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Automation of functional assays by flow injection fluorescence microscopy

Louis D. Scampavia, Peter S. Hodder, Ilkka Lähdesmäki and Jaromir Ruzicka

Bead-injection spectroscopy is a novel technique that uses immobilized eukaryotic cells on microbeads as a renewable biosensor for fluorescence microscopy. The use of a flow injection instrument allows fast functional assays that generate full kinetic characterization of a drug. Because the cell population is automatically replaced for each assay, variability is minimized, thus allowing greater accuracy.

Increasingly, biological R&D is focusing on investigating real-time dynamic events with living cells, and fluorescence microscopy has become a standard tool in such studies. Numerous fluorescent probes are available[1–7] that enable the detection of specific components of complex biological chemistries with excellent sensitivity and selectivity. However, real-time analyses using microscopy have been restricted by three critical factors: (1) precise microfluidic control of reagent addition and removal; (2) inconsistent or irreproducible cellular responses; and (3) automation of microscopy-based experimentation.

First, poor microfluidic control of solutions in the cellular milieu can negate or even corrupt much of the intricate kinetic information generated from a cellular response. Although a variety of perfusion chambers are currently employed in microscopy, some have poorly defined flow profiles that prevent the introduction and removal of reagents in a uniform, reproducible and timely manner. Given that most cell colonies grow in an irregular fashion, media flow around the cells is unique for each experimental set-up, despite the use of a specific perfusion chamber. Moreover, standard kinetic models assume that perfusion generates an instantaneous equilibrium between a reagent (e.g. agonist) and the cellular milieu. However, this condition is frequently not achieved and a hysteresis between the perceived dosing and the biological response occurs owing to diffusion-limited instead of kinetics-limited control of the reagent.

Second, biological interactions are inconsistent owing to physiological stress and changes in the viability and variability of the cellular material. Poor flow-chamber design can induce a shear-stress response from the cells, and poor control of environmental factors at the microscope stage can also distress the cell population. Prolonged exposure to intense radiation [e.g. ultraviolet (UV) or infrared (IR) light] or cytotoxic reagents during an assay can irreversibly damage both the fluorescent probe (e.g. photobleaching) and the biological materials or cells. In both instances, this will limit the number of measurements possible on any selected colony of cells, making sequential stimulations unreliable or complicating data interpretation.

Finally, current advances in fluorescence microscopy have concentrated on the development of improved instrumentation, imaging and computational processing. Unfortunately, the automation of cell and solution handling has yet to be adequately addressed and still

