High-throughput and ultra-high-throughput screening: solution- and cell-based approaches

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The trend towards assay miniaturization for high-throughput and ultra-high-throughput screening continues to spur development of homogeneous, fluorescence-based assays in higher density, smaller volume microplate formats. Recently, first-generation microfluidic devices have been designed for performing continuous-flow biochemical and cell-based assays. These devices provide orders-of-magnitude reduction in reagent consumption, and offer the potential for implementing high-throughput screening in formats that integrate up-front compound handling with unique assay functionality.

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Abbreviations

FCS	fluorescence correlation spectroscopy
FP	fluorescence polarization
FRET	fluorescence resonance energy transfer
HTRF	homogeneous time resolved fluorescence
HTS	high-throughput screening
SPA	scintillation proximity assay

Introduction

Over the past decade, a variety of scientific advances and economic pressures have driven the need for improved drug discovery screening technology [1,2]. These include the growing number of potential therapeutic targets emerging from the field of functional genomics, the rapid development of large compound libraries derived from parallel and combinatorial chemical synthesis techniques, and the ever increasing pressure to reduce development costs while enhancing commercial competitiveness in the pharmaceutical industry. Recent estimates of the number of individual genes in the human genome (~10,000) and the number of unique chemical structures theoretically attainable using existing chemistries (~100 million) suggest that up to 10^{12} assays would be required to completely map the structure-activity space for all potential therapeutic targets [1].

The demand for screening large compound collections against an increasing number of therapeutic targets has stimulated technology development in the areas of assay automation, miniaturization, and detection methodologies. Assay throughput was initially addressed by adoption of the 96-well microplate format and the use of liquid-dispensing and plate-handling robotics for automation [2]. Although this assay format is still widely used, many screening groups are presently moving towards 384-well and higher density, smaller volume plate formats [3,4]. Typical 'high-throughput' screening (HTS) programs in the pharmaceutical industry now operate at throughput rates on the order of 10,000 compounds per assay per day [2], with some laboratories working at 'ultra-high throughput' rates in excess of 100,000 assays per day.

The trend towards assay miniaturization arose simultaneously with the move towards automated HTS as a direct result of the need to reduce development and operating costs. Miniaturization reduces the amount of biological and chemical reagents used per assay, and facilitates higher throughput assay formats by enabling the use of parallel sample processing and multiplexed detection modes. Two general approaches towards further assay miniaturization are being actively pursued: firstly, plate-based systems evolving towards higher well densities and smaller well volumes; and secondly, microfabricated devices designed to perform continuous flow assays.

The goals of automation, miniaturization, and high throughput place an increased emphasis on the development of homogeneous assay formats and the use of high sensitivity detection techniques. Homogeneous 'mix and measure' assays are well suited for HTS as they avoid filtration, separation, and wash steps that can be time-consuming and difficult to automate. Fluorescence-based detection techniques for performing homogeneous assays are becoming increasingly prevalent due to the inherent sensitivity of fluorescence measurements and the industry-wide move away from the use of radioisotopes, although scintillation proximity assays are widely used for many enzyme inhibition and receptor-binding assays.

This review summarizes recent advances and results in homogeneous biochemical (enzyme inhibition and receptor–ligand binding assays) and cell-based approaches to assay miniaturization and HTS. See Hill *et al.* [5] and Zysk *et al.* [6] for additional information on these topics. Screening methodologies based on phage-display techniques or heterogeneous assay formats (e.g. ELISA, filter-binding assays, agar plate assays [7], etc.) have been excluded.

Solution-based biochemical assays

Homogeneous biochemical assays in miniaturized formats are most frequently carried out using scintillation proximity assay (SPA) or fluorescence detection techniques because of the requirement for increased sensitivity as assay volume shrinks. The choice of detection technology employed is dependent on the particular class of assay target being investigated. Binding assays for cell-surface receptors are often carried out using SPA techniques because the high binding affinities and low receptor

densities involved require that the assay be run at very low labeled probe concentrations, whereas fluorogenic enzyme substrates and fluorescent indicators for calcium and other ions are frequently used in enzyme and cell-based assays, respectively. Miniaturized assay formats also require increased emphasis on proper assay design to ensure robustness, reproducibility, and statistical significance of the assay results [8].

