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Microchip-based systems for target validation and HTS

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Microarray and microfluidic device technologies for performing genetic and biochemical analyses are revolutionizing biological research. These technologies are now being applied to gene expression profiling and to primary screening for target validation and lead discovery in the pharmaceutical industry. In this article, we briefly review microchip technology and discuss future development trends.

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▼ Over the past decade, the trend towards miniaturization of chemical analysis systems and the rise of the 'lab-on-a-chip' concept has generated a great deal of interest in the scientific community and popular press[1–3]. Microfabricated analytical devices offer significant potential advantages over standard laboratory instrumentation, including design flexibility and dimensional precision, reduced reagent and power consumption, and multiplexed device design for parallel sample processing[4]. Microchip technologies for biological and pharmaceutical research fall into two general categories: microfabricated arrays of biopolymers for performing solid-phase binding or hybridization experiments[5,6]; and microfluidic devices for performing high-throughput biochemical or cell-based assays[7,8].

Microfabricated array technology

Microfabricated peptide arrays for use in drug discovery experimentation were first described by Fodor *et al.*[9] in 1991. A combination of photolithography and solid-phase peptide synthesis was used to create spatially addressable arrays of peptides for use in multiplexed receptor–ligand binding assays. Similar approaches have since been used to create miniaturized arrays of other biomolecules, such as oligonucleotides and proteins, for applications ranging from DNA sequencing[10,11] and gene expression profiling[12,13] to characterization of the protein complement of cells[14]. Because each site in the microarray consists of a unique oligonucleotide, peptide or protein, these technologies provide a means for conducting highly parallelized experimentation in a highly miniaturized, solid-phase assay format. Sample preparation steps (e.g. amplification and labeling of target nucleic acid molecules), performing the hybridization or binding experiment, and subsequent analysis of the array using isotopic or fluorescence-based imaging techniques are typically carried out manually or in a semi-automated fashion.

Microfluidic device technology

In contrast to microarray technology, microfluidic devices are typically designed to move gases or fluids through a series of process steps to perform solution-phase chemical analysis. A microfabricated gas chromatograph that included a sample injector, separation column and thermal conductivity detector was described by Terry and colleagues in 1979 [Terry, S. *et al.* (1979) A gas chromatographic air analyser fabricated on a silicon wafer. *IEEE Trans Electron Devices* ED-26, pp. 1880–1886]. A variety of other functional components has subsequently been demonstrated in microchips, including microfabricated pumps, valves, mixing ports and filtration devices[15–17]. Scientists are now attempting to incorporate individual fluid-handling components into integrated microfluidic circuits to expand microchip functionality and simplify common laboratory procedures. Recent applications have included microchip devices designed to perform electrophoretic separations of proteins and DNA (Refs 18–20), biochemical and immunoassays[21–24], cell-based assays[25,26], cell-sorting[27,28], PCR reactions[29,30], microfiltration[31], diffusion-based separations[32], and sample injection for mass spectrometry[33–36].

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Application of microchip technologies to pharmaceutical research

The application of microchip technologies to target validation and HTS are areas in which the potential benefits of microchip technology combine well with the needs of the pharmaceutical industry. The effort to screen large compound libraries against the growing number of therapeutic targets emerging from the field of functional genomics is increasing economic pressure to reduce research and development costs, and is driving innovation in drug discovery technology[37,38]. Much of the technological development has been focussed in the areas of assay automation and miniaturization. Miniaturization reduces reagent consumption and facilitates higher-throughput assay formats by enabling the use of parallel sample-processing and multiplexed detection modes. Most HTS is presently carried out in 96-well microplates using robotic plate-handling and liquid-dispensing technology[39], with the microplate format evolving towards higher well-densities and smaller well-volumes[40,41].

Microchip technologies offer alternative approaches to miniaturization, with microarray applications providing the potential for increased parallel experimentation, and microfluidic devices reducing reagent consumption by orders-of-magnitude. The development and commercialization of these technologies is a multidisciplinary undertaking and requires expertise in fields ranging from microfabrication and microfluidics, to surface chemistry and assay development. Here, we briefly review the current status of microchip technology as applied to pharmaceutical research, and discuss future development trends.

Box 1. Company websites

Aclara Biosciences
www.aclara.com
Affymetrix
www.affymetrix.com
Axon Instruments
www.axon.com
BioRobotics
www.biorobotics.com
Caliper Technologies
www.calipertech.com
Cartesian Technologies
www.cartesiantech.com
Cellomics
www.cellomics.com
Ciphergen Biosystems
www.ciphergen.com
Corning
www.corning.com
Eksigent Technology
www.eksigent.com
GeneLogic
www.genelogic.com
General Scanning
www.scanarray.com
Gyros Microlab
www.gyrosmicro.com
Hyseq
www.hyseq.com
Majer Precision
www.majerprecision.com
Mergen
www.mergen-ltd.com
Nanogen
www.nanogen.com
Orchid Biosciences
www.orchid.com
Packard Instruments
www.packardinst.com
Research Genetics
www.researchgenetics.com
Sequenom
www.sequenom.com
Sleicher and Schuell
www.s-und-s.de
TeleChem International
www.arrayit.com

Microarray fabrication

Two distinct approaches have been taken to create miniaturized arrays of peptides, proteins and oligonucleotides on solid supports: (1) immobilization of pre-synthesized or purified molecules; and (2) *in situ* synthesis of peptides or oligonucleotides[5,6]. Both approaches generally require the selection and preparation of appropriately functionalized solid supports (e.g. glass slides that have been derivatized using various silane chemistries) to provide appropriate linker molecules, and chemically reactive groups (typically hydroxyl, amine or carboxylic acid functional groups) that serve as starting points for chemical coupling reactions. One of the key technical challenges to successful implementation of microarray technology has been the preparation of derivatized substrates with uniform surface properties and low nonspecific binding. A variety of prepared slides are now commercially available for researchers who wish to create their own microarrays. For example, Corning (Corning, NY, USA) (Box 1) has recently introduced CMT-GAPS™ amino-silanized slides and TeleChem International (Sunnyvale, CA, USA) offers glass slides derivatized with both organoaldehydes and organoamines to provide a choice in coupling chemistries for attaching oligonucleotides. Sleicher and Schuell (Dassell, Germany) manufacture FAST™ slides, which are coated with a microporous polymer that binds DNA non-covalently but irreversibly.