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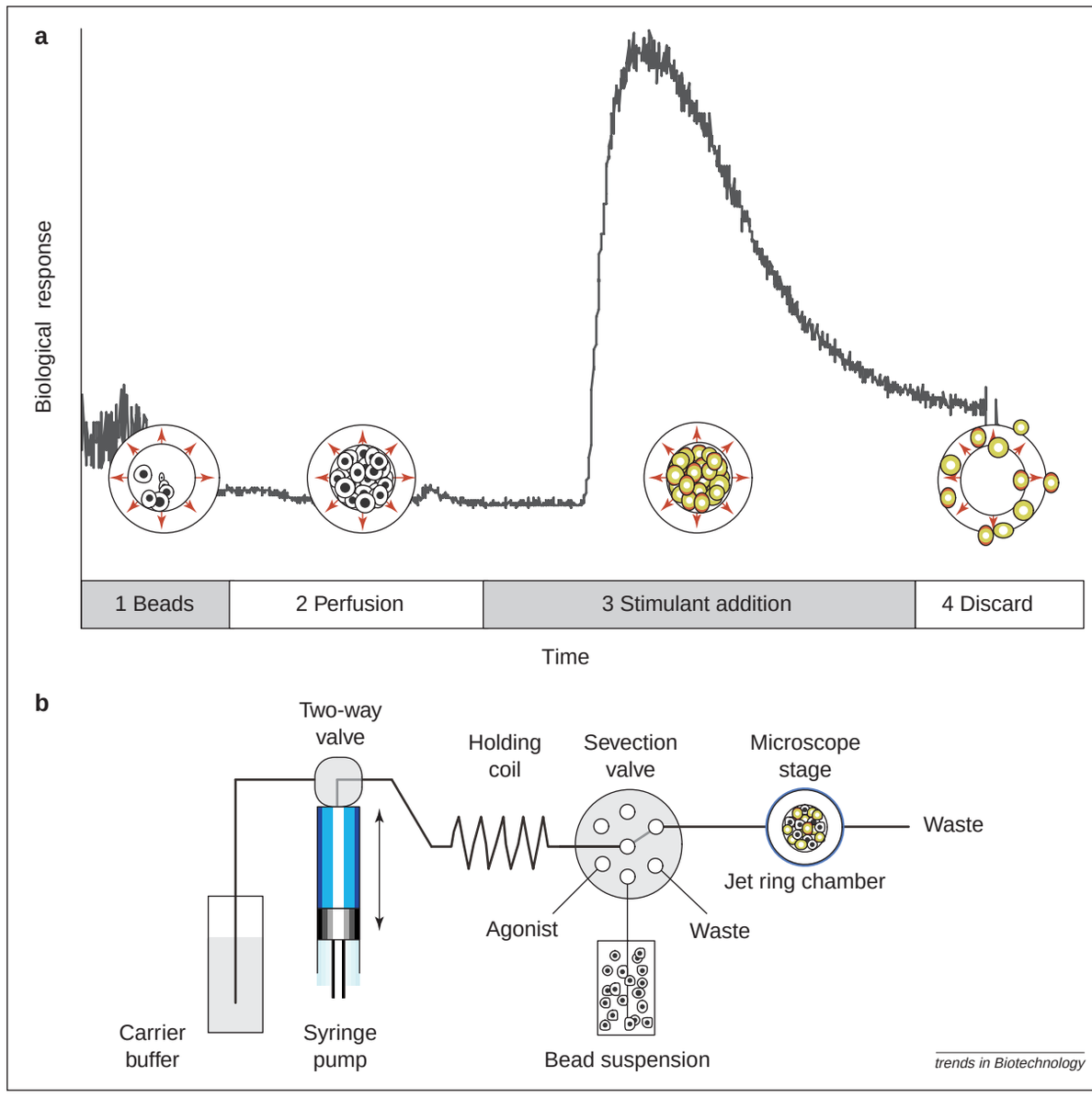

Figure 1

(**a**) The four-step protocol for bead-injection spectroscopy is illustrated relative to a typical biological-response curve from cells. These steps include: (1) the arrival and trapping of the cell-carrying microbeads in the jet-ring chamber; (2) perfusion with buffer and establishment of the signal baseline; (3) the addition of a stimulant followed by a response; and (4) automated discarding of the spent beads. (**b**) A typical flow injection fluorescence microscopy system. A computer (not shown) controls the mechanical components.

depends largely on manual or semimanual processing. In many cases, experimentation still requires the physical placement of cell cultures onto the microscope stage and the manual addition of reagents. Not only is this labor intensive but assay results can be inconsistent because of poor control in the timing of events and the haphazard introduction of reagents.

Bead-injection spectroscopy

Work on flow injection fluorescence microscopy (FIFM) addresses these critical issues and has led to the development of a novel technique known as bead-injection spectroscopy (BIS)[8]. In particular, this work is centered on the automation of drug screening and the evaluation of kinetic affinities to benefit the development of pharmacological and biotechnological products. BIS uses fluidic handling of eukaryotic cells that are immobilized on microbeads, allowing for the automated renewal of cells at the microscope stage. Because the cell population is replaceable, the microcarrier beads serve as a renewable biosensor, a critical element when these bioassays become dysfunctional or are compromised with time. Robustness of the biosensor for multiple assays is thus not a serious issue[8].

BIS is based on the analytical technique known as flow injection (FI), in which all fluidic manipulation of reagents and suspended microbeads are carried out with an FI instrument and the experimental procedure is fully automated. As the FI system brings the stimulant and cells together in a kinetically well-controlled manner, a standard fluorescence microscope can be used to monitor biological interactions in real time. The use of beads also confines the perfusion flow in a predictable fashion, allowing for reproducible reagent addition.

