Miniaturization technologies in HTS: how fast, how small, how soon?

Jonathan J. Burbaum

Miniaturized analytical methods are currently being developed to suit many needs. One area that is evolving with particular speed is HTS for pharmaceutical lead discovery. This review explores the scope and impact of miniaturized methods as applied to modern HTS, and summarizes the prospects for both existing and proposed strategies for miniaturized HTS assay systems.

In recent years, HTS has begun to emerge as a scientific discipline in its own right. Several current reviews[1–3] have addressed the breadth of HTS and new technologies for both automation and screening, so these topics will be treated sparingly.

Why miniaturize HTS?

This review will cover the progress towards miniaturized HTS and will assess its impact on pharmaceutical discovery. However, before delving into too much detail, it is important to define what the goals and impacts of modern HTS are, and what distinguishes HTS from other scientific endeavors. As with many emerging disciplines, HTS means different things to different people. To some, it solicits images of a highly sophisticated robotics laboratory that operates round-the-clock; to others, it implies a hybrid process that combines laboratory science with manufacturing. In either case, the goal of HTS is to harness the discovery potential of large numbers of compounds efficiently, both by reducing time and cost and increasing information content. The goal is not to run more assays than last year (although this is a manifestation of current pressures on the science), rather, the goal is to convert assays plus compounds into information (not simply data) as quickly and as efficiently as possible. From this viewpoint, it follows that the optimal throughput of an HTS operation should be placed in the context of a particular need.

The pharmaceutical industry currently has the most pressing need for improvement in HTS technology. Although this industry has a seemingly insatiable appetite for new pharmaceutical leads, it is under continually increasing pressures, both from competition and from public policy, to reduce the costs of discovery and development. Historically, the pharmaceutical industry has furnished enormous potential, not only for the improvement of human health but also for the realization of significant profit. Over the past three decades, this potential (and its translation into dollars) has propelled rapid advancement, both in the valuation of pharmaceutical companies and in the basic sciences that provide them with their technical foundation. Despite these deep technical and intellectual roots, the pharmaceutical industry has historically relied almost exclusively on screening (primarily of compounds derived from natural sources) to provide or suggest new products. With the explosion of available compounds fueled by combinatorial chemistry and the accelerated identification of new pharmaceutical targets catalyzed by genomics, the ability to analyze potentially fruitful combinations of compounds with targets (i.e. screening) has now become at least partially limiting. This limitation and the financial incentives mentioned above have combined to

Jonathan J. Burbaum, Pharmacopeia, Inc., 101 College Road East, Princeton, NJ 08540, USA. tel: +1 609 452 3712, fax: +1 609 655 4187, e-mail: burbaum@pharmacop.com

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compel the development of miniaturized technologies in HTS.

This viewpoint is widely shared. In a recent issue of the *Wall Street Journal*[4], the following commentary referred to the then-proposed merger of pharmaceutical giants Glaxo Wellcome and SmithKline Beecham:

> 'An explosion in scientific breakthroughs has suddenly created vast research opportunities that are overwhelming drug companies' budgets and management expertise. New gene-sleuthing technology, when combined with high-speed computerized chemistry, is producing countless tempting leads for treating illnesses ranging from AIDS to cancer, from heart disease to depression.'

As evidence, the authors of this article quote the CEO of SmithKline Beecham, Jan Leschly, who says that 'the whole [pace of discovery] is exciting… but it's like kids in the candy shop – there are too many targets… Now the question is, can we afford to do all this?'

Thus, in the drug discovery process, the inherent potential of two massive advances in technology, combinatorial chemistry and functional genomics, is underutilized so long as current analytical capabilities delay the timely and thorough evaluation of all productive combinations of chemicals and targets. The technical strategy, inherent to HTS, is to evaluate 'all' possibilities, since this maximizes the scientific potential involved. Based on the current state-of-the-art technology in computer-aided drug design, the alternative strategy (selection of a subset of 'promising' structures), while arguably effective, is unlikely to yield any true surprises.

Miniaturization of HTS results in three significant benefits that address the pharmaceutical industry's needs: lower costs, faster turnaround and reduced space requirements. Approximately three-quarters of the costs of screening derive from the costs of both chemical and biological reagents (Figure 1a). Miniaturization reduces these costs in proportion to the reduction in volume (Figure 1b). Miniaturization also allows for the compression of more samples onto one plate, reducing the sample-to-sample distance. As sample transport is a significant time factor in screening, whether it involves moving a detector within a plate or moving plates between workstations, the total analysis time per sample is reduced significantly by miniaturizing. Finally, the logistics of assaying large chemical collections has led some companies to centralized screening facilities – a structure that inevitably reduces the efficiency of information transfer both into and out of the screening lab. By miniaturizing assays (presuming that miniaturization of assays leads to smaller facility requirements), it will become more feasible to decentralize, broadening access and improving information flow within the organization.