Immobilization of pre-synthesized or purified molecules

Immobilization of pre-synthesized or purified molecules is carried out either by nonspecific adsorption to the substrate surface or by covalent attachment. Small droplets of the capture-probe set are applied to the substrate surface using quill pin spotters [spotting instruments available from Cartesian Technologies (Irvine, CA, USA) or Biorobotics (Cambridge, UK) and quill pins available from TeleChem or Majer Precision (Tempe, AZ, USA)] or inkjet dispensing technology [Cartesian Technologies, Packard Instruments (Meriden, CT, USA)] to create an array having a unique capture probe at each immobilization site. Covalent immobilization of the probe set is typically carried out by the removal of a chemical protecting group from the derivatized support, followed by the application of droplets of chemically activated molecules. Spotting techniques work well for low- to medium-density arrays (10–10,000 probes per cm^2) but are less suitable for high-density arrays (10,000–100,000 probes per cm^2) owing to the limited spatial resolution of liquid dispensing or spotting technologies, and the wetting properties of the support surfaces.

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Synthesis of peptide or oligonucleotide arrays

In situ synthesis of peptide or oligonucleotide probes is usually performed by spatially selective deprotection of the amino-terminus (for peptides) or 5′-terminus (for oligonucleotides) of the functionalized support, using physical masking or photolithography, followed by coupling with activated building blocks (e.g. amino acids or phosphoramidites). Successive cycles of selective deprotection and chemical coupling are used to create arrays of probes where the oligomer sequence at each synthesis site is determined by the order in which the deprotection masks and activated building blocks are applied. Although photolithographic techniques require photolabile chemical protecting groups, the high spatial resolution achieved make this the method of choice for producing high-density arrays. Fabrication of microarrays containing as many as 96,600 oligonucleotide probes has recently been described in the literature[42].

Commercially available microarrays

Affymetrix (Santa Clara, CA, USA) has pioneered the use of photolithographic deprotection and solid-phase synthesis to create high density oligonucleotide arrays. It currently offers several commercially available arrays, including: the Genechip® Human Genome U95 – a set of five arrays consisting of over 12,000 full length human gene sequences each; application-specific GeneChip® arrays for the study of human cancer, rat toxicology and rat neurobiology; and custom synthesized arrays. Pre-spotted microarrays are available from Hyseq (Sunnyvale, CA, USA), which manufactures the HyChip™ system – a set of universal sequencing arrays with complete sets of DNA sequences that are used in conjunction with a target-specific cocktail of labeled probes to detect differences between a reference sequence and test samples. Other examples include Mergen (San Leandro, CA, USA), which manufactures ExpressChip™ DNA microarrays for gene expression analysis and Research Genetics (Huntsville, AL, USA), which makes GeneFilter® arrays with sets of gene sequences immobilized on nylon membranes for use with isotopic detection techniques.

Variations on the microarray concept include Flow-thru Chips™, which are being developed by GeneLogic (Gaithersburg, MD, USA) and consist of hundreds of thousands of discrete microscopic channels containing immobilized probes. This approach could facilitate hybridization kinetics by providing larger surface-area:volume ratios to minimize diffusion-limited on-rates, and could also enhance detection sensitivity by incorporating more immobilized probes per unit detection volume. The electronic microarray chips being developed at Nanogen (San Diego, CA, USA) use arrays of microelectrodes to control electrical fields at the substrate surface thereby influencing local target concentrations and the kinetics of target hybridization to probes immobilized at individual test sites. Meanwhile, Ciphergen Biosystems (Fremont, CA, USA) is developing a ProteinChip™ system that utilizes arrays of capture probes to isolate and concentrate specific antigens from biological samples. After carrying out the multiplexed solid-phase binding assay, the microarray is scanned using a proprietary surface-enhanced laser desorption/ionization mass spectrometry technique to analyse proteins at the femtomolar level.

Microarrays for gene expression profiling and target validation

Gene expression profiling using oligonucleotide microarrays provides the scientist with a ‘blueprint’ of cellular activity by enabling the monitoring of expression levels for thousands of genes simultaneously. This approach is being used to characterize gene expression patterns in normal cells and to correlate changes in expression levels in particular disease states[13,43–45]. Microarray technologies thus provide pharmaceutical researchers with a powerful new tool for rapidly identifying and validating drug discovery targets. Gene expression experiments are carried out by application of isolated and purified mRNA to oligonucleotide arrays under conditions that promote hybridization between complementary sequences. Detection of hybridization is then carried out either by the direct labeling of the mRNA with fluorescent tags or by the use of labeled detection probes, followed by imaging of the microarray. A variety of imaging instruments is commercially available [e.g. the GenePix™ 4000 scanner from Axon Instruments (Foster City, CA, USA) or the ScanArray systems manufactured by General Scanning (Billerica, MA, USA)] that provide high-resolution, multicolor array images. Correlation of the resulting signals and the known sequence at each probe site provides a direct readout of gene expression level. Examples of recent gene expression profiling studies using microarrays include work on pulmonary fibrosis[43], breast cancer[44] and prostate tumors[45].

Some of the technical issues associated with microarray experimentation and areas of ongoing technology development include: sample preparation time (time required to isolate and label the target RNA or DNA); the need to minimize non-specific hybridization events (e.g. base pair mismatches); and the slow hybridization kinetics observed (typically 60–120 min)[46,47]. Researchers are now beginning to work on microfluidic devices that integrate sample preparation steps (PCR amplification and labeling) with microarray hybridization[46]. Alternative hybridization schemes include enzyme-mediated hybridization for selective discrimination against base pair mismatch[47] and new approaches to detection include coupling of microarrays with MS (Ref. 47). Sequenom (San Diego, CA, USA) is working on high-throughput MS analysis of DNA molecules arrayed on a chip for use in genotyping and SNP analysis.

Microfluidic device fabrication

Many of the fabrication techniques used in microfluidic device technologies were adopted from the semiconductor industry. Most of the recent development work has utilized glass or fused-silica substrates, which exhibit better electrical insulation and optical properties than semiconductors, or polymer materials that reduce costs[48,49].