Moreover, the concentration profile of an injected reagent can be determined accurately by the addition of a reagent that does not interact with cells but that can be monitored through time to generate a tracer (concentration profile) curve. Although BIS has been used in a variety of assays, the governing protocol remains the same for all experiments.

Protocol

Fluorescence-based assays using BIS are performed sequentially as follows (Fig. 1a).

- A representative volume of the cell or bead suspension is aspirated and subsequently injected into a microscope perfusion chamber (e.g. jet-ring chamber[9]), in which the beads are trapped to form a microcolumn.
- The cells are perfused with a physiological buffer and the basal cellular activity is measured to establish a spectrophotometric baseline.
- A sample solution containing a stimulant or reagent is injected into the flow cell, where the resulting biological response can be monitored.
- Finally, the used beads and their immobilized cells are automatically discarded from the flow cell at the end of an assay cycle.

This sequence of events can be automatically repeated as required using the same or new reagents. The entire assay procedure is executed by a flow injection system (Fig. 1b). Fluidic handling and data collection is orchestrated by computer control using commercially available software. However, reproducible assays are dependent on the consistency of the bead packing, the stability of the bead layer and the overall condition of the cell line.

The advantages of BIS

The ability to monitor cell reactions on beads not only allows automated handling of both solid and liquid materials but also provides several other distinct advantages. Cellular responses can be immediately monitored as the stimulant or reagent is introduced, providing temporal information about the initial onset of the response and its rate, overall intensity and duration. The BIS system can also simultaneously monitor stimulant dissociation or elution events and cellular recovery or hysteresis to recovery[10].

Sophisticated experimentation is made possible through automation, which can allow the characterization of complex relationships through a stepwise elution using a strategic selection of auxiliary reagents. A significant advantage of using beads as the cell carrier is that a representative sampling from the cell population can be drawn from the bead suspension. Because each injection of a stimulant reacts with a fresh portion of cells, the observed responses have been shown to be very reproducible[10,11]. The elimination of biological variability within a series of measurements allows for accurate comparisons between stimulants or reagents[10,11].

Functional assays

Ligand or reagent binding to a cell receptor site is not indicative of a subsequent physiological response. The identification and characterization of drug candidates require that the efficacy of a drug be determined. Such assays are referred to as functional assays and are designed to classify a drug as an agonist or antagonist, depending on whether a biological response is invoked or inhibited. Several different functional assays have been carried out using various cell lines and fluorescent probes[12]. BIS has proved to be an ideal tool for automating functional assays and two examples of its use are highlighted below.

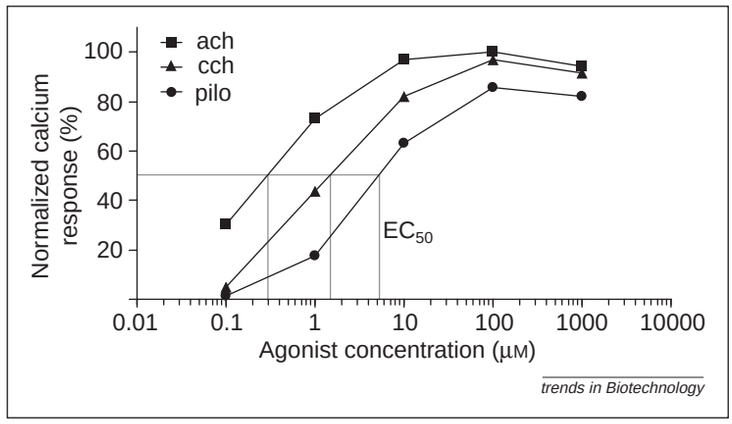

Figure 2

Dose–response curves of different muscarinic receptor agonists have been constructed using bead-injection-spectroscopy data[10]. The dose concentration that elicits half of the full cellular response is known as the 50%-effective concentration (EC_{50}). This value is an important benchmark for comparisons. The EC_{50} of an agonist can be determined quickly, as shown by the dashed lines. Abbreviations: ach, acetylcholine; cch, carbachol; pilo, pilocarpine.