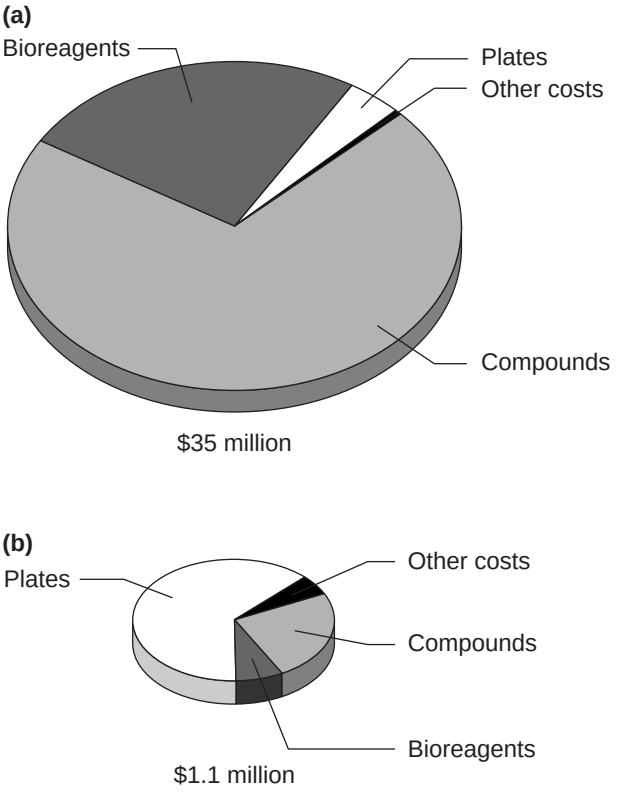

Figure 1. *Cost comparisons for (a) 96-well and (b) 1,536-well assays. Comparative costs are based on a compound library of 106 compounds assayed against 40 targets in 96-well microtiter plates (nominal cost, $3) and in 1,536-well microtiter plates (nominal cost, $25). Bioreagents are assumed to cost $0.20 per 100 μl well, while compounds are assumed to vary in cost depending on their novelty. Newly synthesized compounds subsume the development cost of chemistry ($1 per well per compound) while existing compounds are valued at their replacement cost ($0.25 per well per compound). For the purposes of illustration, compound collections are assumed to be half new and half old.*

The context of miniaturized HTS

Before new strategies specific for miniaturized systems can be defined, a brief review of the history of microtiter plates and their utility in HTS will provide a contextual basis for current advances. The 96-well microtiter plate

was originally designed as a tool for increasing the throughput of viral titer assays in the clinical laboratory[5]. During the 1960s, microtiter-plate technology became accepted as a standard in clinical analysis, essentially without any supporting automation. Nevertheless, the establishment of a standard plate attracted engineering efforts – it generated a complete, high-throughput, serological analysis system comprised of 96-well plates and dedicated to liquid handling. During the next two decades, the availability of both plates and liquid handling led to the development of additional clinical screening assays (notably, ELISA) based on the 96-well plate. In turn, the broader utility of the plate propelled additional innovation in hardware, including automated plate readers and pipetters. The technology then crossed over into pharmaceutical R&D, where the growing need for rapid, low-volume analysis spurred additional growth. In each application, the 96-well-plate system increased sample throughput and reduced the drudgery of repetitive analysis. Thus, the current 96-well-plate-rich environment was seeded by the adoption of a standard container, which focused the efforts of scientists and engineers to develop the assays and supporting hardware to fit the format.

Clinical diagnostics vs pharmaceutical HTS

Given the close historical association of clinical diagnostics and pharmaceutical HTS, it is important to understand how the requirements of the two fields differ. In the case of clinical assays, samples are typically biological in origin and are derived from patients. For HTS assays, samples are typically chemical in origin, and are derived from chemical sample collections or extracts of natural products. For clinical assays, diagnostic quantitation is of chief importance, while for HTS assays, accurate identification of active compounds, together with throughput, is paramount. On a purely technical level, HTS assays share similarities with diagnostic assays; both disciplines use four experimental modes for evaluating test samples (Table 1). There are several differences between these assay modes:

Fingerprint assays involve the separation and analysis of the components of a sample. This is a very broad method for diagnosis [including, for example, Western blot analysis for HIV detection and restriction fragment length polymorphism (RFLP) analysis for genotyping], but it is certainly the last resort as a screening strategy. Fingerprint analysis is slow, because the distinctions between individual fingerprints are based mainly upon time and the samples are handled serially. Moreover, variations in sample conditions over time result in poor reproducibility. In spite of the generality of this approach, for HTS, the long analysis time required for each serially treated sample makes it virtually impossible to achieve the throughput needed to evaluate a sample collection completely.

