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Flow cytometry for high-throughput, high-content screening

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Flow cytometry is a mature platform for quantitative multi-parameter measurement of cell fluorescence. Recent innovations allow up to 30-fold faster serial processing of bulk cell samples. Homogeneous discrimination of free and cell-bound fluorescent probe eliminates wash steps to streamline sample processing. Compound screening throughput may be further enhanced by multiplexing of assays on color-coded bead or cell suspension arrays and by integrating computational techniques to create smaller, focused compound libraries. Novel bead-based assay systems allow studies of real-time interactions between solubilized receptors, ligands and molecular signaling components that recapitulate and extend measurements in intact cells. These new developments, and its broad usage, position flow cytometry as an attractive analysis platform for high-throughput, high-content biological testing and drug discovery.

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Abbreviations

β2AR	β2 adrenergic receptor
FPR	formyl peptide receptor
GFP	green fluorescent protein
GPCR	G-protein-coupled receptor
HT	high throughput
VS	virtual screening

Introduction

Modern drug discovery involves testing of cellular targets against millions of potentially valuable compounds that may bind cellular receptors to effect clinically therapeutic cellular responses. Flow cytometry is a sensitive and quantitative platform for the measurement of particle fluorescence, and is capable of high content characterization of diverse compound bioeffects at the single cell level. Thousands of different assays have been successfully performed on cells or particles, ranging from single-

parameter endpoint assays to complex assays involving multiple cell populations or simultaneous analysis of multiple responses. Flow cytometry has been historically used in a largely manual low-throughput mode, working with individual samples. Here, we briefly overview salient features of this analysis platform that make it so attractive for large-scale biotesting, and outline some of our recent efforts to advance its automation and more efficient application to the drug discovery process.

High-sensitivity measurements in homogeneous, no-wash assays

Flow cytometry enables discrete measurements of optical signals (e.g. fluorescence and light scatter) from single particles such as cells or beads. Sample particles are hydrodynamically focused into a laminar flow so that single particles pass through a laser beam sequentially. The subsequent optical signal characteristics are recorded in real time. A flow cytometer is sensitive to a fluorescent molecule concentration as low as 10–100 pM, enabling hundreds to thousands of molecules to be detected on a cell or bead. Because of the optical configuration, the laser in a flow cytometer only excites a very small volume of the sample fluid immediately surrounding the cell. This diminishes the background signal by limiting excitation of excess, unbound fluorescent molecules in solution. Therefore, homogeneous, no-wash assays are possible in which measurements may be made without the need for removing the excess fluorescent tag to distinguish between free and bound probes [1].

High content measurements

Content in discovery research can be based on performing multiple measurements at a single time. Flow cytometry enables the simultaneous quantitative analysis in individual cells of multiple optical markers of biochemical expression or physiological response. It is thus an inherently high-content measuring methodology. For example, by judicious use of multi-spectral fluorescent tags, multiple cell subsets may be distinguished within a heterogeneous cell mixture, and each can be probed for a quantitative phenotype (e.g. receptor expression and ligand binding), all in a single cell sample. Specially configured research instruments may simultaneously measure up to 14 optical parameters [2*]. A multiplex bead-based suspension array technology has evolved to maximally exploit this feature of flow cytometry [3*]. In one implementation, beads are color coded with varying levels of two spectrally distinct dyes to produce a series of unique bead sets [4]. Each set is engineered to perform a separate assay. A probe labeled with a third fluorescent dye is subsequently used to quantify the assay response

on each bead set. In the most advanced commercial implementation of this technology, as many as 100 ligand-binding assays may be possible in a single sample (<http://www.luminex.com>).