Calcium functional assays

A model experiment based on the functional assay of muscarinic receptor agonists with chinese hamster ovary (CHO) cells illustrates the utility of BIS[10]. The CHO cells selected for this research express type-1 muscarinic acetylcholine receptors (M1)[13], which signal through a G-protein-coupled pathway. To make the cells suitable for assay in the BIS format, they are cultured on microbeads, incubated with the fluorescent intracellular Ca^{2+} probe fura-2 and then suspended in a vial. The BIS instrument automatically aspirates and traps a small volume of this suspension into a jet-ring chamber[9], then delivers agonist to the trapped beads. The jet-ring chamber is a specialized detection-cell design that mechanically traps and releases beads while detecting signals with an inverted fluorescence microscope. After recording the resultant Ca^{2+} stimulation response, the beads are flushed to waste and the assay is repeated with a fresh batch of beads. Using this method, the pharmacological properties of muscarinic receptor agonists are measured. Dose–response curves constructed from the BIS assay can discriminate the partial agonist pilocarpine from the full agonists acetylcholine and carbachol (Fig. 2).

The kinetics of biological response can also be elucidated with BIS techniques[10]. For example, the raw data from the above experiment show dose-dependent differences in the rate of Ca^{2+}-response onset for the different concentrations of the same agonist and the same concentration of different agonists (Fig. 3). These differences result from the complex relationship between the agonist's affinity for the receptor, the

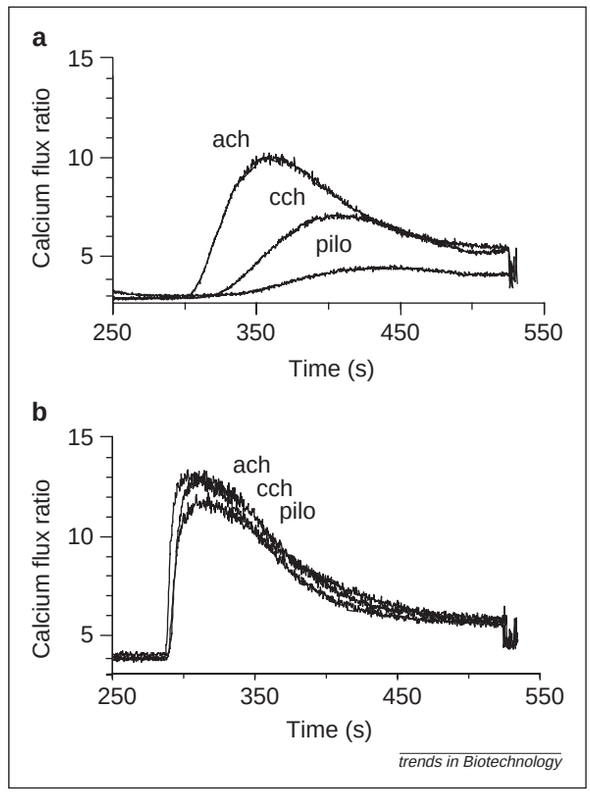

Figure 3

Actual kinetic data obtained from a bead-injection spectroscopy assay using a flow injection fluorescence microscope[10,11]. The overall efficacy of three different muscarinic receptor agonists can be monitored in real time. In low doses (**a**), the agonists vary in the time elapsed before response onset, their rate of response and the overall intensity of the response. However, at a higher dosage (**b**), the agonists become less distinguishable by the cells' response, illustrating the importance of accurately controling the concentration flow profile. Abbreviations: ach, acetylcholine; cch, carbachol; pilo, pilocarpine.

