Miniaturizing screening: how low can we go *today*?

Roger Bosse, Chantal Illy, Jack Elands and Daniel Chelsky

Miniaturization in HTS is perceived as essential by pharmaceutical screening laboratories to accommodate the enormous increase in compounds and targets over the past few years. The two primary goals are to increase throughput while decreasing costs. However, although the desire is there, what is the reality of being able to achieve these goals?

Roger Bosse
Chantal Illy
Jack Elands and
***Daniel Chelsky**
BioSignal
1744 William Street
Montreal, Quebec
Canada H3J 1R4
*tel: +1 514 937 1010
fax: +1 514 937 0777
e-mail: dchelsky@biosignal.com

▼ As advances are made in the efforts to shrink the volume required for each assay point and, thus, the time and cost to screen, the progress in each technology that is required to accomplish these goals is not uniform. While advances in miniaturized screening have been significant, crucial gaps can appear at all stages of the process, making implementation difficult or even impossible. For example, it is not sufficient that an assay can be performed in a reduced volume, as it must also maintain an acceptable data quality, show a proportional reduction in the quantities of reagents required, and an actual reduction in the time taken for the assay. Some complete systems that can produce a high-quality miniaturized screen, while reducing time and cost, do exist today. Finding and assembling such a system only requires doing some homework.

The desire for potent leads

Lead discovery has become highly dependent on the methodical evaluation of chemical collections against a series of biochemical targets. This process has become so important to the pharmaceutical industry that, over the past ten years, most companies have established one or more well-staffed and well-funded centralized screening groups. These are often directed at a senior level, again demonstrating the importance given to this early phase of drug discovery.

The vision, or desire, is to find potent leads for a large number of biochemical targets, which have qualities suitable for medicinal chemistry. To achieve this goal, the number of compounds available for screening has been increased dramatically. In addition to making existing compounds more readily available for screening, new compounds have been acquired through purchases or corporate mergers, or through synthesis, often using combinatorial chemistry. As a result, there has been a dramatic increase in the number of compounds available to the typical screening laboratory over the past decade, going from <50,000 at the beginning of the 1990s up to 300,000 or more[1]. As the number of compounds for screening has increased, so has the number of targets. In addition to the traditional process of slowly uncovering the biochemical relationships in a disease, the cloning and sequencing of various genomes from viruses to humans, has increased the rate of target discovery by at least tenfold[2].

The combined effect of increases in compound and target numbers has required significant improvements in the speed and reliability of the screening process. This has resulted in a demand for automation, integrated screening systems, homogeneous screening formats and a consolidation of effort. Coupled to these demands has been the effort to contain costs, consistent with a highly competitive environment. The perceived solution, particularly for speed and cost reduction, has been to miniaturize the required technologies. High-density, low-volume formats are being sought to reduce the cost of reagents as well as to enable the screening of many more compounds per manipulation.

Efforts to miniaturize have been slow. While compound collections and the number of targets have increased rapidly, the 96-well plate is still dominant as the format of choice[3]. Meanwhile, although the volume is decreasing on average, it is still typically in the 100 μl range. Some screening laboratories have made a serious commitment to 384-well plate formats, fewer are exploring

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1536-well plate formats and only a couple are making a commitment to 3456-well plate formats. The transition to miniaturized screening has been slowly incremental despite the need for these technologies. The reasons for this are evident to those who have tried to use these formats. Several changes in the screening process must occur even before the move can be made to the 384-well plate format. These include high-density plates, low-volume, high-density pipettors, homogeneous screening formats with sensitivity at low volumes, compound libraries in the appropriate format, and detectors capable of reading at higher densities.

Non-microtiter plate formats are also in development as an alternative to shrinking the current format. They will, most likely, displace current technologies in the next five to ten years. However, although these approaches show promise, there are still significant hurdles to overcome and they are not widely available. Therefore, what is available today from a practical perspective is a system using wells that fits into the standard microtiter plate footprint. This format is therefore the focus of this article.