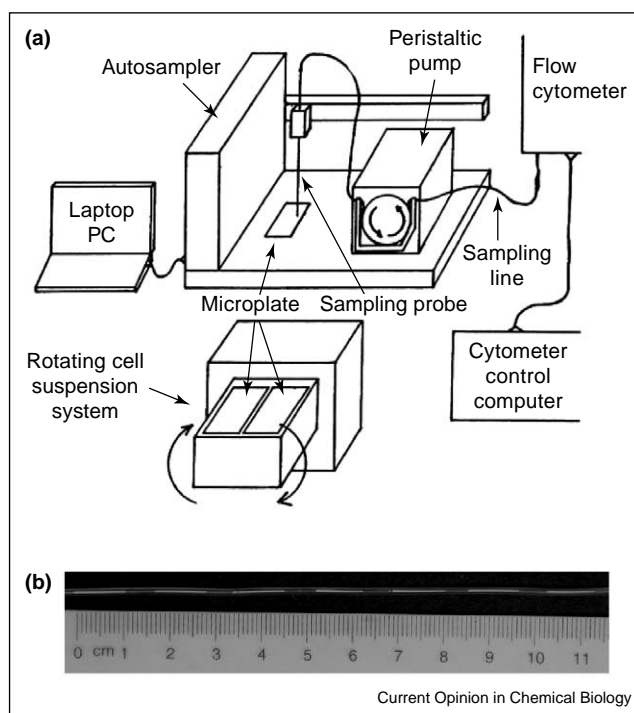
Recent evolution of sample handling in high-throughput flow cytometry

With respect to the serial analysis of individual cells or beads, the flow cytometer has always been considered a high-throughput (HT) analysis instrument. It is now a routine task to analyze from thousands to tens-of-thousands of particles per second. However, flow cytometry has been severely limited in throughput rates for the analysis of multiple discrete samples of cells. Commercial automated sample handling systems are capable of processing about two samples per minute. This is a significant limitation when the objective is to screen a large collection of compounds against replicate cell samples. Over a period of several years, we have evolved two successive generations of sample handling technology for HT flow cytometry to address this issue. The first, plug flow cytometry, uses a reciprocating multiport flow injection valve to execute up to 10 endpoint assays per min, 4 on-line mixing experiments per min and, in secondary screens, a 15-point concentration gradient of soluble compound in ~ 2 min [5–8]. The second generation technology [9], designated HyperCyt[®], uses instead a peristaltic pump in combination with an autosampler to boost endpoint assay performance to rates in excess of 1 sample/sec. HyperCyt[®] has since been more fully developed and characterized as described below.

HyperCyt[®]

The HyperCyt[®] system [9,10^{••}] interfaces a flow cytometer and autosampler (Figure 1a). As the sampling probe of the autosampler moves from one well to the next of a multi-well microplate, a peristaltic pump sequentially aspirates sample particle suspensions from each well. Between wells, the continuously running pump draws a bubble of air into the sample line. This results in the generation of a tandem series of bubble-separated samples for delivery to the flow cytometer. Sample and bubble volumes are determined by the time that the autosampler probe is in a microplate well or above a well intaking air. Commercial alternatives are as much as 30 times slower due both to differences in the way samples are delivered and data are acquired. In HyperCyt[®], the air bubble-separated samples are delivered in a continuous stream to the flow cytometer. Likewise, the data are collected in a continuous stream, with the accumulated data from all wells of a microplate representing a single data file. The time-resolved data, with periodic gaps corresponding to the passage of the sample-separating air bubbles, are analyzed by proprietary software (FCSQuery) developed by one of the authors (BE).

