The Analyst
TUTORIAL REVIEW

Novel flow injection methods for drug-receptor interaction studies, based on probing cell metabolism

Ilkka Lähdesmäki,*[a] Jaromir Ruzicka[b] and Ari Ivaska[a]

[a] *Åbo Akademi University, Process Chemistry Group, c/o Laboratory of Analytical Chemistry, Piispankatu 8, 20500 Turku, Finland*
[b] *University of Washington, Department of Chemistry, P.O. Box 351700, Seattle, WA 98195, USA*

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1 Introduction

1.1 Cellular energy metabolism

1.1.1 Definition and purpose of energy metabolism. The term energy metabolism comprises the chemical reactions and processes that living cells are using to extract energy from nutrient molecules. In the case of animal cells, energy is derived from organic compounds, such as glucose and fatty acids. By subjecting these energy-rich source compounds to a series of metabolic reactions, they are transferred into energy-poor products. The released energy is captured in the form of adenosine triphosphate (ATP), a compound formed from adenosine diphosphate (ADP) and pyrophosphate (P_i) in the following reaction:

$$ADP + P_i + energy \rightarrow ATP + H_2O$$

By including the reverse reaction, ATP hydrolysis, as a part of the overall reaction scheme, biosynthetic pathways can acquire the necessary energy to proceed. Consequently, ATP can be regarded as the cellular energy currency, generated from nutrients by one set of reactions and subsequently used for fueling a wide range of other vital processes in the cell.

1.1.2 Pathways in energy metabolism. A cell can derive energy from proteins, polysaccharides/sugars or lipids.[1] For cultured cells, by far the most important source is glucose in the growth medium. Utilization of glucose for cellular energy production is shown in Fig. 1. It begins by a nine-step pathway called *glycolysis* where one glucose molecule is converted into two molecules of pyruvate, yielding two molecules of ATP. Whereas glycolysis occurs in nearly all living cells and in all conditions, further processing of the pyruvate formed will depend on the type of cell and on the availability of oxygen. In the absence of oxygen, pyruvate is simply converted into lactic acid by the enzyme lactate dehydrogenase. Thus, the overall energy producing reaction will be

$$glucose + 2\ ADP + 2\ P_i \rightarrow 2\ lactic\ acid + 2\ ATP + 2\ H_2O$$

The process is called *lactic acid fermentation* and cells employing it as their main route of energy supply are said to be *anaerobic*. Lactic acid fermentation commonly occurs in immortal cultured cells even in the presence of oxygen, to the extent where such cells usually are predominantly anaerobic under all circumstances.

In the presence of oxygen, pyruvate from glycolysis will be subjected to oxidation and decarboxylation in the *citric acid cycle*, an eight-step cyclic pathway. As a result, carbon dioxide is produced along with two molecules of ATP. Also, several molecules of the dinucleotide NAD^+ are converted into the reduced form NADH. The NADH molecules are subsequently re-oxidized on mitochondrial membranes through the processes of *electron transport chain* and *oxidative phosphorylation*. Oxidation of the NADH molecules is coupled to reduction of oxygen to water and, most importantly, to production of about 26 molecules of ATP. The overall reaction for this *aerobic*

Dr Ilkka Lähdesmäki graduated from Åbo Akademi University, Finland, with an M.Sc. degree in chemical engineering in 1993. He pursued graduate studies in analytical chemistry at the University of Washington and obtained his PhD in 1999. Currently, he is working as a postdoctoral fellow at the Laboratory of Analytical Chemistry at Åbo Akademi University. His research interests are focused on flow injection techniques and the application of fluorescence microscopy to chemical analysis.

Dr Ilkka Lähdesmäki

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pathway of glucose oxidation (glycolysis, citric acid cycle, electron transport chain and oxidative phosphorylation) is

$$\text{glucose} + 6\ O_2 + \sim 30\ ADP + \sim 30\ P_i \rightarrow 6\ CO_2 + \sim 30\ ATP + \sim 36\ H_2O$$

It is easy to see that the aerobic pathway makes much more efficient use of the chemical energy in glucose than the anaerobic pathway does. Therefore, it is not surprising that it is the preferred route of energy generation in all primary cells under sufficient supply of oxygen.