Binding assays involve the detection of intermolecular interactions. Many commonly-employed diagnostic assays fall into this category, including radioimmunoassays and fluorescence polarization immunoassays (such as AxSYM diagnostics line; Abbott). Similar to the fingerprint assay, the binding assay also demands separation of components, although generally in a single-step, all-or-nothing fashion. Thus, the capability of rapid separation combined with the ability to perform the assay in parallel, enables the use of binding assays for HTS. The utility of these assays is clearly established: many drugs that act as receptor antagonists were first identified in assays where the binding of a radiolabeled ligand to its cognate receptor(s) was disrupted by a test sample[3,6].

Table 1. Modes of biological sample evaluation in clinical diagnostics and HTS

Assay mode	Test sample affects	Detect
Fingerprint	Chromatographic support	Detect changes in composition directly
Binding	A ligand receptor system	Separate bound and free
Enzymatic	An enzyme substrate system	Detect conversion of substrate to product
Cell-based	A living cell in culture	Detect changes in cell morphology or biochemistry

Enzymatic assays are usually configured so as to avoid separation. Thus, a synthetic substrate is constructed to generate a colorimetric, fluorescent or luminescent signal in the presence of catalytically active enzyme. The catalytic action of the enzyme acts as a biochemical amplifier to increase the signal from the sample. In diagnostics, this mode is used both directly (such as determining cardiac enzymes in blood samples) and indirectly (such as using

ELISA). Applications of enzyme assays to HTS are quite common, and several recently introduced pharmaceuticals are enzyme inhibitors [examples include: dorzolamide (Trusopt™), a carbonic anhydrase inhibitor; lovastatin (Mevacor™), an HMG-CoA reductase inhibitor; and the plethora of angiotensin converting enzyme (ACE) inhibitors]. Furthermore, because enzyme assays are biochemically well-defined and self-amplifying, they can be carried out in a 'highly' parallel fashion, making these assays particularly amenable to miniaturization.

Cell-based assays are often performed as part of a pathology examination in diagnostics (for example, Pap smears), but they can be even more subjective and time-consuming than fingerprint assays. However, with the advent of cellular engineering, cell-based assays are becoming increasingly utilized in HTS laboratories. By using appropriately constructed cell lines, signaling pathways can be either redirected towards easily-detected proteins or sensed directly using physiological indicators. For example, a biochemical signal that turns on a disease gene can be redirected by cellular engineering to 'switch on' an enzyme whose activity can be easily measured. The main issue with applying cell-based strategies to HTS lies in the speed of assay development. With the recent advances in rapid cell-line generation[7,8], the potential for dramatically shortening the development time for these assays will further increase their utility in HTS campaigns.

What is needed to implement miniaturization?

In general, miniaturization provides the opportunity to do more assays in less time for less cost, with consequent generation of higher quality data. These advantages do not come without strings attached – assays must be adapted to run in smaller volumes, and not all assays can be miniaturized. An example of the latter, which is currently an important discovery tool for pharmaceutical research, is the use of radioactive ligands for evaluating compounds as receptor antagonists[6]. Assay miniaturization minimizes radioactive usage directly, so if a practical strategy for miniaturization of radioactive binding assays were available, the applications and benefits would be immediate. However, measuring radioactivity in miniaturized systems will require new and improved hardware – the current strategy largely involving serial scintillation would take far too long. For example, to scale-down a 100 μl sample by a factor of 100 (to 1 μl), it would take 100^2 (10,000) times longer to count with the same accuracy. In plate terms, 100 μl in a 96-well plate with one second counting per well will take 96 seconds to read. By contrast, 1 μl samples in a 1,536-well plate will take 1,536 × 10,000 seconds to read, or about six months. Therefore, without 'massively' parallel detection systems, miniaturized assays based on radioactivity will be impractical.

New technologies that avoid the use of radioactivity in binding assays have been described (Ref. 9 and references therein). Fluorescence techniques, although comparably photometric, are more scaleable at a given sensitivity requirement because each fluorescent molecule can be excited many thousands of times. This effect amplifies the signal and improves the measurement statistics. In a fluorescence experiment, the intensity of the emission is proportional to the intensity of the excitation, and the measurement can therefore be improved under a given time constraint by using a higher illumination intensity. This fact is evident in other techniques – for example, in fluorescence microscopy extremely small amounts of material can be detected by sophisticated optics and focused light sources. A shift in philosophy, away from radioactivity and towards fluorescence, is already well under way due to cost and safety considerations, and miniaturized formats will directly benefit through improved fluorescence reagents and methodologies.