Typical glass or fused-silica microfabrication steps include:

1 Deposition of a thin chromium or gold film onto the substrate to act as an etch mask.
2 Spin-coating of a light-sensitive polymer film (photoresist) onto the metal film.
3 Exposure of the photoresist to ultraviolet light through an appropriate mask to create a pattern.
4 Development of the photoresist to strip away exposed areas.
5 Removal of the metal film in the exposed areas by etching with KI/I_2 (for gold) or $K_3Fe(CN)_6/NaOH$ (for chromium).
6 Etching of the substrate using a dilute HF/NH_4F solution.
7 Sealing the microchannels by thermally bonding a cover plate of the same material to the patterned substrate[48].

Microchannel dimensions are typically 20–100 μm wide, and 5–30 μm deep. Thermal bonding of the cover plate to the substrate is carried out by bringing the parts into contact and applying heat and pressure to increase compliance and promote formation of covalent Si–O–Si bonds. The process is sensitive to the presence of particulates on the bonding surfaces, hence most fabrication steps are carried out in a clean room. Access holes for addition of reagents or buffers are fabricated in the cover plate before bonding using techniques such as ultrasonic drilling.

Microfluidic device properties

The small dimensions of microfabricated devices give rise to unusual fluidic properties. The Reynold's number (Re) is the ratio of inertial forces to viscous forces acting on a fluid[50]. For very small Reynold's numbers ($Re \ll 1$ for microfabricated devices), the inertial forces are essentially zero and the fluid dynamics are dominated by viscous drag. Fluid flow in this 'low Reynold's number' or 'Stokes flow' regime is characterized by laminar flow profiles (there is no turbulence in the device), by mixing through lateral diffusion and by the effects of surface tension.

Control of fluid flow in these devices has been carried out using a variety of techniques, including pressure-driven flow, and more recently, electro-osmotic flow. Pressure-driven control of fluid flow in microchip devices is often achieved simply by direct connection of external pressure and/or vacuum sources (e.g. programmable syringe pumps or peristaltic pumps). Microfabricated pumps integrated directly into the device have also been demonstrated. Micropump designs using thermopneumatic, electrostatic and piezoelectric actuation have all been described in the technical literature[15–17].

Electro-osmotic flow

In electro-osmotic flow, the flow velocity of the bulk solution (u_{Bulk}) is proportional to the applied electric field (E) and the electro-osmotic mobility (μ_{EO}):

$$u_{Bulk} = \mu_{EO} E$$

Electro-osmotic mobility is in turn proportional to the electrostatic potential at the hydrodynamic plane of shear along the microchannel wall and depends on both the charge density on the channel wall and the ionic strength of the fluid[51]. Electric fields required to generate fluid velocities of approximately 1 mm s^{-1} are typically several hundred volts per centimeter, and are manipulated via electrodes brought into contact with the fluid-filled microchannels. Because electro-osmosis is a surface-driven phenomenon, fluid transport is characterized by 'plug flow' (i.e. the fluid velocity is nearly constant across the width of the channel)[52]. In addition, because the flowstream follows the electric field lines, it is possible to create virtual valves in which fluid flows around corners at intersections of microchannels with essentially no leakage into the other branches of the device[53]. The principal disadvantage of electro-osmotic fluid transport is the tendency for un-wanted separation of components owing to differences in electrophoretic mobility.

Electrophoretic separation

Charged species present in the bulk fluid undergo electrophoresis as a result of the applied electric field, and move with an electrophoretic velocity of:

$$u_{EP} = \mu_{EP} E$$

where the electrophoretic mobility (μ_{EP}) is dependent on the net charge of the molecule and hydrodynamic radius. The net velocity of the charged analyte ($u_{Analyte}$) is thus given by:

$$u_{Analyte} = (u_{EP} + u_{Bulk}) = (\mu_{EP} + \mu_{EO}) E$$

Molecules having different electrophoretic mobilities will travel through the system at different velocities, and hence a discrete injection of a sample containing a mixture of analytes quickly separates into bands of individual molecular species migrating at different rates. Several unique features of the microchip format enable very rapid, high-resolution electrophoretic separations to be performed in these devices[1,2,48,49,53]. The flat velocity profile that is characteristic of electro-osmotic flow essentially eliminates the band broad-

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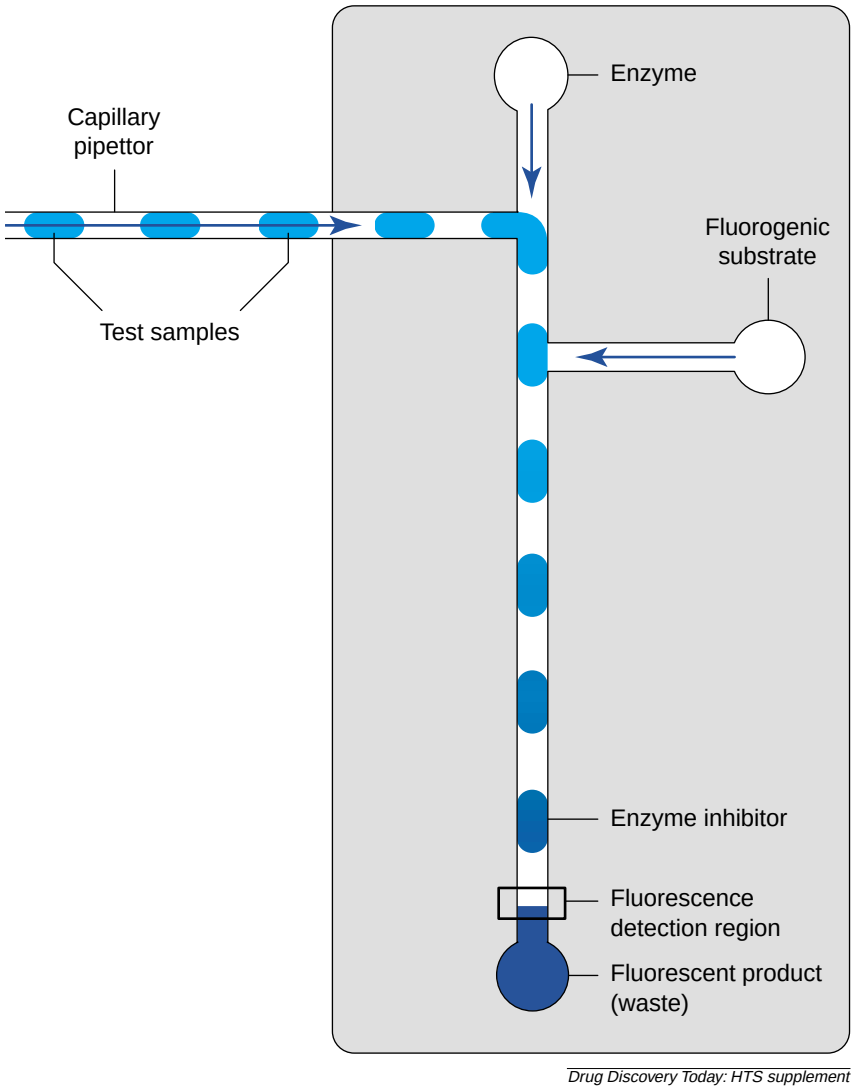

