphysiometer (Molecular Devices, Sunnyvale, CA) was used for this study.

Materials and reagents.

Carrier buffer solution, cell culture and inhibitors. Cytosensor® used the same cell culture, carrier buffer and inhibitor solution as for BIS-LOV experiments described above.

Cell number determination. Cell number was determined by trypsinizing a capsule on the day of the experiment, to remove cells, which were then diluted into trypan blue and counted using a hemacytometer to determine the actual cell number per capsule. This number was halved, since the area of the capsule being interrogated by the Cytosensor® is only half of the total surface area.

Experimental protocol. Approximately 1×10^5 TABX.2S cells were grown in a capsule cup membrane for 24 h prior to experimentation using cell media at 37°C with 5% CO_2 . The cell capsules were placed into the sensor chambers of the microphysiometer and perfused at $100 \mu\text{L min}^{-1}$ with the same 1 mmol L^{-1} phosphate buffer used in the BIS-LOV method. All solutions were thermostated at 37°C with debubblers enabled. The rate of acid extrusion was determined using a 90 s pump cycle, with the cells being perfused for 60 s followed by stopped flow for 30 s. The potentiometric rate measurement is made during the stopped-flow cycle. The typical drop in pH during the pump-off cycle is 0.05 pH units. The system was calibrated bi-weekly with pH standards (Micro Essentials Laboratory, Brooklyn, NY). Slopes did not vary significantly over time, ranging from $62.6\text{--}60.5 \text{ mV pH}^{-1}$ for the eight sensors.

A baseline ECAR was established after loading the cell capsules into the sensor chambers, which typically took 30 min to stabilize. Once stabilized, the solution was changed through a selection valve to a solution containing either 5 mmol L^{-1} sodium azide, or 5 mmol L^{-1} oxamic acid. Cells were exposed to these inhibitors for 12 min, after which they were returned to a control buffer. ECAR extrusion rates were calculated in the typical fashion as described in literature²⁰ for the Cytosensor® microphysiometer

Results and discussion

Calibration of BIS-LOV sensor

This was carried out using blank Cytosensor® beads (without cells) loaded in the central channel. Carbon dioxide scrubbed air was perfused through the carrier solution reservoir. Micro-aliquots ($2.00\text{--}20.0 \mu\text{L}$) of hydrochloric acid solution (1.00 mol L^{-1}) were spiked into different 20.0 mL samples of phosphate buffer, to create a series of calibration standards. Each calibration solution was aspirated ($15.0 \mu\text{L}$, at a flow rate of $10 \mu\text{L s}^{-1}$) from port #6 of the MPV into the holding coil. Then the flow was reversed and the

injected acid was then dispensed at a flow rate of $2 \mu\text{L s}^{-1}$ through port #2 of the MPV into the LOV module. In this way, the calibration solution passed through the central port channel filled with blank Cytosensor® beads prior to flowing through the indicator beads. A flow rate of $2 \mu\text{L s}^{-1}$ was selected to be identical with flow rate used for ECAR monitoring. The calibration curve with a pH range of 7.2 to 7.4 covers the anticipated range for proton extrusion of the cell beads. The area of the peaks recorded during the flow-through period yielded a linear calibration curve, that for 457 nm was $y = 1.62 \times 10^{-11}x + 2.13 \times 10^3$ (linear response $R^2 = 0.9957$) and for 556 nm was $y = -3.69 \times 10^{-11}x + 3.14 \times 10^2$ (linear response $R^2 = 0.9982$), where y is the relative absorbance expressed as peak area and x is the added number of protons. In this way it was determined that 1×10^8 protons corresponds to peak area of 1.6 mA (area) when recorded at the wavelength 457 nm and to a peak area of 3.7 mA (area) at 556 nm.

Basal metabolic ECAR

This was measured using the experimental protocol described on the TABX.2S cell line. The typical response curves are shown in Fig. 3A. This assay yielded ECAR for a single cell to be $(2.46 \pm 0.08) \times 10^7 \text{ H}^+ \text{ s}^{-1}$ at 556 nm and $(2.42 \pm 0.02) \times 10^7 \text{ H}^+ \text{ s}^{-1}$ at 457 nm. An averaged single cell ECAR value from over 20 measurements, carried out at different days using different cultures of the same cell line, was found to be $(2.21 \pm 0.43) \times 10^7 \text{ H}^+ \text{ cell}^{-1} \text{ s}^{-1}$ at 556 nm and $(2.37 \pm 0.23) \times 10^7 \text{ H}^+ \text{ cell}^{-1} \text{ s}^{-1}$ at 457 nm (Table 3).