Miniaturization in HTS is driven both by technical needs and financial imperatives. The question remains: how small? This is not a trivial question, nor one that should be issued as a challenge to technologists – both single-molecule and single-cell measurements have been amply demonstrated, but have yet to be put into practice in a screening laboratory. Rather, this is a question that needs to be answered within the complex milieu of the pharmaceutical discovery process, with its requirements for flexibility, precision and speed.

Miniaturization of pharmacological studies

Taking a current example, the miniaturization of pharmacological studies from single tests to the 96-well format has not progressed as rapidly as the development of modern HTS. Nevertheless, the conversion process is relevant to this review, as it relates to the trend towards the development of 'high-throughput pharmacological screening' (HTPS). An example of this trend is the generation of surrogate assays in ADME/Tox evaluation of new compounds. Traditionally, these assays were performed on

whole animals, providing structure–activity relationships that guided medicinal chemists towards the ultimate clinical candidate. These assays are never performed on the 'true' target of the pharmaceutical compound (humans) for obvious ethical and practical reasons. Therefore, the assay that is used is necessarily an imperfect substitute for the ultimate measurement. Miniaturization is effected either by choosing a smaller animal (such as moving from dogs to mice) or by choosing a different assay (such as cell-based assays). This reduces costs and increases throughput primarily by reducing the quantities of compound needed. However, the choice of assay depends not only on the scale of the assay but also on many other ancillary factors, including subjective factors such as the 'comfort level' of the research group sponsoring the study.

Considering compound absorption through the intestinal lumen and how it often limits the oral bioavailability of new pharmaceuticals, whole-animal studies need to be able to sensitively and reproducibly measure the blood levels of both the primary pharmaceutical and its metabolites. Several years ago, a colorectal adenocarcinoma cell line, Caco-2, was developed into a miniaturized, well-controlled model of the intestinal lumen[10,11]. Although this type of assay is further removed from the true target, it is gaining increasing acceptance as a surrogate model, at least in part, because it is smaller, faster and cheaper to run than an animal study.

The compromises associated with miniaturization are therefore a balance between scientific purity and economic speed.

An emerging process[12] that has promise of bringing pharmacological assays into the fold of modern robotics screening, avoids reporter genes in favor of direct measurements of cell physiology (such as intracellular Ca^{2+} concentration and pH). This is based on the premise that overexpressed reporter genes produce an artificial situation that does not effectively represent pharmacology. Although this technology is relatively well established in research laboratories, its miniaturization would provide more follow-up data quicker for establishing the physiological relevance of putative lead structures.

Progress in miniaturization of primary screening assays

What progress has been made towards miniaturization of 'primary' screening assays, namely those assays for which the activity of a particular set of compounds against a particular panel of targets is largely unknown? These assays are the most sample-intensive assays in pharmaceutical discovery, and the application of new, miniaturized technologies here will have the biggest impact. While the adoption of new technologies in an established process is generally an uphill battle, in this instance the rewards are worth the efforts, and several peripheral technologies are now converging to allow these complex processes to be miniaturized. These technologies include new containers, better modes for liquid handling, improved detectors and specialized assay strategies.

Containers

A new format for miniaturized HTS, based on the original 96-well plate but designed primarily for HTS, contains 1,536 wells on a 2.25 mm spacing in a regular rectangular grid. There are two distinct versions of this format that are commercially available: a deep-well (20 μl) plate from Greiner (Frickenhausen; Germany) and a more conventionally-shaped (2 μl) plate from Corning (Acton, MA, USA) (Figure 2). The Corning plate was co-designed with Pharmacopeia specifically for HTS, and contains additional control wells and alignment features that will enable it to be exploited by high-speed, high-precision instrumentation. Various assays can be carried out effectively in 1 μl volumes and although evaporation rates were a concern, it has been demonstrated that evaporation of water from these plates, while faster than in 96-well formats, can be effectively managed through humidity control and ensuring the plates are covered whenever possible. The