The use of radiolabeled ligands and filtration assays to measure high-affinity binding interactions for cell-surface receptors has largely been replaced by SPA [2,9,10] in HTS applications because the need for a separation step is eliminated. The technique relies on the excitation of a scintillant incorporated into microbeads or microplates upon binding of a radiolabeled ligand to a receptor immobilized on the surface of the bead or plate. Radioactive decay in close proximity to the scintillant-containing surface results in emission of light. Isotopes that produce low energy emission particles that are re-absorbed over short distances (e.g. ^{3}H or ^{125}I) are typically used as the radiolabel to ensure that only ligand bound to the surface is detected [10]. Examples of recent HTS applications based on SPA and related techniques include screening for CCR5 chemokine receptor agonists and antagonists as potential anti-HIV lead compounds [11], identification of ligands for the 12 kDa FK506 binding protein [12], quantitation of cAMP production mediated by activation of seven transmembrane domain receptors [13], and the direct incorporation of a homogeneous binding assay with solid-phase synthesis of small molecule libraries [14•].

Fluorescence-based detection methods are inherently sensitive due to the short duty cycle of most fluorophores (the fluorescence lifetime of fluorescein is ~4 ns) and consequently high emitted photon fluxes that can be achieved even with modest excitation light sources. This property, combined with the variety of different fluorescence modes that can be exploited to advantage in homogeneous assay formats, makes fluorescence detection highly amenable to many HTS applications. The fluorescence techniques most widely used in homogeneous screening assays include fluorescence resonance energy transfer (FRET), fluorescence polarization (FP), homogeneous time resolved fluorescence (HTRF), and fluorescence correlation spectroscopy (FCS).

FRET is the non-radiative transfer of energy between appropriate energy donor and acceptor molecules. Efficient transfer of the donor's excited state energy by this mechanism requires that several spectroscopic and spatial criteria be satisfied. These include significant overlap between the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor, alignment of the transition dipole moments of the donor and acceptor, and an intermolecular separation distance that is small compared to the Forster distance, the separation distance for a given donor–acceptor pair (typically tens of angstroms) at which the energy transfer efficiency is 50% [15]. The efficiency of energy transfer varies inversely with the sixth power of the distance between donor and acceptor, so small changes in intermolecular spacing (e.g. by a few angstroms) can produce large changes in transfer efficiency. This phenomenon has been exploited effectively in the design and synthesis of FRET-based fluorogenic enzyme substrates. Typically, a short peptide corresponding to the sequence for a natural cleavage site of the enzyme is synthesized and labeled at opposite ends with appropriate donor and quencher molecules. In the intact substrate, the donor and quencher are held in close proximity so that the efficiency of non-radiative energy transfer is high and the effective fluorescence quantum yield of the donor is significantly lower than that for the free fluorophore. Enzymatic activity can be monitored by measuring the change in observed fluorescence intensity as the substrate molecule is cleaved by the enzyme and the donor and quencher drift apart. Examples of recent applications include the use of digital imaging for parallel processing of a fluorescent protease assay in 96-well and miniaturized plate formats [16], FRET assays for hepatitis C virus proteinase inhibitors [17], mapping of the S′ specificity of serine proteases [18•], and a competitive assay system for identification of DNA conjugates that bind to single-stranded DNA in which fluorescence is generated by displacement of a fluorophore-tagged DNA strand from the quencher-labeled target strand [19].

FP measurements allow one to measure changes in the rotational diffusion coefficient of small labeled probes upon binding to larger molecules (see Jameson and Sawyer [20] and Nasir and Jolley [21•] for recent reviews). FP is calculated from measurements of the parallel and perpendicularly polarized components of the emitted light intensity when the sample is excited using polarized light. When a binding equilibrium is established between a fluorophore-labeled ligand and a larger molecule, the observed polarization increases. The technique thus provides a rapid and homogeneous analysis of receptor–ligand binding interactions, enzyme-catalyzed hydrolysis of labeled substrates, protein–protein interactions, and so on. Examples of recent applications to HTS include an FP assay for binding to estrogen receptor [22], and an FP competition immunoassay for tyrosine kinases [23].