Figure 1. Schematic illustration of a continuous-flow fluorogenic enzyme assay. Compounds to be tested for activity against the target enzyme are injected into the microchip via an attached capillary, mixed with the enzyme and a fluorogenic substrate, and allowed to react. Enzyme activity is monitored downstream of the mixing point using a fluorescence detector.

ening that is attributable to parabolic flow in pressure-based chromatographic techniques. In addition, the ability to microfabricate an injection port of precisely defined geometry and electrokinetically control sample injection volume minimizes the band broadening associated with sample injection in conventional capillary electrophoresis. Finally, the large surface-area-to-volume ratios in microfabricated channels provide excellent heat dissipation, and enable separations to be carried out at higher electric field strengths.

Hybrid chip designs

Hybrid microchip designs that take advantage of both pressure-driven and electro-osmotic flow offer the best of both worlds for some applications. Pressure-driven flow avoids issues of electrophoretic bias and simplifies assay development because the standard array of blocking reagents can be used to reduce protein adsorption. Meanwhile, application of electric fields still provide electrophoretic separation capability as well as some degree of flow control.

Mixing in microfluidic devices

Mixing of components in microchips is dominated by thermal diffusion owing to the absence of turbulence in low Reynold's number flow regimes[50]. This is true both for pressure-driven and electro-osmotic flow. The time (t) required for a molecule with a diffusion coefficient (D) to diffuse an average distance ($\bar{x}$) is given by:

$$t = \frac{\bar{x}^2}{2D}$$

For a typical small molecule (diffusion coefficient $\approx 5 \times 10^{-6}$ cm^2 s^{-1}), the time required to diffuse 100 μm is ≈10 s. For a protein such as human serum albumin [MW = 68,500; diffusion coefficient = 6.1×10^{-7} cm^2 s^{-1} (Ref. 54)], the time required to diffuse the same distance is considerably longer (82 s). Design of microchip devices must therefore take into account the mixing times required for the reaction components with the slowest diffusion coefficients.

Microfluidic devices for HTS

One of the technical challenges to practical implementation of microchip devices for high-throughput experimentation has been the world-to-chip interface, that is, how to automate sample introduction in order to achieve the required throughput while still taking advantage of the miniaturization achieved though microfabrication. Recently, the first microchip-based systems for HTS have been described[7,8]. Two distinct approaches to the problem of sample introduction are being pursued: (1) the use of microdispensing (e.g. ink-jet or pin-tool transfer) technologies for loading reagents and samples into microchips designed to carry out biochemical reactions and electrophoretic separation of assay components; and (2) continuous-flow assays carried

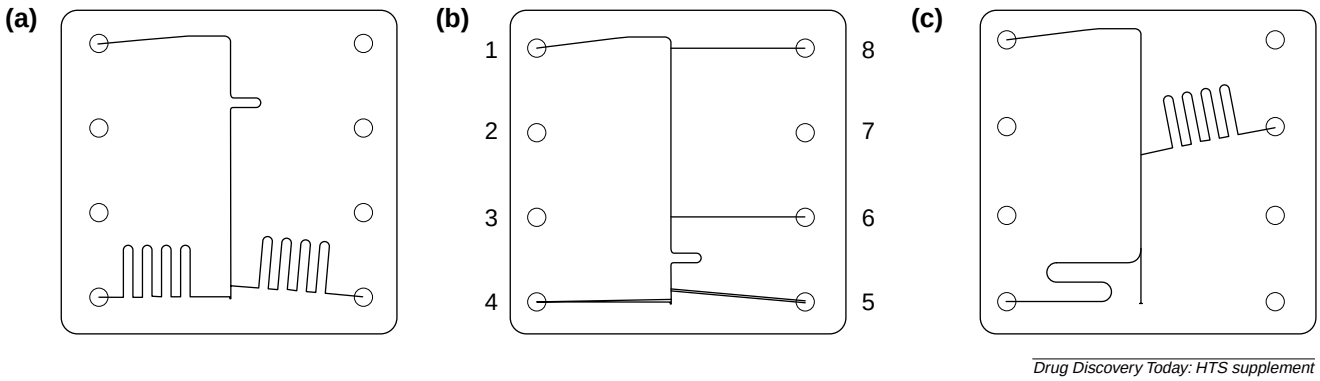

Figure 2. LabChip™ (Caliper Technologies, CA, USA) designs for continuous-flow **(a)** fluorogenic enzyme assays, **(b)** separation-based kinase assays, and **(c)** cell-based assays. The numbering convention for on-chip reagent wells is illustrated in (b). Not all of the reagent wells are used in every chip design. For fluorogenic enzyme assays (a), enzyme and substrate are added to wells 4 and 5, respectively. Test compounds are injected via a capillary attached to the end of the main reaction channel closest to the mixing point for enzyme and substrate, and enzyme activity is monitored with a fluorescence detector positioned near the waste well (well 1). For separation-based kinase assays (b), in-line separation of reaction products and substrates is performed by application of an electric field along the main reaction channel using electrodes positioned in wells 6 and 8. Agonist assays for G-protein-coupled receptors are carried out by placing cells preloaded with a calcium-sensitive probe such as Fluo-3 into well 4 (c) and mixing the cells 'on-chip' with test compounds introduced via the sampling capillary. The device can also be configured for running antagonist assays by probing the cells with a known agonist (well 7) downstream from the point at which they are mixed with test compounds.

out in microfluidic devices that integrate the sample injection process with on-chip reagent mixing and assay functionality.