1.2 Cell response measurements

1.2.1 The concept of cell stimulation. Communication between cells is needed for proper, controlled function of living organisms.[2] Intercellular communication, also called cell signaling, is usually carried out by means of secreted signaling molecules, *i.e.*, stimulants. A stimulant will recognize its target cells by binding to a specific receptor protein that the target cells carry on their membranes. Stimulant–receptor binding will cause a conformational change in the receptor, which triggers off an intracellular cascade of biochemical reactions. This chain of events will eventually result in the expression of a new property in the cell, *e.g.*, production of a new protein. The steps in the cascade reaction are called the response of the target cell to the stimulant in question.

The purpose of a cascade reaction is to relay and amplify the signal inside the cell. Such a reaction is a complicated process and involves many different steps. These include direct activation or deactivation of enzymes, in addition to formation or release of small mediator species, such as Ca^{2+} and cyclic AMP. The mediator species are more usually called second messengers, reflecting their universal role as messengers that pick up the signal shortly after the primary event (stimulant binding) has occurred.

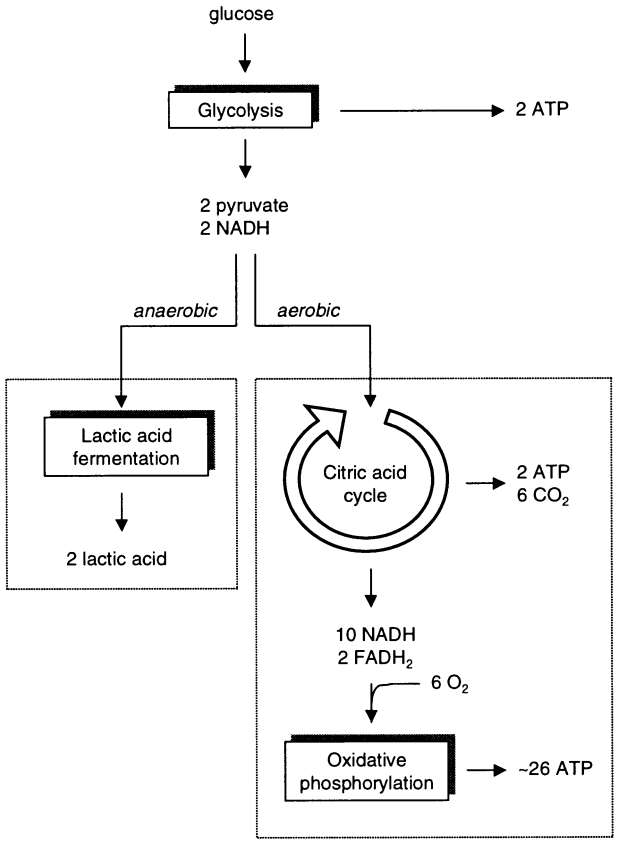


Fig. 1 Metabolic processing of glucose for energy production.

1.2.2 Mathematical models of cell response. Cell stimulation is initiated by a stimulant (drug) binding to a receptor. The binding of a drug *A* to a receptor *R* can be described by the equation

$$A + R \rightleftharpoons AR$$

where AR is the drug–receptor complex formed. Assuming that binding does not decrease [A] to an observable extent and using the law of mass action, the following expression is obtained for [AR]:

$$\frac{[AR]}{[R_t]} = \frac{[A]}{[A] + K_A}$$

where $[R_t]$ is the total concentration of receptors and K_A is the dissociation constant of the drug–receptor complex. The above adsorption isotherm has been observed to describe adequately the binding of a drug to receptors on cell membranes. Further, there is wide agreement that the number of occupied receptors determines the extent of cellular response: in the absence of the drug, no receptors are occupied and no response is observed. When the drug is present in progressively larger quantities, an increasing proportion of the receptors are occupied and an increasing response is observed. Finally, as most or all of the receptors are occupied, the response reaches saturation. However, there is no absolute agreement regarding the exact relationship describing the dependence of the response on receptor occupation. Designating the observed response by E and the maximum response by E_{max}, we can write the following relationship between the response and receptor occupation:

$$\frac{E}{E_{max}} = f\left(\frac{[AR]}{[R_t]}\right)$$

The function f is unknown; its nature will depend on the drug, the cell type, the receptor and the response mechanism triggered off by the drug–receptor binding. Borrowing nomenclature from signal processing, one could say that f is the response function of a system to a stimulus. Since each combination of drug, receptor, cell type and response mechanism constitutes a unique system, the response functions can be very different.