Figure 1

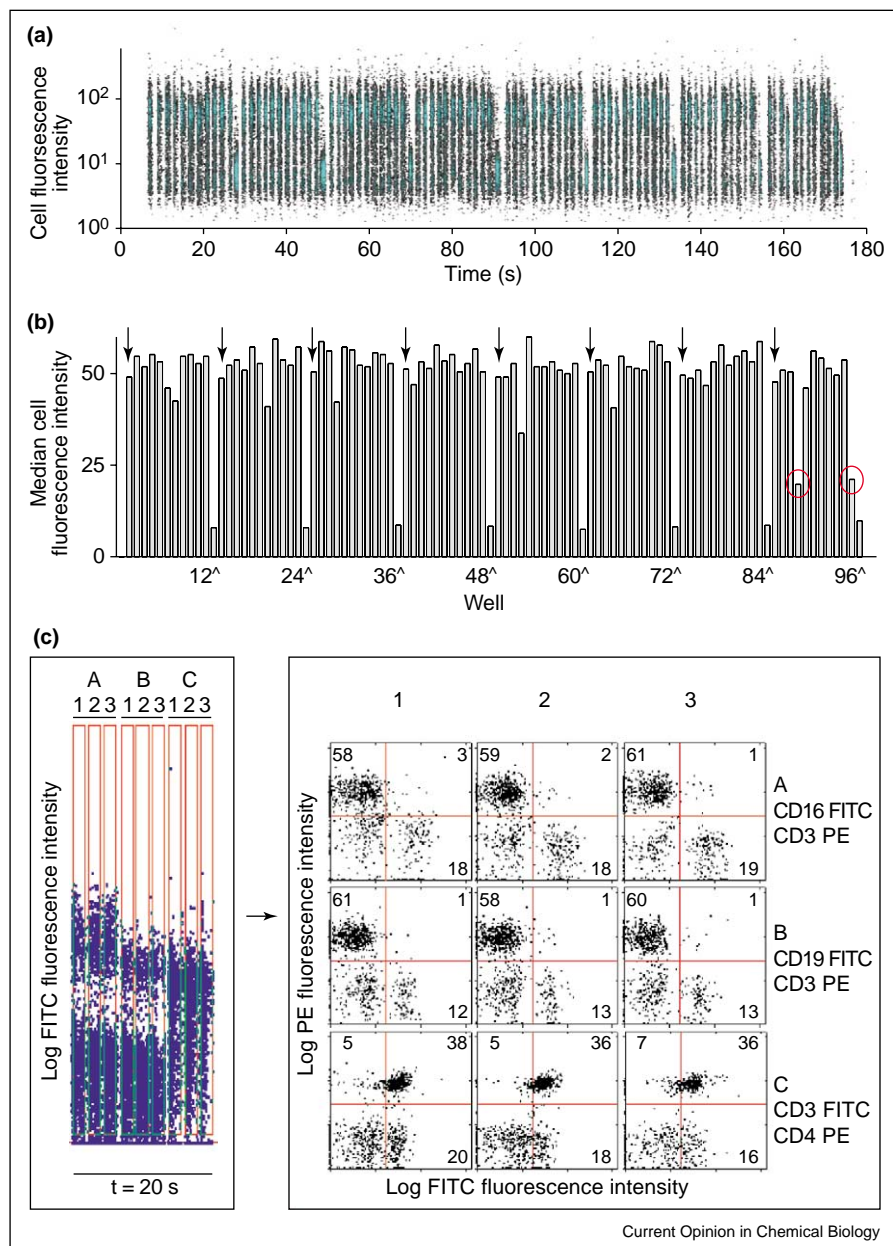


HyperCyt[®]. (a) Configuration for use in endpoint assays. Samples from microplate wells are aspirated by a sampling probe and transported to the flow cytometer through a flexible sampling line. An autosampler moves the probe from well to well while a continuously running peristaltic pump causes the alternating uptake of fluid samples and air bubbles. A rotating cell suspension system periodically inverts microplates to keep sample particles (cells or beads) in suspension during any required pre-analysis incubation steps. (b) A series of fluid samples in transit to the flow cytometer in the sampling line. Each fluid volume (white) is a sample from a unique microplate well. Air bubbles separate individual samples to prevent particle carryover between samples and to facilitate discrimination of individual samples in the time-resolved flow cytometry data. In this example, each sample spans ~ 1 cm in the sampling line which corresponds to a volume of $\sim 0.5 \mu\text{l}$.

HT endpoint assays, the fast track

Maximum throughput rates are achieved when HyperCyt[®] is applied to the analysis of endpoint assays. This is due to the inherently low level of particle carryover (see below) that allows sequential sampling of wells without an inter-sample probe rinse step. A current HyperCyt[®] system application of this type is the screening of combinatorial chemical libraries to detect small molecule ligands for the human formyl peptide receptor (FPR). In a representative 96-well assay, compounds are assessed for the ability to block binding of a fluorescently-tagged high-affinity peptide ligand to cell membrane FPR. The resulting 96 time-resolved clusters of data (Figure 2a), collected in less than 3 min, are analyzed to determine the median fluorescence intensity of peptide bound to cells from each well (Figure 2b). The assay response range is defined by replicate control wells containing unlabeled blocking peptide (100% block, numbered wells at bottom)

Figure 2



High-throughput endpoint assays with HyperCyt[®]. **(a)** Time-resolved cell fluorescence data from a 96-well microplate in a screening assay for small-molecule FPR ligands. Each data cluster represents fluorescent ligand binding to cells from a separate well. Blue-green color indicates high cell density. **(b)** Median fluorescence intensity of cell fluorescence in each well from data illustrated in (a). In control wells, fluorescent ligand binding is blocked with a 100-fold excess of unlabeled ligand (numbered wells at bottom) or is not blocked (arrows at top). The red circles indicate compounds that block peptide binding by 70% or more. **(c)** Immunophenotyping. Left, one parameter output vs time. Right, replotted high content two-parameter output.