The basal values obtained are within the reported ECAR range for mammalian cells. It should be noted that Cytosensor® data varies from a frequently reported^{18,19} value of $1 \times 10^8 \text{ H}^+ \text{ cell}^{-1} \text{ s}^{-1}$ to as low as $1.3 \times 10^7 \text{ H}^+ \text{ cell}^{-1} \text{ s}^{-1}$,⁷ and as high as $6.5 \times 10^8 \text{ H}^+ \text{ cell}^{-1} \text{ s}^{-1}$.²⁰ These variations are due to difference in cell lines, variances in cellular metabolism or decreased number of live cells actually present in the Cytosensor® chamber. Changes in the calibration slope of the transducer due to variations in the cell count are another plausible cause of these differences. It should be kept in mind, however, that the main purpose of most ECAR measurement is to determine changes between cellular proton extrusion rates from the basal state to the stimulant or inhibitor state. Repeatability of the relative ECAR changes is thus more significant for drug screening or comparisons than absolute values.

Change of metabolic rate in the presence of inhibitors

Azide is a cytochrome C oxidase inhibitor that shunts glucose metabolism toward an anaerobic pathway, resulting in increased lactic acid extrusion. Oxamic acid, has an opposite effect as it blocks conversion of pyruvate to lactate resulting in a decrease of proton extrusion. A typical response of TABX.2S cells shifts in the presence of azide (5 mmol L^{-1}) to a new metabolic pathway resulting in an increase of proton extrusion by $77\% \pm 3\%$ as recorded at 556 nm and $70\% \pm 4\%$ at 457 nm (Fig. 3B and Table 3). Addition of oxamic acid (5 mmol L^{-1}) on the other hand, decreases the proton extrusion by $38\% \pm 11\%$ as measured at 556 nm and $33\% \pm 4\%$ at 457 nm (Fig. 3C). Additional measurements on 24 runs are summarized in Table 3.

Response of the BIS-LOV system

The response to ECAR is dependant on the number of interrogated cells, the buffering capacity of the surrounding solution, the response function of the pH indicator and stopped flow duration, where extruded protons accumulated within the column of cell beads. In this work, only the stopped flow period has been optimized for mouse hepatocyte cells over a 120 to 380 s (Fig. 3D) period. This is an important variable, since decreasing buffer capacity will result in poor reproducibility, exacerbated by the acidobasic properties of the injected stimulant.

Comparison of Cytosensor® to the BIS-LOV technique

The Cytosensor® was operated in a standard fashion as recommended by the manufacturer. In this comparison, all other components such as the cell culture, carrier buffer and inhibitor solution were identical for the Cytosensor® and BIS-LOV measurements. Typical readouts and extrusion rates were performed for cells in a basal state, and stimulated states using sodium azide and oxamic acid as shown in Fig. 4A, B and C and Table 4. The results obtained for both the Cytosensor® and BIS-LOV are summarized in Fig. 5A and B. The ECARs results from the BIS-LOV agree within the error interval with the Cytosensor® data. The acid extrusion rates under basal conditions made by the BIS-LOV module and the Cytosensor® are normalized to 100% to obtain comparable acid extrusion rate (Fig. 5B). The percentage changes of the ECAR by addition of metabolic inhibitors (sodium azide and oxamic acid)

correspond to the normalized extrusion rate under basal condition.

Conclusion

The above results confirm the feasibility of using an immobilized pH indicator as a transducer for ECAR monitoring of cell cultures in basal and altered metabolic states, yielding data that tracks well with the data obtained by the Cytosensor® system. While this methodology is still in preliminary stages of development it highlights the following advantages to the BIS-LOV approach:

(1) A relatively large number of cells (1×10^5 to 1×10^6) can be interrogated automatically within a small packed column (40 μL) in a sterile and enclosed environment.

(2) The amount of cells can be easily varied, as required by a experimental needs.