The reality

Microplates

Microplate availability is the first obstacle to changing over to high-density formats. Despite their appearance as a low-technology tool, high-quality plates must meet very stringent requirements for plate flatness and rigidity, well positioning, well depth, and color intensity (whiteness) and uniformity. In addition, the plates must be produced in several varieties to accommodate different screening formats, including polypropylene and polystyrene, white and black, clear, clear bottoms with white or black side walls, round, V and flat-bottom, deep, standard- and shallow-well, and low- or standard-profile. As the well density increases, the demands for uniformity increase as well, particularly with respect to adherence to specifications for flatness, well depth and well position. Two approaches have been taken with the 1536-well plate, a 10–15 μl variety with round or square wells available from several manufacturers, and a low volume (1–2 μl), shallow-well plate, designed by Corning (Acton, MA, USA), in collaboration with Pharmacopeia (Princeton, NJ, USA). While the larger-format wells have been more popular because of the limitations in liquid handling of the smaller formats, the Corning plate is the only plate to significantly shrink the volume of screens in the 1536-well plate format. An alternative for microliter volume assays is the 3456-well NanoWell plate (Aurora Biosciences, San Diego, CA, USA)[4].

If the intention is to reduce the volume to 10–15 μl, with no special requirement for a high plate density, an intermediate approach is available in the form of a low-volume 384-well plate from Greiner (Frickenhausen, Germany). With conical, shallow wells, in a standard height plate, this offers the ability to use small volumes with an easy interface to 96-tip or 384-tip liquid handlers. An added, probably unexpected, benefit is the substantially improved signal obtained because of the close proximity of the sample to the detector. This effect has been observed with detectors including the Packard Instrument Company's (Meriden, CT, USA) TopCount (radioactivity and luminescence) and AlphaQuest (AlphaScreen technology) microplate analysers. Other options that will give similar results are the low-volume 96- or 384-well HE plates from LJL BioSystems (Sunnyvale, CA, USA) or the low-volume 96- or 384-well ProxiPlate microplates from Packard Instrument Company.

Liquid handling

Automation, primarily in liquid handling, has made significant improvements in recent years. The most important change has been the addition of reliable 96- and 384-tip pipetting stations that incorporate on-board plate handling capabilities. Several of these stations have also been developed to handle lower volumes, even down to 0.25 μl, with reasonable accuracy, either into dry or wet wells. A hands-on evaluation of such instruments has been published by an HTS laboratory[5]. It was found that reproducible low-volume pipetting at high speed is actually available today from at least one or two of the instruments tested. With increasing density of the format, the uniformity of the tips becomes more important. This can be accommodated by fixed tips such as those offered by Tomtec (Hamden, CT, USA) or by Robbins Scientific (Sunnyvale, CA, USA). Low-volume, narrow-profile disposable tips are also available that can be used with 1536-well plates. However, these have only recently achieved the uniformity required for reproducible results.

The increase in the rate of plate handling using a 384-tip system compared with the 4- or 8-tip devices, designed primarily for dilutions that predominated ten years ago, is substantial. The addition of plate stackers and a device to shuffle the plates between stackers and liquid handlers, such as on the PlateTrak (CCS Packard; Torrance, CA, USA), the CyBi-Well 384/1536 (CyBio AG, Jena, Germany) and the Quadra 384 (Tomtec), have added even more capability. These on-board systems, even when incorporated into a larger integrated system, have the advantage of dedicated support and speed.

In addition to traditional pipette tip-based systems, many alternative systems have been developed. The most popular concept has been 'inkjet' style dispensing which can deliver volumes down to 20 nl into sub-microliter wells[6–8]. However, these have several limitations. Only a small number of tips per instrument are typically available. While the inkjets dispense rapidly, they require substantial time to load, dispense and wash between additions of different reagents. They are most efficient, therefore, when they can be used to load once with multiple dispensings of the same reagent.