Sensitivity in fluorescence-based assays is often limited by background signal arising from assay reagents or containers. This problem can sometimes be overcome by using time-resolved techniques. Fluorescence lifetimes of rare earth chelates (e.g. europium) are often milliseconds in duration, whereas background fluorescence due to reagents and plastics is typically on the order of nanoseconds. Use of pulsed excitation sources and gated detectors (or phase modulation techniques) thus allows the background fluorescence to decay before sampling of the fluorescence signal of interest takes place. HTRF is a hybrid technique that takes advantage of the long

fluorescence lifetimes of europium crytates and the large apparent Stokes shift (the difference between the peak excitation and peak emission wavelengths of a fluorophore) obtained by exploiting energy transfer between the europium donor and suitable acceptors [24,25]. Examples of recent applications of HTRF to screening applications include tyrosine kinase assays (see Kolb *et al.* [26••] for a recent review), studies of IL-2–IL-2R interactions [25], and screening against a novel tumor necrosis factor receptor in a 384-well microplate format [27].

One of the emerging detection techniques for HTS applications is FCS (see Auer *et al.* [28••] for a recent review). Typically, these measurements are carried out using confocal optics to provide the highly focused excitation light and background rejection required for single molecule detection. Confocal optical systems are intrinsically amenable to miniaturization because the output signal is dependent on the detection volume (typically femtoliters) and concentration of fluorescent probe, rather than on the total assay volume. At nanomolar tracer concentrations, only a few fluorophore molecules are present in the detection volume at any given time, giving rise to temporal fluctuations in signal as molecules diffuse in and out of the signal collection region. Molecules of different size, and hence different diffusion coefficient, remain in the detection volume for different lengths of time. Smaller molecules yield shorter fluorescence pulses; larger molecules yield longer fluorescent pulses. Autocorrelation analysis of these time-dependent fluorescence signals provides information about the diffusion characteristics of the fluorescent particles. As binding of a fluorescent probe to another molecule results in a change in its effective diffusion coefficient, the technique can be used to monitor binding interactions as well as other molecular events. A similar analysis of flourescence intensity fluctuations (fluorescence intensity distribution analysis [FIDA]) yields information on changes in fluorophore quantum yield or spectral shift, and can also be used to monitor binding events when the binding interaction influences these properties. Measurement times of ~1 s are required to acquire enough data to carry out the autocorrelation analysis. Present limitations of the technology include difficulties in integration with microplate handling systems because of the short working distances of the microscope objectives typically used, and the stringent optical requirements placed on the fabrication of the microplates themselves. Recently, Koltermann *et al.* [29] described the application of dual-color FCS to carry out homogeneous assays for restriction endonucleases (EcoRI, BamHI, SspI, and HindIII) in a simulated high-throughput screening mode.

Chemiluminescence (the process of photon emission upon decay of an unstable chemical intermediate) [30,31] and electrochemiluminescence (the process of photon emission upon decay of an unstable chemical intermediate associated with certain electron transfer reactions) [32] techniques offer the potential for achieving detection sensitivities rivaling those obtained with radioisotopes by virtue of the absence of scattered excitation light and background fluorescence inherent in other fluorescence-based techniques. Electrochemiluminescence seems particularly promising because reagents can be regenerated and thus sustain high photon flux rates over longer periods of time. Examples of recent screening applications include an assay for acetylcholinesterase activity [33].