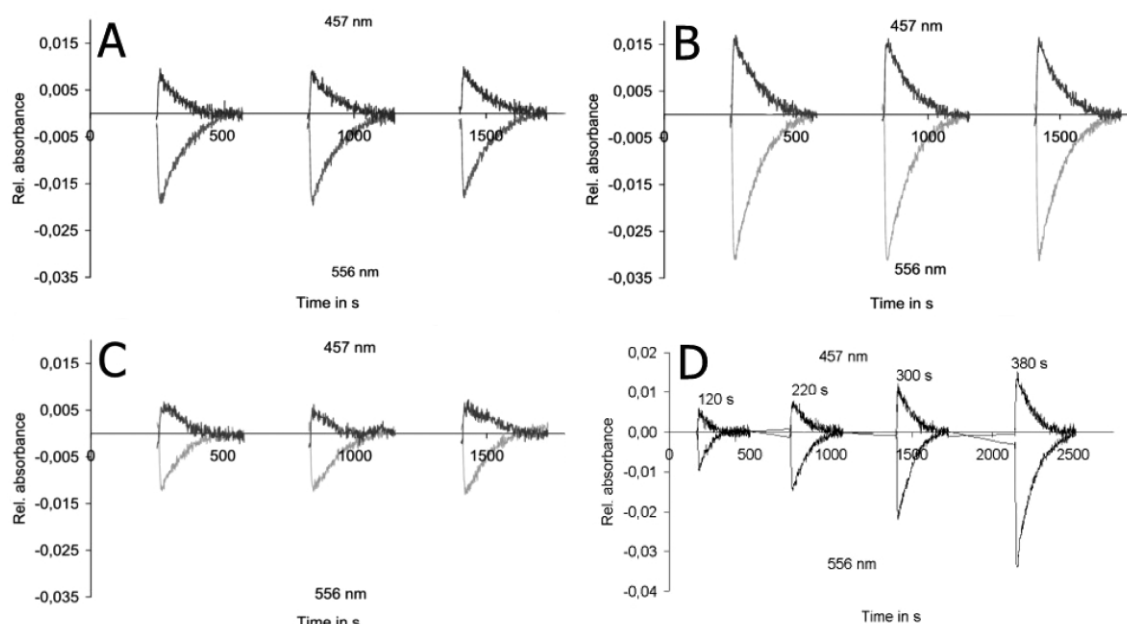


Fig. 3 Typical peaks made by the Lab-on-Valve system (stop flow period: 220 s); A: basal condition, B: stimulated by 5 mmol L⁻¹ sodium azide, C: stimulated by 5 mmol L⁻¹ oxamic acid, D: with different stop flow periods.

Table 3 ECARs measurements of mouse hepatocyte cell line TABX.2S, carried out by BIS-LOV technique

Source of error	Type of measurement and cell condition	Wavelength	Average of H ⁺ cell ⁻¹ s ⁻¹ in $\times 10^7$	SD	RSD (%)	n
Within run	Calibration by acid	556 nm	—	—	2.00–8.38	4
		457 nm	—	—	1.02–9.74	4
	Basal condition	556 nm	2.21	0.43	19.54	20
		457 nm	2.37	0.23	9.80	22
	Stimulated by sodium azide	556 nm	3.44	0.65	18.88	21
		457 nm	3.76	0.65	17.30	24
	Stimulated by oxamic acid	556 nm	1.50	0.62	41.58	22
		457 nm	1.53	0.17	11.34	23
Between run (same culture)	Calibration by acid	556 nm	—	—	0.09–5.14	4
		457 nm	—	—	2.76–5.86	4
	Basal condition	556 nm	2.12	0.41	19.41	4
		457 nm	2.35	0.29	12.18	4
	Stimulated by sodium azide	556 nm	3.16	0.26	8.18	4
		457 nm	3.63	0.52	14.21	4
	Stimulated by oxamic acid	556 nm	1.53	0.39	25.62	4
		457 nm	1.46	0.07	5.09	4
Between culture	Basal condition	556 nm	2.33	0.30	12.76	2
		457 nm	2.39	0.06	2.29	2
	Stimulated by sodium azide	556 nm	3.73	0.89	24.00	2
		457 nm	3.94	0.44	11.25	2
	Stimulated by oxamic acid	556 nm	1.38	0.05	3.32	2
		457 nm	1.56	0.14	9.07	2

(3) The entire system, including solution reservoirs are thermostated at 37 °C, with the option of air or mix-gas perfusion. This allows the cells to remain viable for longer periods of time.

(4) Cell culture can be replaced in a reproducible manner at any time during a series of experimental runs. Thus if the cells are adversely affected by a stimulant or toxic substance, the disabled cells can be discarded and replaced by a new column that is sampled from the reservoir of identical cell beads.

(5) The transducer, comprising of indicator beads, can be renewed as well in an automated fashion. In contrast, the transducer surface of the Cytosensor® must be cleaned with alcohol between experimental runs, and cell culture must be manually mounted between the membranes of Cytosensor® cup.²⁰

(6) The sensitivity of the BIS-LOV ECARs measurement in the present instrument configuration (0.03 pH units at 556 nm and 0.07 pH units at 457 nm) is adequate for many acid excretion studies.

(7) Proton extrusion rate for mammalian culture measured by BIS-LOV is in a good agreement with literature data.