They are also relatively delicate and clog easily, making compound addition the most difficult application. Other approaches, such as pin tools, avoid some of the limitations of inkjet devices such as tip number and clogging. Pin tools, however, require direct contact with the assay well or its contents, and lack variable volume control. For these reasons and others, they are not well used outside of genomics applications where their limitations are far outweighed by their low cost, reliability and speed[6].

Homogeneous screening formats

As screening volumes reduce, the ability to perform separation steps also decreases. While at least one plate-washer is available (Embla 1536; Skatron, Sterling, VA, USA) that can handle 1536-well plates, it does so by alternating between dispense and aspiration heads, creating a potential bottleneck in any high-throughput screen. Filtration, even for 384-well plates, is simply not available. Therefore, homogeneous screening formats are essential to miniaturization, requiring only a series of additions to perform the screen. While a variety of homogeneous formats are available, most cannot be formatted beyond 384-well plates. This is primarily because of the available signal. Although the signal does not necessarily decrease linearly with volume, losses in the signal are often more than can be tolerated. Fortunately, several homogeneous technologies are effective in the 384-well plate format and a few are effective with 1536-well plates.

One of the simplest approaches to high-density screening is the use of luminescence. With low background levels, a relatively high signal, few causes for interference, and several available detectors, luminescence has been shown to work in 1536-well plates. A good example is a cell-based reporter gene assay[9] performed in a final volume of 3 μl. Compounds were added in dimethylsulfoxide (DMSO) and dried to produce a thin film using a vacuum chamber. Assay medium containing activators was then added in 1 μl volumes using a Hydra 384 (Robbins Scientific) followed by the addition of 5000 cells in 1 μl. Five hours later, 1 μl LucLite (Packard Instrument Company) was added and the signal was detected, using a Tundra camera (Imaging Research, St Catharines, Ontario, Canada). The data obtained was of a high quality with results said to be consistent with the same compounds tested in a 96-well plate format.

Scintillation Proximity Assays (SPA; Amersham, Uppsala, Sweden), Homogeneous Time Resolved Fluorescence (HTRF; Packard Instrument Company), and FlashPlate (NEN Life Science Products, Boston, MA, USA) microplate assays can all be formatted to 384-well plates, but with reduced performance. Amersham has introduced a new version of SPA beads with a red-shifted emission called LEADseeker, coupled to a charge-coupled device (CCD)-based detector, which can be used with 1536-well plates. However, the current beads settle very quickly, posing a challenge for accurate pipetting. Another new technology for non-radioactive homogeneous binding assays is the FMAT system (PE Biosystems, Foster City, CA, USA). This approach uses laser scanning of a small volume to detect binding of a fluorescent ligand to cells or beads at a higher concentration than in the surrounding medium[10]. This is available commercially as a dedicated instrument for 96- and 384-well plates. Pharmacopeia, however, has demonstrated 1536-well plate capability, with a prototype instrument using very similar technology. Because of the nature of the detection, the signal is not significantly affected by changing to the higher-density format.

Fluorescence resonance energy transfer (FRET) has been used successfully in 1536-well plates in a screening demonstration using a protease assay[11]. A peptide substrate was constructed with a fluorophore at one end [4-dimethylaminoazobenzene-4′-sulfonyl chloride (DABSYL)] and a quencher at the other end [5-(2-aminoethylamino)-1-naphthalene-sulfonic acid (EDANS)]. Cleavage of the substrate results in increased fluorescence. The screen was run by preloading each assay well with compounds dissolved in a volatile solvent, followed by a drying step, resulting in a thin film on the bottom of each well of the Corning 1536-well plate. Substrate (0.5 μl) and enzyme (Plasmepsin, 0.5 μl) were added using an inkjet style dispenser followed by detection on a SAIC (San Diego, CA, USA) SpeedReader CCD-based detector. Results were essentially identical to those obtained with the same compounds screened in sixteen 96-well plates[11].