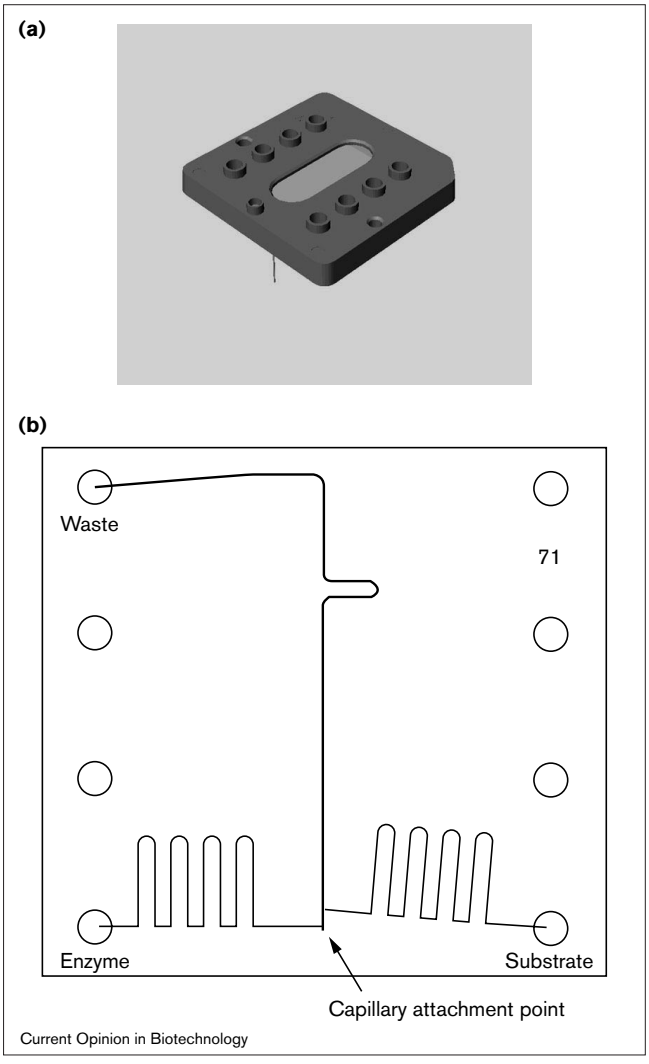
Cell-based assays

Cell-based assays for HTS fall into three broad categories: second messenger assays that monitor signal transduction following activation of cell-surface receptors; reporter gene assays that monitor cellular responses at the transcription/translation level; and cell proliferation assays to monitor the overall growth/no growth response of cells to external stimuli.

Second messenger assays typically involve measurements of fast, transient fluorescent signals that occur on the timescale of seconds. A variety of fluorescent molecules have been described that respond to changes in intracellular Ca^{2+} concentration, membrane potential, pH, and so on, and have been used to develop second messenger assays for receptor stimulation and ion channel activation [34••,35••]. Screening methodology for ion channel drug discovery has been aided recently by the development of hydrophobic voltage-sensitive probes and FRET-compatible microplate instrumentation [36].

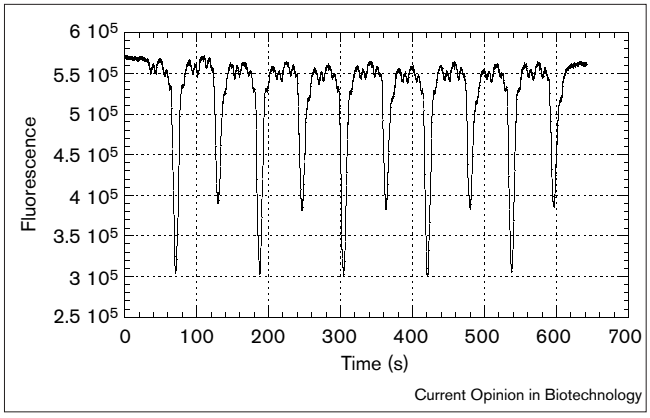
Reporter gene and proliferation assays typically involve incubation steps of several hours, followed by colorimetric, fluorescent, or luminescent read-out. Overall throughput is increased by running many assays in parallel and optimizing the throughput of the detection step. Reporter gene techniques involve splicing the gene for an enzyme or green fluorescent protein into the cell line of interest in such a way that activation of the target gene results in expression of the reporter gene product as well, which can then be used as part of a colorimetric, fluorescent, or luminescent read-out of gene activation. An example of a luminescent reporter gene read-out would be coexpression of luciferase to catalyze the light-emitting luciferin reaction. Dias *et al.* [36] have developed a reporter gene assay for steroid receptor ligands using a translational fusion of Cre recombinase and the ligand-binding domain of the human glucocorticoid receptor that was transfected into mammalian cells along with a loxP/luciferase reporter gene. The recombinase function was dependent on ligand binding to the reporter, and Cre-mediated recombination resulted in constitutive expression of luciferase. Other examples of recent reporter gene applications include a luciferase reporter gene assay for detection of protein kinase C inhibitors [37,38], and a dual-luciferase reporter gene assay for measuring translational coupling efficiencies of recoding mechanisms, such as frameshifting or readthrough [39]. In the latter example, a recoding test sequence was spliced between renilla and firefly luciferase