Even though the cellular response functions could be substantially different, experimental observations have shown that in many cases the response and the *free* drug concentration are related by a hyperbolic function:

$$E = \frac{E_{max}[A]}{[A] + b}$$

where b is a fitting constant. Owing to the form of the equation, the fitting constant b happens to equal the concentration of free drug needed to produce half-maximum response. For this reason, it is often called EC_{50}. Further, using the more common notation C for free drug concentration (in place of [A]), we can rewrite the response equation:

$$E = \frac{E_{max}C}{C + EC_{50}} \quad (1)$$

A much more thorough discussion of the theory of cell responses is provided elsewhere.[3,4]

1.2.3 Quantification of cell response. The response activated in a cell by stimulation is a complex process consisting of several phases: stimulant binding, signal transduction through the cell membrane, intracellular response cascade and the final event. The extent of the response triggered by a drug can be monitored at any of these levels. In addition, certain side effects or consequences of the response process can be utilized. Several different instrumental methods are used for cell response studies, the most common ones being radioactive labeling and scintillation, fluorescence spectroscopy and potentiometry.

During the past decade, fluorescence microscopy and flow cytometry have achieved a dominant position among these methods, owing to the recent development of many reliable and easy-to-use fluorescent indicators.[5]

Traditionally, response quantification has relied primarily on monitoring the concentration of intracellular messengers. The observed changes in messenger concentration provides an idea of how strongly or how fast the cell is stimulated by different stimulant levels, or by different stimulant species. Of all the different second messengers, intracellular Ca^{2+} has been a particularly popular target for monitoring since it is a common messenger and reliable, easy-to-use fluorescent methods have been developed for measuring it.

1.2.4 Measurement of cell stimulation through metabolic acid generation. In recent years, another method for response quantification has been emerging, which is based on the metabolic effects of the response. When a response cascade is activated, one of the general consequences will be altered consumption of energy in the cell. Since the energy metabolism generates acidic side products (lactic acid, CO_2), any changes in the metabolic activity will be reflected in the rate of metabolic acid production, and subsequently in the rate of acid extrusion. The link between cell stimulation and acid extrusion is utilized in the Cytosensor Microphysiometer, a commercial instrument that quantitates cell responses by measuring acid extrusion rates.[6] Cells are held in a small compartment that is continuously perfused by a flow of buffer solution. To measure the acid extrusion rate the flow is stopped, in which case acid produced by the cells accumulates in the compartment and changes the pH of the solution. A sensitive electrochemical pH sensor is used to detect the pH change, and acid extrusion rate is calculated as

$$\text{acid extrusion rate} = \frac{\Delta\text{pH}}{\Delta t}$$

Since the rate correlates with the magnitude of cell response, the instrument can be used for quantitating responses of the cells to stimuli. Exposure of cells to a stimulant is achieved by injecting a known volume of stimulant solution into the perfusion buffer stream, in other words, using flow injection. Based on the fairly extensive microphysiometry literature, acid extrusion seems to be a very general response parameter that can be used to study the responses of many cell lines and stimulant–receptor systems.[6,7]

The advantage in measuring a metabolic parameter, as opposed to traditional, specific parameters such as [Ca^{2+}], [cAMP] or membrane potential, is that it provides a more universal way of probing the response. The downside of the approach is that the connection between the response and the measured parameter is more complex than it is in the case of the traditional parameters. Both are due to the fact that metabolic parameters are a consequence of the whole response cascade whereas the traditional ones are involved in a specific, integral step in the response.