(8) ECARs measurements by BIS-LOV are based on an actual calibration by means of a standard acid addition, which can be an automated process easily incorporated into standard experimental protocols. The Cytosensor® are based on calibration and has been reported to vary for 50.9 mV pH⁻¹ ²⁰ to a theoretical value of 61 mV pH⁻¹.¹⁹

The BIS-LOV instrument as stated is in its infancy and further improvements are needed. Full automation for loading indicator and cell beads into the LOV module needs to be developed. Success in this endeavor will allow the BIS-LOV to be adapted to rapid screening of therapeutic agents with minimal manual intervention. Since the between run error (Table 3) contributes the largest portion of variance to the measurement, improvements in the cell handling, such as these, will greatly increase the power of these measurements. Also, reducing or eliminating the conduit space (< 8 µL) between cell and transducer beads (Fig. 1A) will decrease the dispersion of the acidified buffer providing greater sensitivity in the measurement. Although the present apparatus is composed of commercially available components, the setup still requires onsite adaptations. However, since the Cytosensor® is no longer commercially available, there is a pressing need for an instrument that can promote and advance ECAR experimentation for cellular studies and drug discovery.

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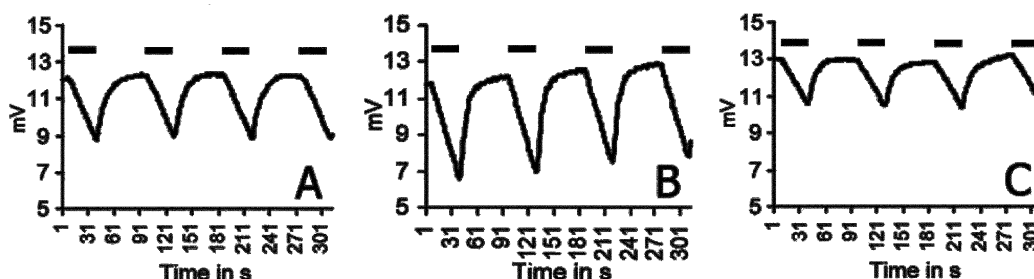


Fig. 4 Typical readouts made by Cytosensor® (stop flow period: 30 s indicated by black bars); A: basal condition, B: stimulated by 5 mmol L⁻¹ sodium azide, C: stimulated by 5 mmol L⁻¹ oxamic acid.

Table 4 ECARs measurements of mouse hepatocyte cells (line TABX.2S) carried out by Cytosensor®.

Source of error	Cell condition	Average of H ⁺ cell ⁻¹ s ⁻¹ in × 10 ⁷	SD	RSD (%)	n
Within run	Basal condition	2.73	0.05	1.88	24
	Stimulated by sodium azide	3.80	0.11	2.92	24
	Stimulated by oxamic acid	2.10	0.05	2.29	24
Between run (same culture)	Basal condition	2.67	0.28	10.49	4
	Stimulated by sodium azide	3.83	0.60	15.62	4
	Stimulated by oxamic acid	2.15	0.46	21.45	4
Between culture	Basal condition	2.82	0.04	1.44	2
	Stimulated by sodium azide	3.87	0.20	5.16	2
	Stimulated by oxamic acid	2.10	0.15	7.35	2

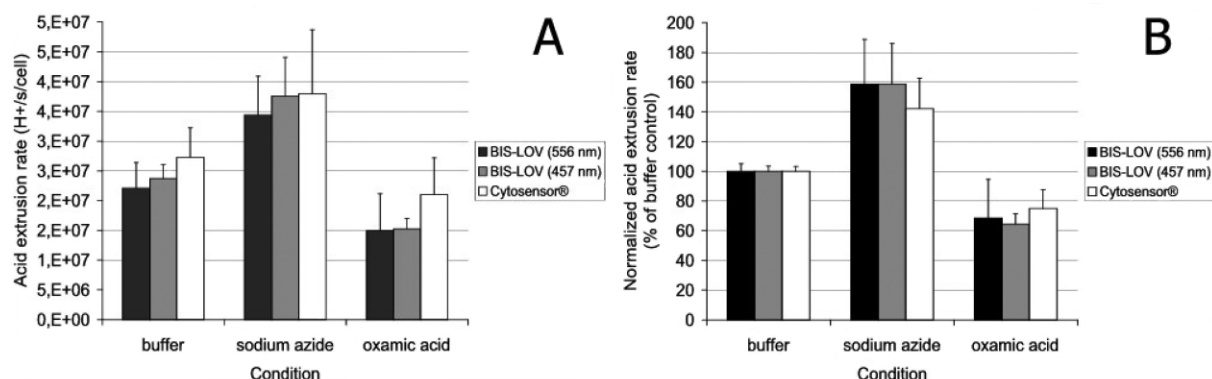


Fig. 5 Comparison of the results made by the BIS-LOV module to the Cytosensor®. Changes in extracellular acidification rates (ECARs) of TABX.2S cells upon addition of metabolic inhibitors (sodium azide and oxamic acid). A: Comparison of the absolute ECARs, B: comparison of the normalized ECARs.

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