Figure 1



(a) A microfluidic device for running continuous-flow enzyme inhibition assays. Test compounds are injected via a fused-silica capillary attached to the microchip. **(b)** Schematic layout of a continuous-flow enzyme inhibition assay chip.

Figure 2



Continuous-flow enzyme inhibition data for a phosphatase assay. Fluorescence was monitored downstream from the mixing point for enzyme and substrate as a series of inhibitor and blank injections were carried out using a 20 s injection cycle. Percent inhibition is calculated from the difference between the steady-state baseline and substrate-only fluorescence background, and the amplitude of the change in fluorescence signal corresponding to the presence of an inhibitor in the sample injection plug.

reporter genes, and the ratio of the resulting luciferase activities was used as a measure of the efficiency with which the ribosome made the transition from one open reading frame to the next.

Many scientists are turning to imaging techniques to extract higher information content (e.g. spatial distribution of protein expression) from their screening assays. Conway *et al.* [40] recently described quantification of G-protein-coupled receptor (GPCR) internalization using a GPCR–green fluorescent protein hybrid. Carrithers *et al.* [41] have developed a melanophore-based screening assay for erythropoietin receptors. Miraglia *et al.* [42] describe a laser scanning system for imaging the bottom of microplate wells to carry out homogeneous microvolume cell- and bead-based assays for HTS.

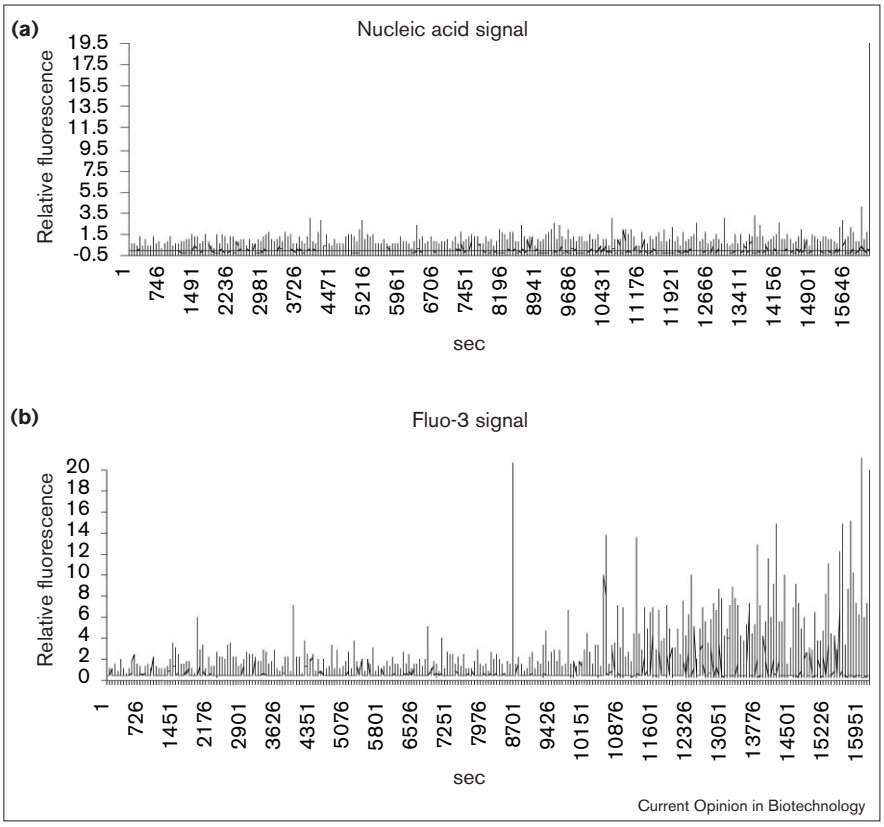
Bedard *et al.* [43] recently described a colorimetric cell proliferation assay for human cytomegalovirus (HCMV) antiviral susceptibility adapted to microtiter plate format. In this assay, virus-induced cytopathic effects on cell viability and proliferation were monitored by following the reduction of the tetrazolium salt WST-1 to a water soluble formazan product, which is directly quantified by measuring the absorbance at 410 nm.