1.2.5 Measurement of cell stimulation through metabolic oxygen consumption. Owing to the central role of oxygen in the aerobic energy metabolism, oxygen consumption measurements can be used to study biochemical events where some part of the aerobic pathway is affected.[8,9] Several good methods exist for measuring oxygen consumption of cultured cells in suspension: the amperometric Clark electrode,[10,11] the use of a phosphorescent oxygen indicator in the cell suspension[9,12] and electron paramagnetic resonance spectroscopy.[8] However, there do not seem to be reports on using any of these methods on adherent cell cultures. This may be due to the fact that adherent cells are most commonly cultured as monolayers on flat, solid substrates. The limited number of cells in a monolayer will be small compared with the volume of the bathing solution, which makes it difficult to detect oxygen consumption within reasonable time periods.

Many cell types are naturally adherent, and therefore adherent cultures lend themselves well to studies of cellular responses to biochemical effector molecules and drugs. As described above, this is done by following a suitable parameter, such as intracellular Ca^{2+}, pH, acid extrusion rate or membrane potential, while exposing cells to the effector agent. Inclusion of oxygen consumption among the monitored parameters could provide a significant contribution to the obtainable information. The contribution could be especially important for studies of primary cells since their metabolism is aerobic and hence there is a wide range of cases where oxygen measurements could potentially be applied.

1.3 Bead injection

Flow injection analysis (FIA) is an established analytical technique.[13] Its traditional use lies in the field of chemical assays, but in recent years the technique has also been used to develop methods for live cell assays. Bead injection (BI), in turn, is the latest advancement of the flow injection techniques. It features the use of microbeads to create a small, disposable sensing layer that can be renewed for each individual assay.[14] The following review describes how unique characteristics of FI and BI can be utilized to design tools for probing cell metabolism, and how these tools can be used to measure cell responses.

2 Acid release and oxygen consumption measurements by BI

2.1 Acid release

The concept of acid release measurements and its utility for cell response quantification have been demonstrated by the development and success of the Cytosensor Microphysiometer.[6] The greatest strength of this technique is that it measures a parameter, metabolic turnover, which occurs in all animal cells. Also, although there are some signal transduction pathways that do not alter energy metabolism, the method has proved to be applicable to an extremely wide range of cells and stimulants.

Rather than carrying out acid release measurements with a special electrochemical instrument, it would be desirable to implement the measurements in a more universal platform, such as fluorescence microscopy. However, the implementation is complicated by the fact that acid release measurements involve probing an *extracellular* quantity. The complication with extracellular measurements arises from the difficulty of detecting effects of the modest cellular acid generation on the large volume of buffered solution surrounding the cells. In order to be able to measure the acidification, the following requirements need to be met:

(1) The buffering capacity of the bathing solution must be low, or at most intermediate level (10 mM or less), otherwise the secreted acid will not produce observable pH changes.

(2) The volume of solution around the cells should be as small as possible in order to prevent excessive dilution of the secreted acid. Also, the movement of the solution needs to be restricted to prevent dilution.

(3) The pH sensing element needs to be brought as close to the cells as possible.

The Microphysiometer achieves all the different items above by immobilizing a large number of cells in an agarose matrix and sandwiching the agarose 'sheet' between a physical support and the surface of an electrochemical pH sensor.[6] For construction of an analogous fluorescent technique, the obvious

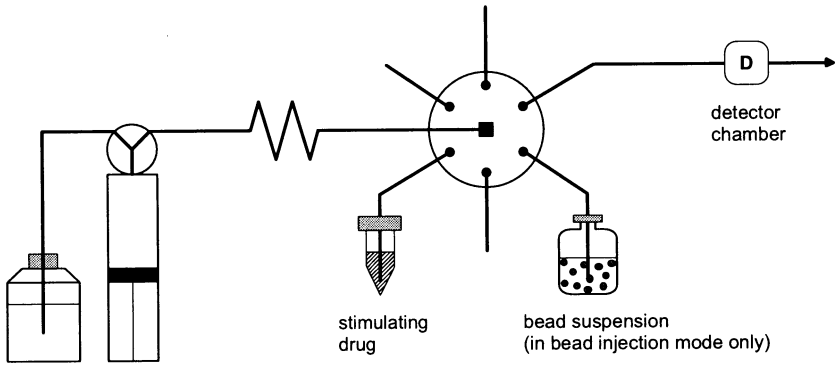
solution would be to place cells in a thin-layer flow through chamber and include a fluorescent pH indicator in the bathing solution. The approach does indeed work but suffers from the problems of a limited signal-to-noise ratio (owing to the thin layer of fluorescent indicator) and the necessity to include the fluorescent indicator in the carrier solution and also in all stimulant solutions (the authors' unpublished observations).