Single-use microfluidic devices

The first approach is currently being pursued by Aclara Biosciences (Mountain View, CA, USA). Multiplexed LabCard microfluidic devices and optical systems that enable up to 12 microfluidic processes and electrophoretic separations to be performed in parallel have been described[8]. Reagents are dispensed into the chip and allowed to react, and the extent of enzymatic activity is subsequently monitored by high-speed (<20 s) electrophoretic separation of the fluorescently-tagged substrate and product molecules. Separation-based assays for proteases, phosphatases, kinases and phospholipases were carried out in volumes as small as 300 nl in the microchip, or in larger volumes (1–5 μl) in a standard microplate, and then transferred to the microchip for analysis. Aclara has developed a series of electrophoretic tags (e-Tags™) for labeling substrate molecules that shift the electrophoretic mobility of the molecule so that multiplexed assays can be run in a single separation channel. Examples of a four-plex kinase assay have recently been presented (Wu, J. *et al.*, Multiplexed capillary electrophoresis-based assays on single-use plastic microarrays for HTS. *Annual Meeting of the Society for Biomolecular Screening*, 6–9 September 2000, Vancouver, BC, Canada). This approach enables two levels of multiplexing: running multiple assays in each separation channel and the fabrication of multiple separation channels in each device. Aclara is working on multiplexed LabCards with up to 96 individual separation channels. Work on G-protein-coupled-receptor assays is also under way (Boone, T. *et al.*, HTS in single-use microfluidic devices. *ScreenTech 2000*, 28 February–3 March 2000, Monterey, CA, USA). Aclara is developing the CellChip™ system in collaboration with Cellomics (Pittsburgh, PA, USA). In this system, microfluidic channels deliver reagents to wells containing immobilized cells for high-throughput, high-content screening (Kain, S. *et al.*, Chips-to-Leads: The CellChip™ system for high throughput lead optimization. *Annual Meeting of the Society for Biomolecular Screening*, 6–9 September 2000, Vancouver, BC, Canada).

The principal advantages of single-use, disposable microfluidic devices that are loaded by external sample dispensing mechanisms include the elimination of sample cross-contamination between assays and the potential for performing long incubation reactions if necessary. With Aclara's Oasis™ LabCard technology, evaporation from reaction wells is compensated for by replacement fluid entering from side channels (Wang, C. *et al.*, Nanoliter AlphaScreen Assays in Oasis™ LabCard: A novel miniaturization solution. *Annual Meeting of the Society for Biomolecular Screening*, 6–9 September 2000, Vancouver, BC, Canada). Assay reaction times of 10–100 min have been demonstrated in these devices[8].

The consumption of assay reagents in single-use, disposable microfluidic devices is determined by the limitations of the reagent dispensing technology employed. These range from tens of nanoliters for 'drop-on-demand' technologies, to several microliters for conventional liquid-pipetting robotics, and the potential for miniaturization inherent in the microchip format is not therefore fully exploited by the disposable microchip approach. In addition, system throughput is limited both by the rate at which reagents and samples can be dispensed,

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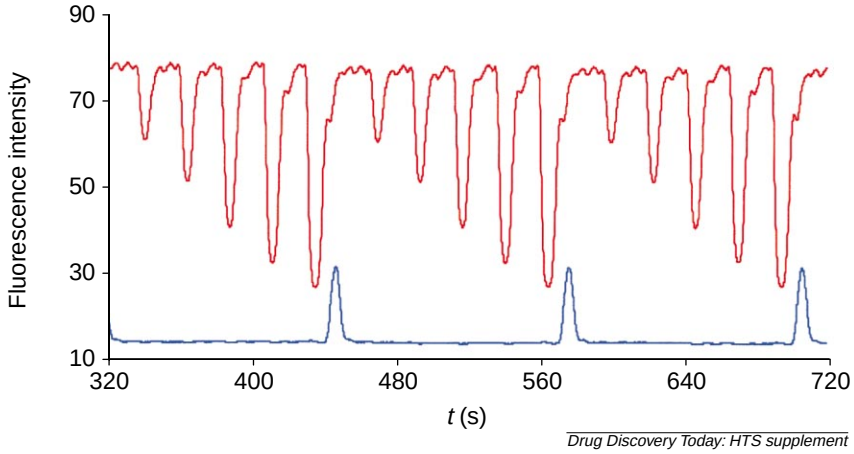

Figure 3. Continuous-flow enzyme inhibition data for a phosphatase assay running in a LabChip™ (Caliper Technologies, CA, USA) microfluidic device. Fluorescence was monitored downstream from the mixing point for enzyme and substrate as a series of five inhibitor concentrations or buffer blanks were injected using a 12 s injection cycle. Each sample was injected six times per experiment, and the experiment was repeated using three different chips. Percentage 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 injection plug. Final concentrations of reagents on the chip (after dilution and mixing) were 8 nM phosphatase and 6 μM difluoro-methylumbelliferyl phosphate substrate. Inhibitor concentrations ranged from 2.5 μM to 40 μM in the microplate. The lower trace shows the periodic injection of a marker dye detected on a second optical channel that was used to correlate sample injection plugs with specific locations on the microplate. Good reproducibility both for within-run replicate sample injections and for chip-to-chip performance was observed, with CV <10%.

and by the time required to replace and position the microchip, placing a premium on multiplexed device designs.

Microfluidic devices for continuous-flow assays

Caliper Technologies (Mountain View, CA, USA) is pursuing an alternative approach based on the concept of a continuous-flow assay. Continuous-flow processes have a potentially higher throughput than batch processes and integration within microchip devices is more straightforward[30,55]. For HTS applications, this means that significant throughput should be achievable even in relatively simple devices, while system stability and performance should be improved by eliminating the need to swap and re-equilibrate chips, re-align optical detection devices, etc.

A LabChip™ device that integrates sample injection directly from a microplate source with a continuous-flow enzyme inhibition assay is illustrated schematically in Fig. 1. The presence of an inhibitor in the series of injected compounds is indicated by a transient decrease in the steady-state fluorescence baseline resulting from turnover of the fluorogenic substrate.