or buffer (0% blocked, arrows at top). Test compounds that block peptide binding by 70% or more are considered 'hits' (circles), subject to further evaluation. The assay is homogeneous in that cells, compounds and fluorescent peptide are added in sequence and the wells are subsequently analyzed without intervening wash steps. Ligands with inhibition constants (K_i) ranging from nanomolar

to tens-of-micromolar have been detected with this assay (BS Edwards, unpublished data).

We have validated cell-based HT endpoint assays for ligand binding, surface antigen expression, immunophenotyping and cell adhesion [10^{••}]. For example, at analysis rates of 1.5 s/sample a multi-parameter (high content)

lymphocyte immunophenotyping microassay in 96-well plates provided immunofluorescence results comparable to manual analysis (Figure 2c) [10^{••}]. Accurate quantitative measurements have been demonstrated over a four-decade range of fluorescence intensity using input cell concentrations of 1–20 million/ml and source well volumes of 5–15 μ l. Typical sample volumes of 1–2 μ l allow scarce quantities of test cells or reagents to go a long way.

On-line microfluidic mixing for analysis of soluble compounds

We have described two novel microfluidic mixing approaches for use in conjunction with HyperCyt[®]. These approaches have been characterized with fluorescent cell calcium responses to sampled peptides as well as with a novel bead-based assay (biotin displacement of fluorescein-tagged biotin on streptavidin beads) that provides a fluorescence response analogous in time and kinetics to a cell calcium response [11]. The first approach uses the pulsatile delivery of the HyperCyt[®] peristaltic pump to generate mixing of particles with fluid compounds sampled from microplate wells [12]. The second approach uses a 100 micron magnetic ‘flea’ contained within the HyperCyt[®] delivery tubing and a magnetic stirrer [13[•]]. Soluble compounds can be sampled from microplate wells and processed at rates up to 10 samples/min [13[•]].

HyperCyt[®] cell sorting extends microfluidic mixing

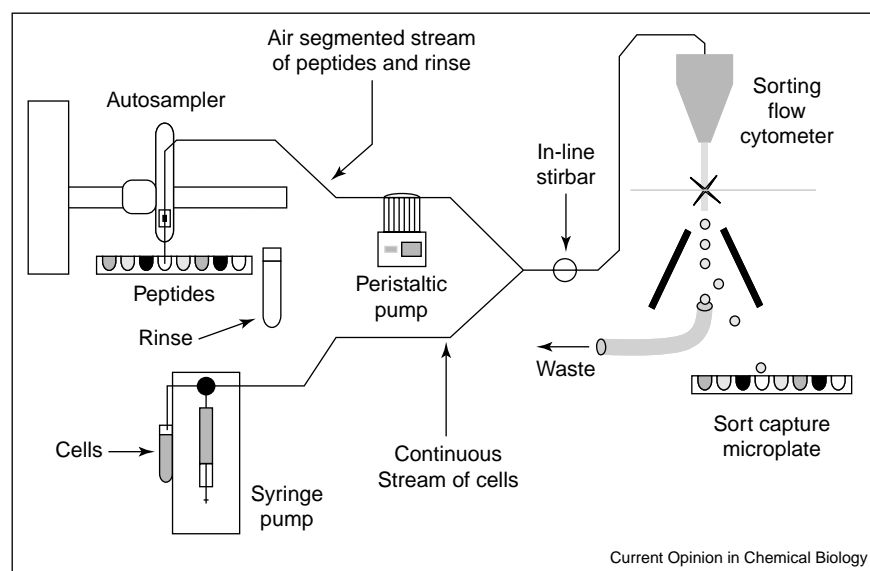
We interfaced HyperCyt[®] with a MoFlo flow cytometer and achieved on-line mixing combined with sorting of