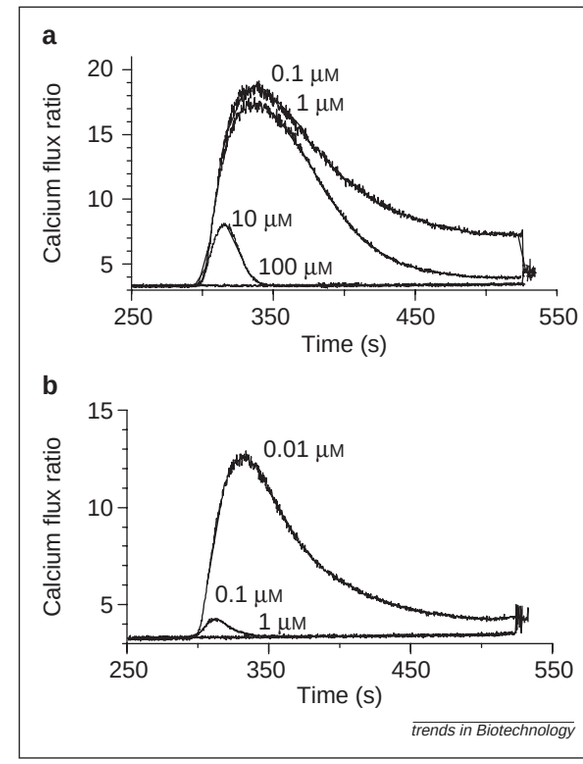

Figure 4

Bead-injection spectroscopy is used to measure muscarinic receptor antagonism in real time[11]. The carbachol-induced intracellular-Ca^{2+} response is attenuated by the presence of antagonists: pirenzepine (**a**) or atropine (**b**). The concentrations of pirenzepine required to diminish the carbachol stimulation are higher than those of atropine.

kinetics of the cellular response and the concentration of agonist present in the jet-ring chamber[10,14,15].

Another BIS assay measures the influence that pirenzepine, a muscarinic receptor antagonist, has on the Ca^{2+} response of the CHO cells. This is achieved by assaying a series of samples in which each successive sample is a mixture of carbachol at a constant concentration (10 μM) and a varying concentration of pirenzepine[11] (Fig. 4a). As the sample is delivered to the trapped cells, the carbachol and pirenzepine compete for receptor sites on the cell surface in real time. The results of this assay show an increase in the Ca^{2+}-response decay that is dose dependent. Using the same assay parameters, BIS can measure the efficacy of atropine, another muscarinic receptor antagonist. Atropine extinguishes the Ca^{2+} response at a lower concentration, thus exhibiting its higher affinity for the muscarinic receptor (Fig. 4b)[11].

pH functional assays

In the energy metabolism of any living cell, the generation of acidic products (e.g. lactic acid, CO_2) is part of the metabolic process. Consequently, acid extrusion and intracellular pH measurements can be used to study those events that perturb the rate of cellular metabolism, such as stimulation of cells by a chemical agent[16]. Intracellular pH measurements have been carried out by staining cells with a fluorescent pH-indicator, such as 2′,7′-bis-(2-carboxyethyl)-5/6-carboxyfluorescein (BCECF, a mixture of -5- and -6- isomers), and monitoring the pH-dependent fluorescence by means of a fluorescence microscope or a spectrofluorimeter[17,18]. Alternatively, acid-extrusion measurements have been made with a Cytosensor Microphysiometer®, a specialized instrument based on potentiometric pH detection[16].

One advantage of the BIS technique is that the intracellular pH or the acid extrusion can be measured with the same instrument[19]. Intracellular pH is probed by growing cells on beads and then loading them with an intracellular fluorescent pH indicator. The FI instrument is then used to deliver the beads to the jet-ring chamber, where they are trapped and packed into a microcolumn. The cells are exposed to a stimulant and the change in fluorescence intensity is proportional to the actual change in pH.

By comparison, acid extrusion can be probed by first immobilizing an insoluble fluorescent indicator on the microbeads and then growing cells on these labeled beads. The beads are again handled by the FI instrument in a similar fashion and the response is monitored through fluorescence detection. Consequently, no instrument modifications are needed to switch between intracellular pH measurements and acid extrusion measurements. The only difference between these two

measurement modes is the placement of the indicator, either inside the cells or outside and adjacent to the cells' outer membrane.