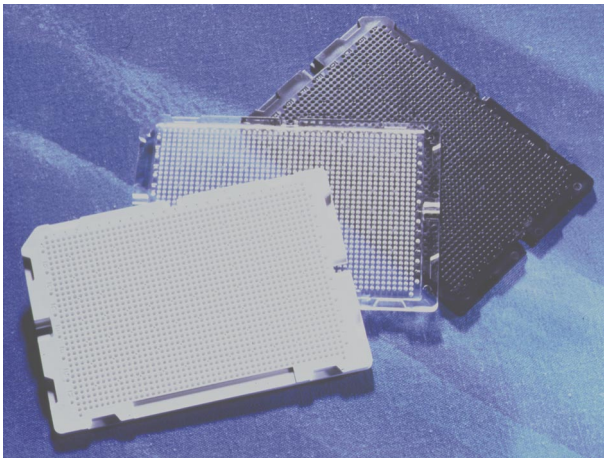

Figure 2. *1,536-well microtiter plates. The aspect ratio matches that of the traditional 96-well plate.*

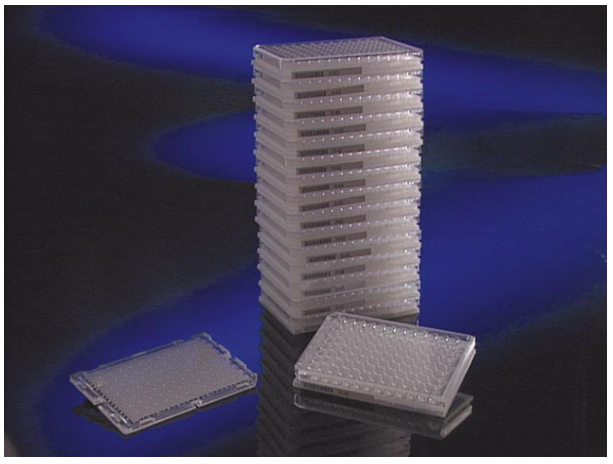

Figure 3. *Illustration of space and reagent savings afforded by 1,536-well plates. On the right is a stack of sixteen 96-well microtiter plates, while on the left is one 1,536-well plate. Each 1,536 plate permits as many tests on one plate as the entire 96-well stack, while the volume of sample in the entire 1,536 stack (at 1 μl per well) is half that in the 96-well stack, in 1% of the total volume.*

changes in surface effects, which relate to evaporation and also plate-binding capacities, are relatively modest: comparing the 96-well format with the Corning plate, for example, the surface-area-to-volume ratio changes by only three- to fourfold.

The Corning 1,536-well format has a well density 16-fold higher than a conventional 96-well plate, with working assay volumes ~100-fold smaller. In this format, shallow wells are evenly spaced at one-quarter the spacing of a 96-well plate, which allows a pre-existing compound array in 96-well format to be reformatted to 1,536 in a straightforward manner. Furthermore, at this density, currently available fast-readout CCD (charge-coupled device) chips, which typically contain a 512 × 512 pixel array, can be used as parallel detectors. At this density, one well in a 1,536 plate represents a 10 × 10 array (100 pixels), which is suitable for the tolerances that are required in the smaller format. This miniaturized format allows for more efficient storage, such that there is nearly a 50-fold savings in storage space in the 1,536 format (Figure 3).

Several miniaturization strategies that allow densities higher than 1,536 have been explored. At Pharmacopeia, 'field format' assays[13] have been explored in which beads containing photochemically-releasable library compounds are distributed in an immobilizing matrix containing reagents that detect the presence of active inhibitors. These assays can, in principle, allow for extremely high throughput, but are limited to relatively soluble compounds that act independently. Successful application of field format assays to peptide libraries has been described using melanophore cells, which are pigmented amphibian cells that can couple a wide variety of G-protein-coupled receptors to pigmentation changes[14].

Solutions to the additivity/compound separation problem generally involve the physical isolation of single compounds, exploiting solid-phase library-synthesis techniques. One solution has been presented by the Affymax group[15]; a screening assay was carried out using a bead-based combinatorial library arrayed on a machined plate containing 0.3 μl wells. A bead slurry, together with enzyme and agarose (to control evaporation), was 'squeegeed' into the plate and test compound released photochemically. Enzymatic activity was detected using a liquid overlay of fluorogenic substrate.

An alternative, 'well-less' strategy is to isolate samples by patterning surfaces with hydrophilic/hydrophobic arrays. Clearly, this strategy presents additional problems with evaporation and sample handling, but assay set-up and execution can be as straightforward as film processing. At Harvard, this strategy has been successfully employed to evaluate both cell proliferation[16] and growth inhibition[17] in droplets of 0.05–0.2 μl. Here again, the presence of the bead in the assay can present significant technical difficulties, but the achievable throughput and reagent savings may offset this shortcoming.