responding cells (Figure 3). A relatively long sample aspiration time (10 s) followed by aspiration of a series of inter-sample rinse volumes (3 for 3 s each) enabled screening of cellular FPR responses at a rate of 3 peptide concentrations/min with \sim 10 000 cells analyzed for each concentration. The rinse cycle between samples was essential to minimize fluid carryover of the soluble test ligands, an important consideration in on-line mixing assays (see below). When cells highly responsive to peptide were collected by sorting, and expanded in culture for 12 to 30 days, the sort-purified cells showed enhanced sensitivity to low peptide concentrations and more sustained responses to all stimulatory peptide concentrations compared with the unsorted cell population from which they were derived [14[•]]. This screening approach enables identification and isolation of individual cells with a responsive phenotype, allowing subsequent analysis of the conferring genotype (e.g. to rescue and identify transfected genes from a genetically diverse DNA library). A major technical advance, derived from related fluidics studies [15], changed the configuration of a flow nozzle to be compatible with the air bubbles between samples created by HyperCyt[®].

Sample carryover

HyperCyt[®] is a microsystem where microfluidic dimensions are at work in transporting samples to the flow cytometer. The HyperCyt[®] engineering team has adapted microfluidic principles to the problems of mixing and carryover [12,13[•],16[•]]. Mixing in HyperCyt[®] requires breaking laminar flow and can be performed at sample

Figure 3



HyperCyt[®] configuration for mixing and sorting to isolate ligand-responsive cells. An air-segmented stream of peptides, sampled from microplate wells by an autosampler, joins a continuously flowing stream of cells at a ‘Y’ junction. The two conjoined streams are mixed by a rotating in-line micro-stirbar *en route* to the flow cytometer. Cells with elevated intracellular Ca^{2+} are sorted into microplate wells and expanded in culture for subsequent reanalysis. Reproduced from [14[•]] with permission. Copyright © 2004 by Sage Publications, Inc.

delivery rates >1 sample/sec. However, separation of samples by air bubbles in microfluidics leads to two types of carryover, of differing significance. Particle carryover from sample to sample is minimized by bubbles because particles tend to travel in the middle of the stream. Practically speaking, in an endpoint assay where the assay contents are premixed and developed in the well, a sample is contaminated with ~1% of the particles from the prior sample. For higher stringency, a rinse volume can be easily added between samples at the cost of reducing throughput. Of greater significance, particularly for on-line mixing assays is fluid carryover. In this case, the assay (such as a cell response to a drug compound) develops on-line and the compound from each sample is left behind as a thin film along the wall of the sample line. We have recently developed a fluid carryover model for predicting inter-sample rinse cycles to optimize the balance between carryover and sample throughput in the flow cytometer (JW Bartsch *et al.*, unpublished data). Minimizing fluid carryover presents an engineering opportunity to design a microsystem with optimal surface area and composition to achieve on-line mixing assay throughput approaching that of endpoint assays.

Integration of virtual screening

In an academic research group such as ours, resources for large scale, HT screening operations are somewhat more limited than in biotech or pharmaceutical companies. Here, rather than brute force screening of vast combinatorial chemical libraries, it is much preferred if not essential to perform targeted screens on smaller focused libraries. Compound subsets may be identified by virtual screening (VS) (e.g. by computational docking of ligands onto crystal structures). A limitation of working with most G-protein-coupled receptors (GPCRs), including the FPR, is the absence of crystal structures. We have therefore adopted an alternate approach. By homology modeling of rhodopsin, the only mammalian GPCR successfully crystallized to date, we generated a pharmacophore model for the FPR that predicted the existence of distinctive agonist and antagonist binding sites (CG Bologa *et al.*, unpublished data). Using this model, we have screened *in silico* a large (>450 000) small-molecule library from Chemical Diversity Laboratories, Inc (<http://www.chemdiv.com>) to produce a library subset enriched with putative FPR-active compounds. In initial HT flow cytometry screening of a different, structurally random collection of 880 off-patent drugs and alkaloids from Prestwick Chemical Inc. (<http://www.prestwickchemical.com>), we observed a hit rate of only 0.1%, comparable to the industry average for HT screening. By contrast, we achieved hit rates more than an order of magnitude higher in the physical screening of the compound collection pre-selected by VS (BS Edwards, *et al.*, unpublished data). We anticipate these initial screening results will enable further refinement of the model for improved future VS efficiency.