Trends in miniaturization: microplates versus microfluidic chips

Although at present most HTS is still carried out in 96-well plate format, the move towards 384-well and higher density plate formats is well underway [2–4,44]. Instrumentation for accurate, low-volume dispensing into 384-well plates is commercially available, as are sensitive plate-readers that accommodate this format. A variety of assay types have now been demonstrated in 384-well plates [16,45]; however, reformatting of 96-well compound plates into the higher density format can become a significant bottleneck to implementing screens in this mode [1].

Progress continues to be made with higher density plate formats as well. Dias *et al.* [36] have implemented their recombinase/luciferase reporter system for use in 864-well plates. As few as 560 cells per assay well were sufficient to measure dose-response curves for ligand binding to the glucocorticoid receptor. Maffia *et al.* [46] carried out luciferase reporter gene assays in human T cells using a 1,536-well (3 microliter) plate format. Mere *et al.* [47] describe FRET-based biochemical and cell-based assays carried out in a 3,456-well format. Recently, Oldenburg *et al.* [48] described a 9,600-well (0.2 microliter) assay system in which both

Figure 3



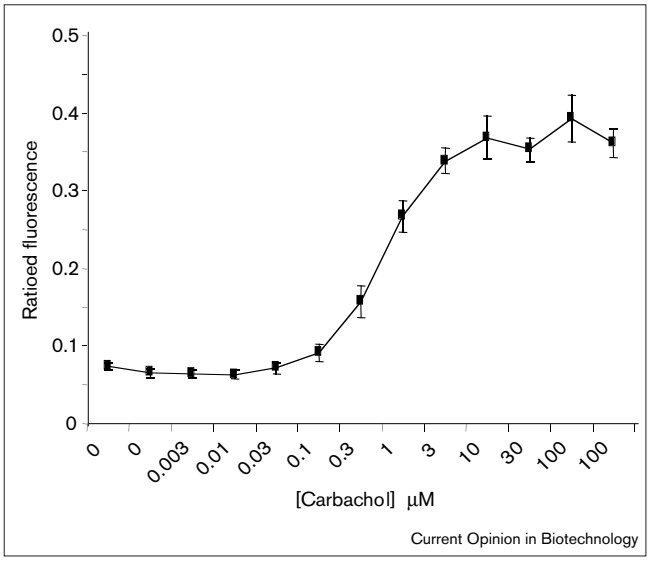
Continuous-flow assay data for a fluorescence-based Ca^{2+} flux agonist assay carried out in a microfluidic device. Cells were preloaded with the Ca^{2+}-sensitive indicator Fluo-3 and a nucleic acid stain. 40 microliters of m1-WT3-CHO cells at 10 x 10^6 cells/ml were added to the on-chip reagent well. Carbachol was sampled from a microplate every 30 seconds. **(a)** The fluorescence signal arising as nucleic-acid-stained cells flow past the detector. **(b)** The fluorescence signal arising from the intracellular Ca^{2+}-sensitive dye Fluo-3.

combinatorial and discrete compound libraries were screened against a matrix metalloproteinase target.