Alternative container formats for bead-based assays have also been described[18,19]. A 9,600-well plate (0.2 μl volume) has been molded from special, low-fluorescence plastic. The pyramidal-shaped wells of this plate, together with the pointed intrawell boundaries, means that individual beads segregate into defined wells, much like the Japanese pachinko games. These wells then provide a container for subsequent biological evaluation. Evaporation at this volume becomes more significant, but assays based on both fluorogenic enzyme–substrate combinations and bacterial growth have been demonstrated. Finally, a 3,456-well plate (1 μl volume) has been disclosed by Aurora Biosciences[20]. This plate is said to control evaporation through a novel, inverted-well design, but it is not yet available commercially.

Fluidics

Microliter-volume control of diverse fluids is an important milestone in assay miniaturization. Several groups[21,22] have demonstrated fluidics for assay set-up and validation, and it is likely that present needs will establish a competitive environment that will drive further development of microliter fluidics. There are two distinct problems for fluidics in miniaturized HTS systems: reformatting (moving compounds into miniaturized formats) and dispensing (placing bioreagents into microwell plates). The key requirement for a dispenser, on the one hand, is the capability to place nearly identical droplets of aqueous solutions into contact with each test sample without cross-contamination – a requirement that is now met using technologies developed for ink dispensing in high-speed printing applications. The key requirement for reformatting, on the other hand, is to handle sample stocks, generally dissolved in organic solvents (such as DMSO), without contamination. This requirement typically imposes a washable, or disposable, sample-handling system running in parallel.

The need for high-speed reformatting is evident. In a recent evaluation of assay strategies for miniaturization beyond 96-well plates by researchers at Glaxo Wellcome, a thorough evaluation of a complete screening solution (including reformatting of compound collections, establishment of assay fluidics and detection) based on 96-, 384- and 864-well plates was described[23]. All three formats could be read by commercially available readers after slight modifications, but the reformatting process limited throughput.

Detection

Miniaturization requires changes in detection strategies, away from radioactive methods and towards methods based on fluorescence. Presently, radioactivity is used in about half of the assays employed in HTS. Many, if not most, of these assay types have an analogous fluorescence approach, although it is inevitable that particular assays will prove to be difficult or impossible to adapt. Based on currently available data, we expect that fluorescence methods will be feasible in >90% of HTS assays. This optimism is due in part to the versatility of fluorescence techniques – applications of simple ('prompt') fluorescence to HTS are common, but tend to be limited by background effects, both from the biological milieu and from photophysical effects such as light scattering. As discussed above, these methods can readily be scaled to microvolumes. This fact has allowed the measurement of single-molecule fluorescence, using an instrumental technique known as fluorescence-correlation spectroscopy[24]. Other techniques, such as time-resolved fluorescence[9,25,26] and ratiometric dyes, can be used to improve the reliability of fluorescence measurements.

Other new detection strategies based on fluorescence have been reported recently. At Novartis (Basel, Switzerland), a technique has been developed that involves performing assays on planar waveguides[27]. This technique, named FOBIA (fast optical biospecific interaction analysis), employs evanescent wave-derived fluorescence to excite surface-adsorbed molecules selectively. To date, FOBIA has only been used to analyze hybridization of fluorescently-labeled oligonucleotides and surface-adsorption of antibodies, but it is an interesting strategy for the future. Another approach to ultrasensitive fluorescence detection is the use of multichannel glass fibers that more efficiently couple photonics to detection, as fewer lenses are used and laser light is addressed individually to each well[28]. This sensitive, parallel technique holds promise for rapid kinetics; it has been applied to kinetic analysis of PCR reactions.

Alternative detection strategies based on fluorescence polarization[29], fluorescence correlation[24] and direct measurement of protein–protein interactions[30] all have potential in these miniaturized formats. However, fluorescence polarization suffers from the drawback that it does not increase the sensitivity of the measurement with respect to background (as measurement of polarization presumes measurement of fluorescence), although it does provide the advantage of being a direct and fairly universal probe for binding. Fluorescence-correlation spectroscopy is more sensitive, but is applicable only for screening within a relatively narrow range of concentrations. Saturation of the observation field with molecules becomes limiting at higher-than-optimal concentrations, while slow data accumulation becomes limiting at lower-than-optimal concentrations.

As fluorescence is an inherently miniaturizable detection strategy, the advent of more-advanced detection strategies in miniaturized formats, primarily based on fluorimetry, are imminent. Once researchers can carry out more tests without compromising their experimental protocol, the true power of miniaturized formats will be realized. The evolutionary track parallels that of modern genomics, where changes in speed and cost have enabled new and more powerful applications of the technology (such as comparative and functional genomics).