The assay throughput in such a device is determined by the amount of sample plug-dispersion in the flowstream, which in turn determines how frequently samples can be introduced. Optimization of throughput involves identifying sources of sample plug-dispersion such as: Taylor-Aris dispersion[56–58]; imperfections in the connection between the capillary and the microchip; the geometry used in laying out the pattern of microchannels[59]; analyte adsorption to the walls of the microchannels[60]; and adjusting the device design and fabrication technique to minimize as many of these contributions as possible. The maximum achievable throughput will vary for

Table 1. Percentage inhibition data[a]

	Chip identification											
	07159971Q1			07159971Q14			07159971Q14			Chip-to chip average		
Inhibitor concentration (μM)	Mean	STD	CV (%)	Mean	STD	CV (%)	Mean	STD	CV (%)	Mean	STD	CV (%)
2.5	27.2	2.4	9	31.7	1.4	4	31.0	1.9	6	30.0	2.4	8
5.0	45.7	3.5	8	49.9	1.9	4	50.2	2.8	6	48.6	2.5	5
10.0	67.9	4.4	6	69.0	2.0	3	70.1	3.9	6	69.0	1.1	2
20.0	84.9	4.5	5	87.5	2.2	3	88.4	4.8	5	86.9	1.8	2
40.0	96.3	4.5	5	96.6	2.3	2	97.8	4.8	5	96.9	0.8	1

[a]Percentage inhibition data for the experiment described in Fig. 3. Five concentrations of inhibitor were injected from the microplate. Each sample was injected six times per experiment and the experiment was repeated using three different chips. Excellent reproducibility was observed, both for 'within-run' replicate-sample injections and for 'chip-to-chip' performance, with coefficients of variation (CV) of <10%.

different chip designs and operating conditions owing to differences in sample transit time and the relative contributions of sample plug-dispersion sources.

The microchip design and data for a continuous-flow phosphatase inhibition assay, carried out as already described, are shown in Figs 2a and 3, respectively. A marker dye is periodically injected into the microchip and is continuously monitored on a second optical channel to enable correlation of sample injections with locations on the microplate and also to provide a means for detecting flow problems in the microchip. Typical sample injection volumes range from 1 to 4 nl. The volumetric reagent consumption rate in this chip design is ≈0.15 nl s^{-1}. Sample injection cycles of one compound tested every 10–15 s in fluorogenic enzyme assays provide a single-channel device throughput of up to 4300 assays per 12 h of operation, and a total reagent consumption of <2.3 nl per assay. Higher throughput has been achieved in feasibility studies by: (1) deliberately overlapping sample injection plugs (six second injection cycles generate 7200 assays per 12-h day – this is analogous to the strategy of screening against mixtures of compounds in microplate assays); or (2) by multiplexing the device design (four parallel sample injectors with 10–15 second injection cycles, generating up to 17,300 assays per day). Throughput of up to 28,800 assays per day should be achievable using existing four-channel microchip designs with a sample injection cycle of 6 s.

Work is under way on microchip designs with higher degrees of multiplexing. Prototype screening systems are currently being tested that enable the user to load the microchip with reagents and carry out screening experiments using 96- or 384-well plates. Post-run data analysis software provides a calculation of percentage inhibition for each sample tested, as well as a 'hit table' based on a user-defined inhibition threshold. Good reproducibility for within-run response to replicate