The BI technique has been successfully applied to address the problems and develop a viable method for fluorescent acid release detection.[15] The setup used in the work is shown in Fig. 2(a). The cells are grown on microcarrier beads that have been labeled with a fluorescent pH indicator. An aliquot of beads is packed into the flow-through chamber so that they form a disposable layer containing both the pH sensor and the cells. The response of the cells to drug exposure is measured by stopping the flow in the absence and the presence of the drug and determining the rate at which the stagnant solution acidifies in each case. The degree of the response is calculated as the ratio between the observed acidification rates. Since the beads can easily be renewed, each exposure can be carried out on a fresh set of cells. One run (consisting of bead loading, washing, stop period 1, washing, stop period 2, washing and bead disposal) takes 20–30 min to complete, depending on the flow rate. The observed pH changes during 3 min stop flow periods are of the order of 0.1 pH units. Results from a complete dose–response series [Fig. 3(a)] show a dose dependent behavior that corresponds well with results obtained on the same type of cells using the Microphysiometer.[16]

Cells can be grown on microcarrier beads using standard cell culture media and procedures. Therefore, the use of beads as the growth support (as opposed to monolayer cultures on culture plates) does not add cost or complexity to the work involved in preparing cells for the assay. Furthermore, microcarrier culturing has proved to be a method providing good growth rate and viability for most cell types.[17] Consequently, the use of beads as the growth support does not compromise the integrity of the results obtained.

2.2 Oxygen consumption

Detection of cellular oxygen consumption is achieved through measurement of the extracellular oxygen concentration, *i.e.*, by probing another quantity of the bulk solution surrounding the cells. The requirements for the O_2 detector configuration are much the same as the requirements for acid release detection listed earlier: the main concerns are restriction of the volume and movement of the extracellular solution, and the proximity of the sensing element to the cells. Consequently, the bead injection apparatus described above can be turned into an oxygen consumption sensor merely by replacing the fluorescent pH probe by a fluorescent oxygen probe. The operation of such



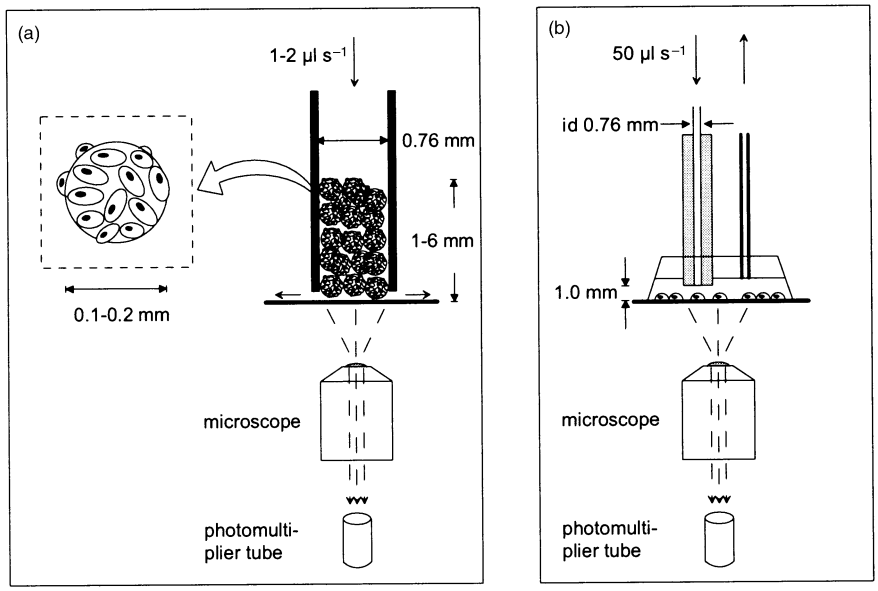


Fig. 2 The flow injection set-up. Detector configurations in the different modes of operation: (a) the jet ring flow-through chamber, used in the bead injection mode; (b) the inverted radial flow chamber, used in the traditional sequential injection mode.