Enzyme assays

Several groups[18,19,23] have demonstrated enzyme-based assays in miniaturized formats. At Pharmacopeia, enzyme assays have been used to test the limits of miniaturization in 1,536-well plates and to provide a basis for assessing our miniaturized HTS system as a whole. Combining an active-enzyme-based screen with an active-production library, synthesized and fully characterized at Pharmacopeia[31], it has been possible to compare directly screening results from 96-well with 1,536-well formats (Figure 4). In this experiment, an active library, arrayed in 96-well plates, was taken through the entire 1,536-screening process, from reformatting (moving samples from 96-well to 1,536-well plates), through dispensing (two reagents, enzyme and substrate, added in sequence), to analysis (field fluorimetry, at a fixed time after initiation). The results shown in Figure 4 represent a single time point in the course of the enzymatic reaction, but comparable results have been observed using a kinetically-based analysis (against carbonic anhydrase, data not shown).

Cell-based reporter assays

Functional cell-based assays in miniaturized formats are increasing in importance as primary screening assays. These cell-based assays can take several days from initiation to analysis, which puts additional constraints on assay design, but by carefully controlling humidity and airflow, it is possible to grow cells in 1,536-well plates (Figure 5). When cells containing a luciferase reporter gene are plated and then stimulated, luciferase activity can be detected photometrically. At this scale, the bottom surface of the well will support a few thousand cells at confluence (about 20-fold fewer than conventional plates), but it is probably sufficient to provide a statistical population of cells for a screening assay. Smaller assays (such as at the single-cell level) will present challenging experimental problems for HTS because of stochastic variation in cell populations; these variations will translate into 'noise' in a screening assay.

A novel strategy to carry out functional, reporter-gene assays has recently been described by scientists at the University of California at San Diego and Aurora Biosciences[7]. In this assay, the well-studied bacterial enzyme β-lactamase has been conscripted for use as the reporter. When combined with a ratiometric β-lactamase substrate that localizes in the cytoplasm, a fluorogenic reporter gene assay is possible. There are several advantages

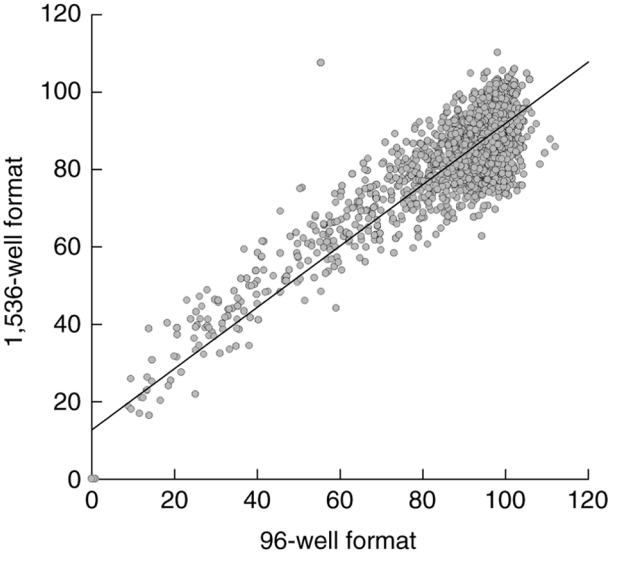

Figure 4. *Correlation of activity in a protease assay between 96-well and 1,536-well formats. The aspartyl protease plasmepsin was assayed using the fluorogenic enzyme substrate DABCY-(γ-Abu)-ERMFLSFP-(EDANS), which generates a fluorescence signal (λ_{ex} 350 nm, λ_{m} 490 nm) when cleaved (scissile bond is the Phe-Leu bond above). The assay was carried out as follows: sixteen 96-well microtiter plates containing active library compounds in columns 2 through 12 dried on the surface of each well were prepared, diluted tenfold to a screening concentration, and assayed according to published procedures[31]. Some of the compound stock remaining was reformatted from the 96-well plate to white, 1,536-well plates (Corning) and dried. The 1,536 assay was performed using the same reagent concentrations as the 96-well assay, except that 0.5 μl of assay buffer containing the substrate was added using a bioreagent dispenser, the library compounds were allowed to dissolve, and a second dispense step of 0.5 μl enzyme was performed to the same wells. After 10 min incubation, the plate was placed into a field imager (SAIC SpeedReader, with filters: λ_{ex} 350 nm, λ_{em} 510 nm) and an image (0.25 sec exposure) was collected. Data from the 1,536-well plate were directly correlated back to the data in the 96-well based assay to derive the plot shown. Relative protease activity is shown as a percentage of control activity along each axis. The intercept on the y (1,536) axis is positive because empty wells (rather than known inhibitors) were used as controls; the intercept therefore represents the residual (background) fluorescence in the 1,536-well plate. The correlation between the two formats is good (r = 0.92) and the active compounds observed in either format are comparable.*