The evolution and implementation of microplate-based screening in smaller volume, higher density formats (1,536-well plates and beyond) faces a number of technical hurdles. The need to control evaporation in open systems having high surface area to volume ratios becomes increasingly important as well volumes are reduced. Some assays may be difficult to implement in these formats due to sensitivity to final dimethylsulfoxide concentration and the limitations inherent in present-day low-volume liquid-dispensing technologies.

The recent development of microfabricated fluidic devices for carrying out biochemical and cell-based assays has the potential for triggering a complete paradigm shift in the evolution of HTS technology, and is currently being actively pursued in both academic and industrial laboratories. Microchannels with dimensions in the 10–100 micrometer range are fabricated in glass or fused-silica substrates using photolithography and etching techniques borrowed from the semiconductor industry. These devices make use of electrokinetic pumping and hydrodynamic pressure to control nanoliter-scale fluid flow, and offer the potential for achieving orders-of-magnitude reduction in reagent consumption, parallel sample processing for higher throughput, implementation of unique assay read-outs, and improved data quality. The feasibility of a variety of different assay types has been demonstrated in prototype

Figure 4



Continuous-flow assay data for a fluorescence-based Ca^{2+} flux assay. The ratio of Fluo-3 : nucleic acid fluorescence signals from Figure 3, averaged over 30 s intervals is plotted. Each point represents data collected for 50–100 cells.

devices, including binding assays [49,50•], enzyme assays [51,52•,53•], and cellular assays [54,55].

At Caliper Technologies Corporation (Mountain View, CA), first-generation microchip devices that integrate direct compound sampling from microplate sources with continuous-flow enzyme inhibition assays have been designed and tested. An illustration of one such device is shown in Figures 1a and b. Test compounds are injected via a fused-silica capillary attached to the microchip, mixed with the target enzyme and fluorogenic enzyme substrate, and allowed to react as the mixture flows along the main reaction channel. Typical injection volumes range from 1 to 4 nanoliters using sample injection cycles of 10–30 seconds. Reaction of the enzyme with fluorogenic substrate yields a baseline fluorescence signal that exhibits a transient decrease if the test compound inhibits enzyme activity (Figure 2). Prototype systems are currently being tested that allow the user to load the microchip with reagents and carry out screening runs of several thousand compounds.

Development efforts are presently focused on improving the robustness of the system and moving new types of assays into the continuous-flow microchip format. Kinase assays based on electrophoretic separation of reaction products from substrates, and cell-based assays using Ca^{2+}-sensitive fluorescent indicators and 50–100 cells per data point (Figures 3 and 4), are two additional examples of assays that have been successfully demonstrated in feasibility experiments. New microchip designs that integrate liquid-handling and compound dilution with assay functionality (e.g. designs that allow the user to sample directly from a dimethylsulfoxide source and carry out compound dilution in the chip) offer the potential for eliminating many of the microplate preparation steps that presently create operational bottlenecks in screening laboratories.

Conclusions

The need to increase the throughput of drug-discovery screening operations while reducing development and operating costs is continuing to drive the development of homogeneous, fluorescence-based assays in miniaturized formats. The use of 384-well and higher density plates and commercially available plate-handling robotics has made HTS a reality, and has allowed some screening groups to achieve ultra-high throughput rates in excess of 100,000 samples per day. In parallel with the drive towards higher throughput and miniaturization, there is increasing emphasis on assay design and information content to ensure that the resulting data provides reliable guidance in the drug development decision making process.

The evolution of microplate technology towards higher density, smaller volume formats continues to advance. This strategy may, however, be approaching practical limits inherent in the use of conventional liquid-dispensing technology and current microplate fabrication processes, and because of the difficulty of controlling evaporation in open systems as well volumes get smaller.

The development of new microchip-based screening technologies represents a 'quantum leap' in assay miniaturization. The combination of nanoliter-scale liquid-handling, integrated devices for compound dilution and assay functionality, electrophoretic assay read-out, and state-of-the-art fluorescence detection techniques has the potential to revolutionize the drug discovery screening process.

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- •• of outstanding interest

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