a setup was recently demonstrated by studying the effects of amobarbital and carbachol on the oxygen consumption of Chinese hamster ovary (CHO) cells.[18]

3 Intracellular pH measurements by BI

It is obvious to raise the question of whether *intracellular* pH measurements could be used to quantitate metabolic acid production. All acid generated by the cellular metabolism is, naturally, produced within the cell. Since the cell membrane shields the generated acid against dilution by diffusion and convection, only very small amounts of acid should be needed to create observable changes in pH. Intracellular pH measurements in themselves are not new. They have been used extensively in different physiological experiments, particularly to study pH regulation mechanisms in cells[19–22] after artificially induced acid or alkaline loads. There are also some cases where intracellular pH has been used as a quantitative indicator of responses to chemical stimulation.[23–30] However, intracellular pH changes observed in response to chemical stimulation are not necessarily just a consequence of altered metabolism: changes in pH can be an integral part of the response cascade,[31,32] in which case the role of pH is roughly analogous to that of Ca^{2+} as a second messenger. In other words, intracellular pH seems to be related to cell responses in a more complex, or more varied, manner than intracellular [Ca^{2+}] or acid release is. It may be for this reason that intracellular pH has not been considered as a means of measuring metabolic acid production.

Recently, the use of intracellular pH for acid production measurements has been suggested. Following the example of Hodder and Ruzicka,[33] who used bead injection in intracellular Ca^{2+} assays, Lähdesmäki *et al.*[15] measured changes in the intracellular pH of CHO M1 cells when the cells were exposed to different levels of carbachol. In order to probe intracellular pH, the cells were grown on microcarrier beads and a fluorescent pH indicator was loaded into the cells prior to the experiments. The observed pH changes were found to be dose dependent, and to conform well to eqn. (1) [see Fig. 3(b)]. It was observed that intracellular pH responses are more difficult to interpret than acid release responses. On the other hand, they were also found to contain more information about the cellular pH regulation.

It should be stressed that since dilution and extracellular solution volume do not present a problem in intracellular measurements, the use of a BI setup is not required for such measurements. However, because cells can be stacked into a 1–3 mm thick plug, the BI approach does provide a signal-to-noise ratio clearly superior to traditional intracellular assays where cells are observed as a monolayer on a microscope coverslip. Further, it allows quick renewal of the observed cells, so that each assay can be performed on a fresh set of cells.