The challenge of informatics

To fully realize the potential of flow cytometry to meet the challenges of throughput, and content, informatic systems need to be integrated with the data collection efforts. Our group has provided persuasive evidence that acquiring a multi-well plate of samples as a single file is the right approach to data acquisition where each cluster of events represents the contents of a single well [9,10^{••},12,13[•],14[•]]. This challenge is extended by multiplexing, where each cluster of events might represent 10 or more assays. In addition, the data need to be accurately tracked back to the contents of the well, each well potentially containing a member of a chemical library. The data then need to be output in a format compatible with cheminformatic analysis of the assay result, the activity of the compound. These issues are a source of current progress in our continuing development of HT flow cytometry systems and methodologies.

Bead-based approaches to unravel GPCR complexity

GPCRs are the biggest superfamily in the human genome, representing more than 800 proteins (http://www.gpcr.org/htmls/entries_org.html) organized in five families [17], that transmit extracellular signals into cells via intracellular G-protein heterotrimers [18]. Each GPCR could couple to as many as 1020 G-protein heterotrimers (27 α , 5 β , and 12 γ subunits), relatively few of which are characterized. Between 40% and 50% of the currently marketed drugs target GPCRs [19,20], an indication of the potential for many new important targets among uncharacterized GPCRs. We have developed several bead-based platforms for use with soluble GPCRs that have permitted probing of functional GPCR molecular assemblies with flow cytometry. Our initial efforts in this area involved the creation of a series of FPR constructs and characterization of their interactions with G-protein beads, beads derivatized with chelated nickel and displaying hexahistidine-tagged G-protein heterotrimers [21[•]]. We used a FPR-GFP (green fluorescent protein) construct to study ternary complex for a family of ligands, a FPR- α 2 fusion protein for exploration of kinetic disassembly mechanism and $\alpha\beta\gamma$ affinity, and the wild type receptor and FPR-GFP on beads as sensors for receptor availability in solution [21[•]]. In a recent extension of this approach to the β 2-adrenergic receptor (B2AR), we derivatized beads to discriminate B2AR-GFP assemblies sensitive to full and partial agonists and antagonists [22^{••}]. The dose-response curves of ternary complex formation revealed maximal assembly for ligands previously characterized as agonists and reduced assembly for ligands previously characterized as partial agonists. We also used two types of derivatized beads in a duplex (the simplest form of multiplex) assay to simultaneously determine whether a test compound was an agonist, antagonist or inactive [22^{••}]. This assay format is clearly compatible with HT small-volume drug discovery [23].

Conclusions and future challenges

Flow cytometry is rapidly evolving as a platform for automated HT high content measurement of compound bioeffects in cell- and bead-based systems. In the HyperCyt[®] system, we have demonstrated the automated, accurate analysis of endpoint assays at rates of 40–60 samples/min, on-line mixing assays at up to 10 samples/min, and mixing assays in conjunction with cell sorting (10 000 cells screened per assay) at 3 samples/min. Homology-based VS techniques have been implemented to improve physical screening hit rates, effectively achieving increases in assay throughput in the order of magnitude range. Multiplexing of cell- and bead-based assays offers the promise of additionally increasing throughput by a similar factor (or more) (see also Update). However, there are several challenges that remain to allow HyperCyt[®] to perform at high rates for extended periods in a discovery environment. Bead-based systems are in progressive development to enable systematic studies of functional GPCR molecular assemblies, an approach anticipated to accelerate the comprehensive understanding of GPCR diversity. The introduction of soluble GPCR molecular assemblies in this arena has the potential of opening the door for other membrane molecules, and other signaling and response pathways. We believe these flow cytometric approaches may prove to be superior both in understanding assemblies and mechanisms and, when combined with HyperCyt[®] and VS, in small molecule discovery. Moreover, these approaches have the potential to replace radioligands and to outstrip the sensitivity and throughput of plasmon resonance spectroscopy. Because flow cytometers are widely available in nearly all research institutions, the biological and instrumental platforms reviewed here should be readily accessible to a broad spectrum of investigators.

Update

A novel cell-based multiplexed screening approach has been recently reported in which fluorescent color-coded cells expressing different GPCRs were simultaneously screened for intracellular Ca²⁺ responses to a library of compounds [24]. The cell mixtures were sampled and delivered to the flow cytometer by a proprietary automated technology and analyzed as time-resolved data files in a fashion similar to that used by the HyperCyt[®] system. The screening strategy discriminated agonists, partial agonists and antagonists.

Acknowledgements

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