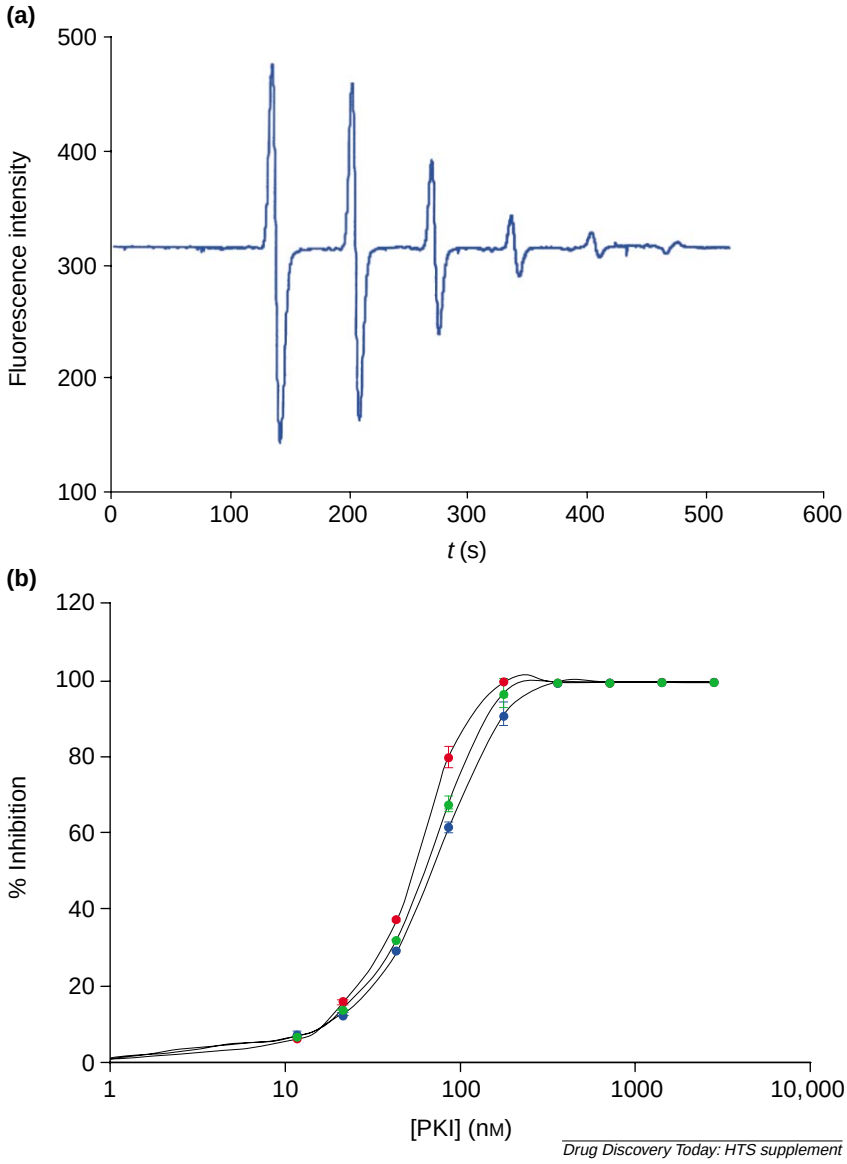

Figure 4. Data from a continuous-flow electrophoretic mobility-shift assay running in a LabChip™ (Caliper Technologies, CA, USA) microfluidic device. **(a)** Sample injections containing different concentrations of a protein kinase inhibitor were made via the sampling capillary into the chip, where the kinase was continuously mixed with its fluorescein-labeled peptide substrate and ATP. An electrical field is applied by placing electrodes in wells 6 and 8 of the chip. Each injection of an inhibitor results in a characteristic 'peak-and-dip' perturbation of the steady-state fluorescent signal owing to the electrophoretic separation of a zone containing excess substrate from a zone containing reduced product, compared with that present in the steady-state reaction mixture. The relative positions of the peak and dip depend on the polarity of the electric field and the relative charges of the substrate and product species. The amplitude of the signal perturbation has been correlated with the concentration and potency of the injected inhibitor. Final concentrations of reagents on the chip (after dilution and mixing) were 100 nM kinase, 10 μM fluorescein-labeled peptide substrate and 50 μM ATP. Inhibitor concentrations ranged from 31.25 nM to 500 nM in the microplate. **(b)** Dose–response curves for a kinase inhibition experiment carried out as described above. Percentage inhibition was calculated by comparing the peak amplitude (blue circles), amplitude of the dip (red circles), and peak-to-dip amplitude (green circles) of the signal perturbation, with that induced by a 100% inhibition control. The estimated K_i from this experiment was 32.5 nM. Abbreviation: PKI, protein kinase inhibitor.

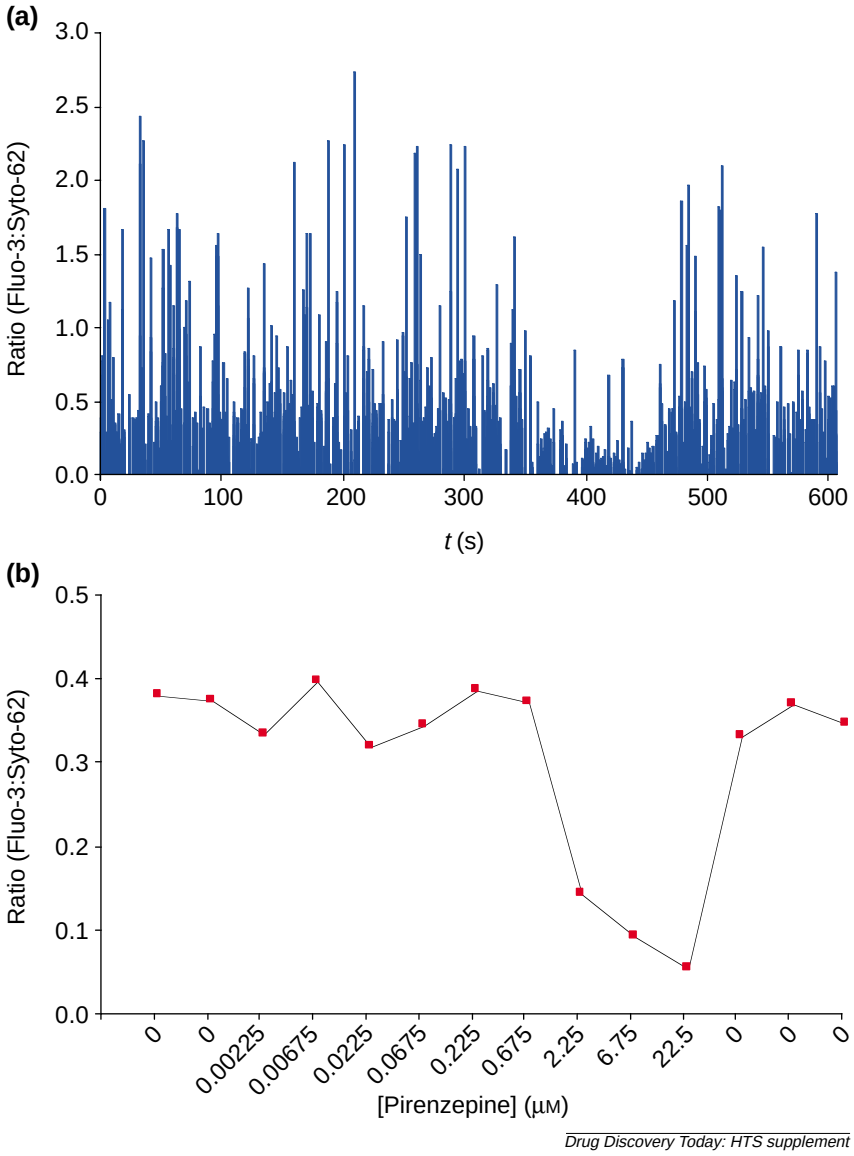

Figure 5. Data from a continuous-flow cell-based assay running in a LabChip™ (Caliper Technologies, CA, USA) microfluidic device. CHO-m1-WT3 cells were preloaded with the calcium sensitive dye Fluo-3 and the nucleic acid stain Syto-62. Cells were then added to well 4, and a 3 μM solution of the known agonist carbachol was placed in well 7. Antagonist samples were injected from a microplate every 30 s, mixed with cells and carbachol on-chip, and fluorescence was monitored as the cell suspension flowed past the detector. Ratiometric measurements of Fluo-3 and Syto-62 fluorescence were used to control variations in cell number and excitation light intensity in the detection spot. Fluorescence data was processed and averaged for ≈50 cells to obtain each assay data point. **(a)** Fluo-3:Syto-62 fluorescence ratio plotted versus time. **(b)** Fluo-3:Syto-62 fluorescence ratios calculated and averaged over time windows corresponding to increasing concentrations of pirenzepine.

injections of known inhibitors (as well as for chip-to-chip performance) has been observed in short duration experiments (Table 1). Development efforts are currently focussed on improving the robustness and performance of systems in full-day screening trials.

Assay development issues that can arise when transferring assays from a conventional microplate format to the continuous-flow microchip format include: adsorption of the target enzyme or other assay components to the microchannel walls; mismatch of buffer composition and fluid viscosities between solutions placed on the chip and in microplates; and, for slow turnover enzymes, the limited reaction time available on the chip (typically 30–60 s with the present chip design and operating conditions). However, protein adsorption can often be reduced or eliminated either through the addition of zwitterionic reagents to the assay buffer[24,49], or by the use of polymer coatings (H. Yang and S.A. Sundberg, unpublished). In the case of slow-turnover enzymes, reaction rates can sometimes be increased, without detrimental effect to assay performance, simply by increasing the enzyme concentration. Alternatively, flow rates in the microchip can be decreased, or the length of reaction channels increased, to extend the available reaction time. The latter approach, however, compromises assay throughput because dispersive forces have longer times to act on the sample injection plugs traveling through the system, which consequently must be spaced further apart.