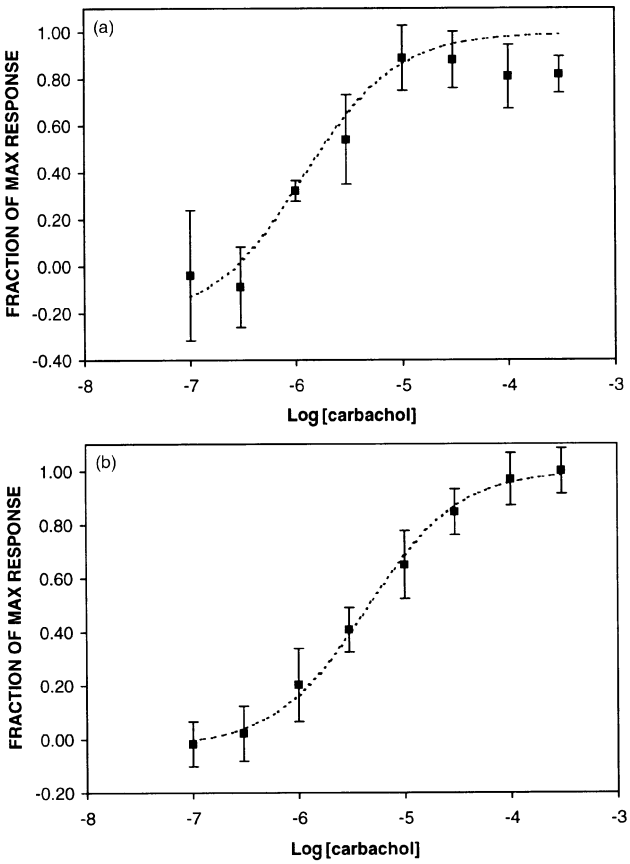


Fig. 3 Dose–response curves for carbachol action on CHO M1 WT3 cells. (a) Response evaluated by extracellular acidification rate. (b) Response evaluated by changes in intracellular pH. Reprinted from ref. 15, entitled 'Measurement of cellular stimulation through monitoring pH changes by bead injection fluorescence microscopy', Copyright 2000, with permission from Elsevier Science.

4 Utilization of precise drug delivery in metabolic measurements

4.1 Functional tracer curves

The combination of flow injection and fluorescence microscopy was first applied to cell stimulation measurements by Ruzicka *et al.*,[34] who studied Ca^{2+} release in baby hamster kidney cells as a result of carbachol stimulation. A radial flow through chamber [see Fig. 2(b)] was used to achieve swift delivery and removal of the stimulant. One of the main conclusions from their work was the importance of tracer curves in elucidating the kinetic relationship between stimulant exposure and observation of response from the cells. A tracer curve, in this case, is an injection of a fluorescent tracer chemical in place of the stimulant. The fluorescent chemical is delivered on to cells exactly as the stimulant would be and therefore the fluorescent signal from the tracer yields the precise stimulant exposure profile. Some concerns have been voiced about the reliability of tracer curves in cell response measurements: it has been suggested that although a tracer curve reports the delivered concentration profile in the bulk solution, there might be a more or less stagnant diffusion layer right next to cell surfaces where the concentration changes in a different way than it does in the bulk.

To address these concerns, a concept of functional tracer curve was devised based on injecting a bolus of a weak acid (NH_4Cl) on cells and monitoring the intracellular pH by means of a fluorescent pH indicator.[35] The idea was borrowed from physiological pH measurements in cells where injections of a weak acid or a weak base are routinely used to create an artificial alkali or acid load in the cell.[19] As the injected plug of NH_4Cl comes in contact with the cells, the lipophilic cell membrane will repel the charged NH_4^+ ions whereas the neutral NH_3 fraction will permeate the membrane. Once NH_3 enters the cell, the intracellular fluid becomes more alkaline and the alkalinization is detected as a change in the fluorescence intensity of the pH indicator. Since a signal can only be observed after NH_3 has been 'extracted' into the cell, the time dependence of the functional tracer must include diffusion through the assumed stagnant surface layer, in addition to diffusion through the cell membrane.

Comparison of a regular fluorescein tracer curve and an NH_4Cl functional tracer curve (Fig. 4) revealed that there was no noticeable time difference between their leading edges. Based on this observation, it can be concluded that the

concentrations in the bulk solution and the solution–cell membrane interfaces are the same at all times and that a regular tracer curve gives flawless information about the stimulant exposure profile.

Another functional tracer curve was developed to characterize the rates of cross-membrane transport processes and the pathways of energy metabolism. Prior to recording the curve, the cells were perfused with glucose-deficient buffer. This slows their metabolism and considerably reduces metabolic acid production. When a plug of normal, glucose containing buffer is then injected on the cells, acid production should increase as a result of the more intense metabolic activity. The ensuing decrease in intracellular pH will be detected as a change in the fluorescence intensity of the pH indicator. When the glucose containing plug is removed, an opposite change should be seen. The method was adopted to flow injection from microphysiometry,[6] where glucose starving is routinely used as a kind of instrument calibration procedure.