An example of this is shown by the changes in intracellular pH and acid extrusion when CHO M1 cells are stimulated with 100 μM carbachol (Fig. 5). Exposure to carbachol results in subtly increased acid extrusion, as well as in a transient acidification of the cytosol. Both changes have been found to be dose dependent, allowing the construction of a dose–response curve and the subsequent determination of the efficacy of carbachol as a stimulant of the CHO M1 cell line[19]. Carbachol was found to produce a 50% effective concentration (EC_{50}) value of 4 μM from the BIS measurements of intracellular pH. By comparison, an EC_{50} value of 1 μM was measured from the acid-extrusion experiments using the BIS system. These values compare well with reports of carbachol having an EC_{50} value of 3 μM, based on Cytosensor Microphysiometer® measurements[20].

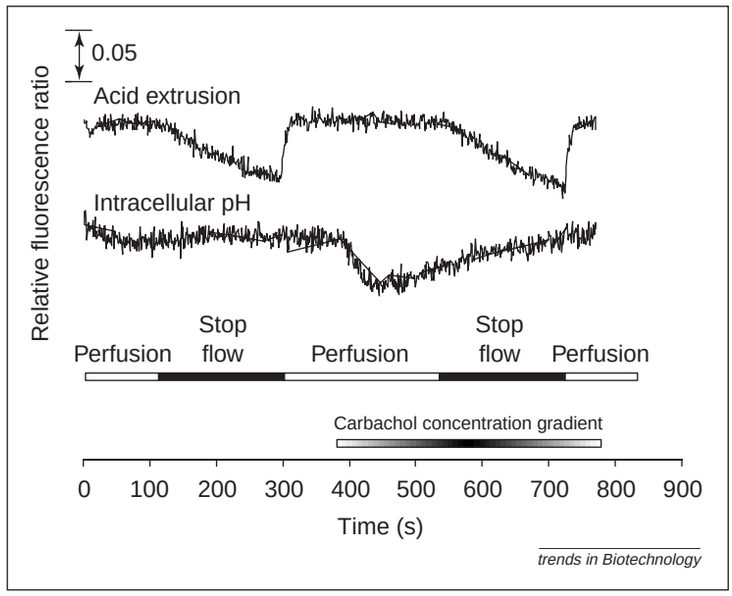

Figure 5

Response of chinese hamster ovary M1 cells to 100 μM carbachol in terms of intracellular pH and acid extrusion[19]. The top bar shows the flow injection protocol; the bottom bar indicates the concentration exposure to carbachol, with darker shading representing higher concentrations. The 'stop flow' step allows the extracellular acidification rate to be monitored in the acid-extrusion experiment.

Future outlook

One advantage of using cell-carrying microbeads is that a renewable biosensor is now possible for microscopic purposes, in which multiple assays using fresh cells allow a significant improvement in assay repeatability. The FIFM apparatus provides fast functional assays with full kinetic and temporal drug characterization. This kinetic information includes dose-dependent variances in the response onset, rate, maximum intensity, duration and possible hysteresis. Consequently, a variety of computational analyses are possible from a single assay. Moreover, BIS is based on the response of a population of cells, resulting in a better signal-to-noise ratio and less variability in the data.

As demonstrated above, functional assays can have different meanings for the definition of a cellular response. For example, the efficacy of the agonist carbachol was found to be dependent on the biological pathway investigated. Cytosolic-Ca^{2+}-flux assays produced an EC_{50} value of 1 μM, whereas intracellular pH and acid extrusion assays produced EC_{50} values of 4 μM and 1 μM, respectively. With minimal procedural modifications, the BIS–FIFM system demonstrates how assays can be tailored to provide the pharmacological information that is most relevant to the cell-line, agonist and physiological or biochemical response required.

BIS is a truly versatile technique that provides users of fluorescence microscopy with a plug-and-play system for automation that is simple to master and adapt for a variety of analyses. In particular, we envisage its use in measuring bioligand kinetics (association and dissociation rates) with receptors on living cells. This technique could also be exploited to estimate the number and types of receptors found on a population of cells using standard fluorescence-based immunoassays. Central to all BIS applications is that no instrumental modifications are needed: only changes in the chemistry are required to apply BIS technology to several different problems.

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