Kinase assays based on electrophoretic separation of the reaction product from the enzyme substrate (Coffin, J. *et al.*, High throughput screening on microchips: the mobility shift approach. *Annual Meeting of the Society for Biomolecular Screening*, 6–9 September 2000, Vancouver, BC, Canada), and cell-based Ca^{2+} flux and transmembrane potential assays (Farinas, J. *et al.*, Cell-based assays for drug screening in the LabChip™ system. *Annual Meeting of the Society for Biomolecular Screening*, 6–9 September 2000, Vancouver, BC, Canada) have also been demonstrated in the continuous-flow format. A schematic illustration of a LabChip™ device for performing separation-based assays, and examples of kinase assay data are shown in Figs 2b and 4, respectively. A different layout, designed to perform Ca^{2+} flux assays, is illustrated in Fig. 2c. Examples of

data for an antagonist assay are shown in Figs 5 and 6. Data was processed and averaged for approximately 50 cells to obtain statistically significant measurements of changes in intracellular Ca^{2+} concentration. Injection cycle times of 20–30 s per assay are being used at present. Higher throughput should be achievable by using higher flow rates (to move the same number of cells past the detector in a shorter period of time) and faster data acquisition rates (to monitor their response accurately).

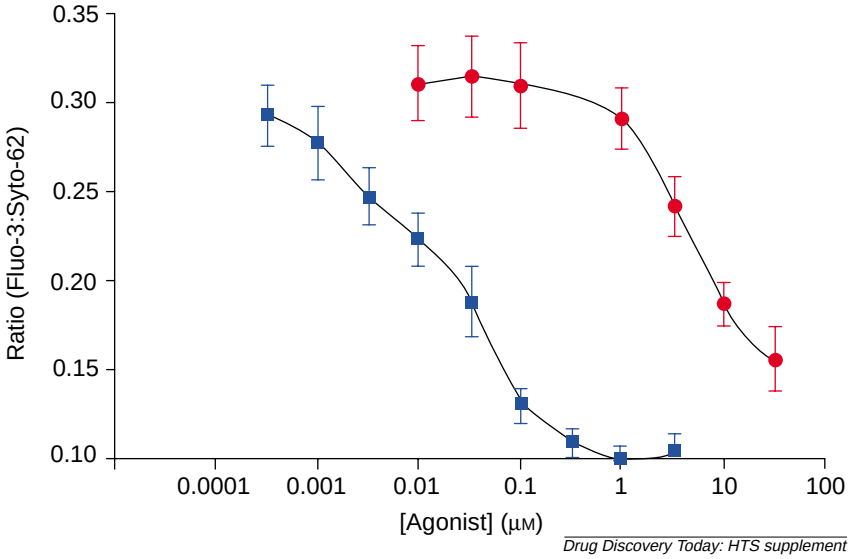

Figure 6. Comparison of pirenzepine (red circles) and atropine (blue squares) dose–response curves. The IC_{50} for atropine was 2-log lower than pirenzepine as predicted from published competitive binding data[61] (atropine K_i = 0.29 nM, pirenzepine K_i = 33 nM).

Concluding remarks

The application of microarray and microfluidic device technologies to target validation and primary screening for drug discovery is already providing pharmaceutical scientists with powerful new research tools. Current development efforts in the microarray field are focussed on improving the quality of oligonucleotide microarrays, increasing the density of probe sites, and developing hybridization and detection methods for improved sequence discrimination and sensitivity.

The feasibility of running assays in microchip-based HTS systems has been demonstrated for a variety of assay types. Prototype microfluidic devices feature orders-of-magnitude reductions in reagent consumption compared with microplate assay formats, and enable implementation of novel separation-based assay formats. Development efforts are focussed on 'ease-of-use' issues and system robustness, as well as on increasing assay throughput and demonstrating extended microfluidic chip functionality. At Caliper Technologies, next-generation LabChip™ designs are already being tested that integrate compound dilution with assay functionality. These designs enable users to sample directly from DMSO daughter plates and carry out compound dilution on the chip, and could help to eliminate microplate preparation steps that create operational bottlenecks in screening laboratories. Other microfluidic device formats are also being tested. For example, Gyros Microlab (Uppsala, Sweden, USA) is testing microfluidic assay technologies based on a compact disc format that exploits centrifugal forces to drive fluid flow.

New approaches to microchip-based separation techniques are also an active area of investigation and could find application in pharmaceutical research. Microchannel column packing materials are being studied by researchers at Sandia National Laboratories (and elsewhere) for use in capillary electrochromatography applications (Fintschenko, Y., *et al.*, On-chip packed channel separations. *Proceedings of the Micro Total Analysis Systems 2000 Symposium*, 14–18 May 2000, Enschede, The Netherlands; Oleschuk, R. *et al.*, Utilization of bead based reagents in microfluidic systems. *Proceedings of the Micro Total Analysis Systems 2000 Symposium*, 14–18 May 2000, Enschede, The Netherlands). Eksigent Technologies (Livermore, CA, USA), a new company spun out of Sandia National Laboratory, is using electrokinetic pumps to perform 'HPLC-on-a-chip' (Paul, P., *et al.*, Electrokinetic pump applications in micro total analysis systems: mechanical actuation to HPLC. *Proceedings of the Micro Total Analysis Systems 2000 Symposium*, 14–18 May 2000, Enschede, The Netherlands).

Scientists at companies like Aclara, Caliper Technologies and Orchid Biosciences (Princeton, NJ, USA) are already working on microfluidic devices for gene expression profiling and SNP analysis (Wu, J. *et al.*, Multiplexed capillary electrophoresis-based assays on single-use plastic microarrays for HTS. *Annual Meeting of the Society for Biomolecular Screening*, 6–9 September 2000, Vancouver, BC, Canada; Shiue, L. *et al.*, Automated nanoliter scale genotyping. *3rd International Meeting on Single Nucleotide polymorphism and Complex Genome Analysis*, 8–11 September 2000, Taos, NM, USA).

Looking further out on the development horizon, microfluidic chips could one day integrate mRNA or DNA sample preparation, amplification and labeling steps with high-density microarray analysis for ultra-high-throughput gene expression profiling, while microchip-based screening systems will exploit novel miniaturized compound storage formats for desktop HTS capability.

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