Comparison of the regular tracer curve and the glucose-based functional tracer showed clear kinetic differences (Fig. 5): the functional tracer was lagging slightly behind the regular tracer. One reason for the lag is that in contrast to ammonia, glucose does not enter cells by diffusion through the cell membrane but by the action of carrier proteins located on the membrane.[36] Another reason is that in order to see a change in the cytosolic pH, glucose not only needs to enter the cell but also to go through glycolysis to produce lactic acid. Consequently, the observed kinetic differences give an idea about the rates of these

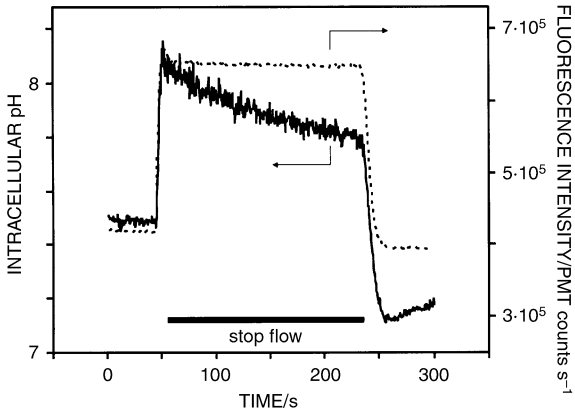


Fig. 4 Regular tracer curve (injection of fluorescein, dashed line) and functional tracer curve [injection of $(NH_4)_2SO_4$, solid line]. Reprinted from Fig. 3 in ref. 35, entitled 'Flow injection microscopy as a tool for two-parameter monitoring of cellular responses', Copyright 1998, with permission from Springer Verlag.

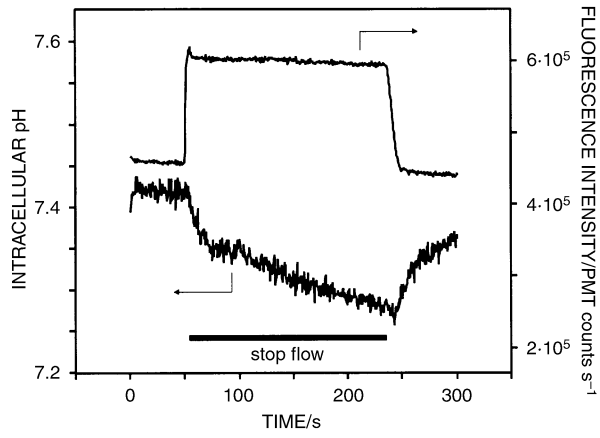


Fig. 5 Regular tracer curve (injection of fluorescein, upper curve) and functional tracer curve (injection of glucose, lower curve). Reprinted from Fig. 2 in ref. 35, entitled 'Flow injection microscopy as a tool for two-parameter monitoring of cellular responses', Copyright 1998, with permission from Springer Verlag.

two processes. Since glucose intake and lactic acid/CO_2 production are common features in all animal cells, this experiment can be used as a universal tracer curve for metabolic responses.

In conclusion, functional tracers prove the value of tracer curves in cell response measurements, and also provide opportunities for their more diverse use. The usefulness of all tracer curves, conventional and functional, is based on comparing the tracer and the cellular response, *i.e.*, comparing two separate measurements. Reliable comparison is only possible owing to the excellent reproducibility that is characteristic of the operation of flow injection systems.

5 Conclusions

The ability to measure parameters connected with the energy metabolism in living cells is important in its own right for general cell physiology. Recently, metabolic measurements have gained additional importance since it has been shown that some of them can be utilized to evaluate responses of cells to chemical stimulation.

Owing to their inherent excellent reproducibility, as well as their ability for swift and efficient solution delivery and removal, flow injection techniques lend themselves well to the development of tools for probing the energy metabolism in living cells. Bead injection, the latest in the line of flow injection techniques, has proved especially useful and provides several advantages specifically for extracellular metabolic measurements. The most important of these are: (1) a relatively large number of adherent cells (on the order of 10^4) can be packed in a small (*ca.* 1–3 μL) volume, in a closed environment, (2) rapid renewal of inspected cells, where each assay can be conducted on a fresh set of cells, and (3) the sensing element is placed right next to the cells. Combining these advantages of bead injection with the universality of fluorescence microscopy allows the construction of a platform for probing several different parameters.

Future research in the area of metabolic response measurements could, perhaps, be enhanced by the use of a fluorescent temperature probe developed by Engeser *et al.*[37] As mentioned recently,[38] the probe may make it possible to measure the metabolic activity in a cell based on the heat released in energy generation.

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