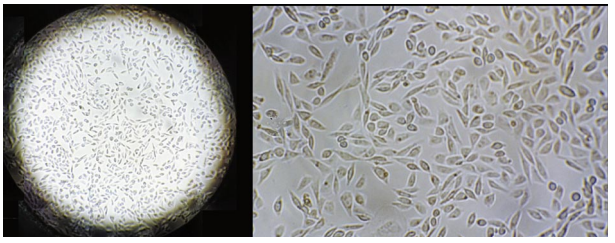

Figure 5. *Cell culture in 1,536-well microtiter plates. Mammalian cells can be seeded and grown in tissue-culture treated polystyrene plates. Shown are HEK293 cells after 24 h of growth at 37°C and 95% humidity in a specially constructed tissue-culture chamber.*

to this approach; first, β-lactamase is not normally found in the mammalian cytoplasm, which means that the detection limit is as low as the non-enzymatic decomposition rate of the substrate. Second, because individual cells can be loaded with the fluorescent substrate, FACS (fluorescence-activated cell sorter) selection of cell lines for assay development is possible. Third, because the detection is by ratiometric fluorimetry of an enzymatic activity, absolute concentrations of the cytoplasmic fluor can be determined, and enzymatic amplification of the signal can significantly improve the sensitivity of the assay. The major technical disadvantage to this approach is the high cytoplasmic concentrations of the synthetic substrate needed (~0.1 mM, which is similar to the intracellular concentration of ATP); it could conceivably affect cellular physiology and lead to non-correlative results. However, this disadvantage may be overcome in the future by the use of brighter fluors or improved (lower K_m) substrates for β-lactamase.

Table 2. Progress in HTS assay miniaturization

Assay class	Possible?	Demonstrated?	Limitations
Enzymatic	✓	✓	Fluorometric
Cell-based reporter gene	✓	✓	Class of reporter gene
Receptor binding (homogeneous)	✓	~	Speed of readout
ELISA	✓	~	Reproducibility of washing protocol
Proximity-based binding assays	~	~	Sensitivity
Receptor binding (radiometric, separations-based)	~	~	Sensitivity/washing

Summary and conclusions

Miniaturization of assays is a continually evolving, historical process that follows the progress of technology. In other words, if an assay *can* be miniaturized, why *wouldn't* the smaller format be used? To provide a subjective answer to the question: the return on investment (both in capital and in time) that weighs the costs of changing established practices against the benefits of new technology, must be analyzed. The 1,536-well miniaturized format is clearly feasible as the next evolutionary step in screening, so the question for conversion is rather audaciously phrased: miniaturization to 1,536 *can* be done, so *when* will it overtake the 96-well format as the preferred screening format?

In the current environment, it is relevant to ask: what is the lifespan of the 1,536 format? In truth, of course, no one knows, but we are able to speculate: based on recent estimates of the number of drug targets and the number of human genes, it seems reasonable to approximate that there will be 10,000 potential drug targets or counter targets that will be amenable to HTS. Similarly, there are probably ~100 million compounds that could be synthesized for screening using existing chemistries. Given these assumptions, the total database for structure–activity is about 10^{12} tests (a few terabytes of information), or (a relatively modest) 650 million 1,536 plates. This estimate is very approximate, but the numbers are clearly achievable (for comparison, the human genome contains about three billion nucleotides). Thus, the 1,536-well plate will probably suffice for the foreseeable future of HTS, and will lead the way towards a future of 'database mining' for lead identification.

Regardless of the future prospects for any particular format, miniaturization in HTS is rapidly transforming the outlook for lead discovery in pharmaceutical research. Currently, several diverse assays have been demonstrated in miniaturized formats, but the needs of HTS are very broad. There is now a 'critical mass' of interest in new screening technologies that will drive innovation and invention in the area.

As a progress report, Table 2 summarizes the assays that have been demonstrated in miniaturized formats thus far.

In conclusion, to answer the questions posed by the title of this article:

- How fast? – It depends on the current benchmark, but a faster assay is generally an improved assay, not only because of the competitive nature of drug discovery but also because of concern for the stability of biological reagents.
- How small? – Again, it depends. Miniaturization places additional constraints on assay design. In the near future, volumes of about 1 μl (e.g. the 1,536-well plate) will be adequate for many primary screening applications. In the more distant future, it is likely that additional applications for HTS will become evident and these applications will affect the choice of the 'next' format.
- How soon? – Based on the answers to the first two questions, it is safe to say that miniaturization is a process, and a 1,536-well-based screening system is a constantly evolving and adapting target. Consequently, it is simply a matter of time and is based upon the needs and budgets of individual companies within the industry.

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