Fluorescence polarization[12] in 1536-well plates has been made possible by the Acquest (LJL BioSystems) instrument. Based on the change in rotation rate of a small tagged molecule when it changes from the free to the bound state, fluorescence polarization is gaining popularity as a non-radioactive, homogeneous assay system. It has been shown to be useful for many assays[13] including tyrosine kinase, protease and binding assays, including a 1536-well assay for peptide binding to the cyclin dependent kinase 2–cyclin E complex[14].

A new technology on the market, designed specifically for low-volume assays, is AlphaScreen (BioSignal, Montreal, Quebec, Canada). Based on the proximity of two beads, a highly amplified signal is generated through a cascade of chemical events. When the donor bead is exposed to 680 nm light, singlet-state oxygen is generated, and this diffuses a short distance before decaying to the ground state. If the acceptor bead is in close proximity, the singlet-state oxygen reacts with chemiluminescent molecules to produce energy, which is transferred to fluorophores in the same bead. The resultant light is emitted at approximately 600 nm, a shorter wavelength than that of the incident light. This, coupled with time-resolved detection, results in very low background levels. In a typical assay, such as examining TNFα binding to its receptor, a high signal with a signal–background ratio of >10 is maintained even as the volume of the assay is reduced from 75 to 3 μl (Fig. 1). In a functional

assay example determining cAMP levels in forskolin-stimulated CHO cells, it can be seen that the quality of the data does not suffer significantly when moving from a standard 25 μl, 384-well format (Figs 2a, 2b) to a 6.6 μl, 1536-well format (Figs 2c, 2d). In both instances, a high signal is maintained and the pharmacology remains unchanged.

Compound libraries

One of the most significant limitations to low-volume screening is the dispensing of compounds into the assay. It is standard practice to add compounds, pre-dissolved in DMSO, directly to the assay well. Although common ten years ago, the practice of diluting compounds using aqueous solvents prior to addition to reduce the final concentration of DMSO, is disappearing. Too many compounds were found to 'fall out' of solution when even a small quantity of water was introduced at compound concentrations above 100 μM. Therefore, it is more common, and a better practice, to add compounds diluted in pure DMSO directly to the assay. As a consequence, keeping final DMSO concentrations at 1–10%, depending on the tolerance of the individual assay, becomes difficult when the total assay volume is only five or ten microliters. At a 10 μl assay volume, for example, a compound volume of 0.25 μl would result in a final concentration of 2.5% DMSO. This concentration is generally only acceptable for cell-free assays.

The use of an inkjet liquid handling device can solve the DMSO problem by limiting the volume of addition to only a few nanoliters. These systems, as discussed above, can be slow, susceptible to clogging, and generally less reliable than standard tip-based devices. However, if care is taken to ensure solubility of compounds and if the compound addition step is carried out prior to the initiation of screening so as not to impact on the timing of the assay, such devices can be a very good alternative to pushing standard tips to the limits of their accuracy. Another approach, which is gaining popularity, is the use of a volatile solvent such as methanol to enable the addition of compounds in a larger volume, followed by air-drying to create a thin film on the bottom of the well. The compound then goes into solution when the assay reagents are added. The problem with this approach is that compounds with high clogP values (hydrophobic) tend to go into solution very slowly and can be under-represented in the screen. Several screening laboratories claim that non-volatile additives in the original solution will enhance resolubilization of the compounds in the assay. However, none have yet volunteered the nature of their secret additives.

An additional concern for high-density compound screening is that most corporate compound collections are currently formatted in 96-well plates. Several companies that currently routinely screen using 384-well plates have taken the step of establishing a second set of compound plates at that density. This enables a single 384-tip addition of compounds directly from the final dilution plate to the screen. Likewise, in changing to 1536-well plates, compound formatting into these plates will be necessary to take full advantage of the higher density. Although a single 1536-tip addition is not yet possible (except perhaps with a pin tool device), attempting to add compounds from four or 16 different compound plates for each assay plate in a high-throughput environment would create serious logistical and time-delay problems.

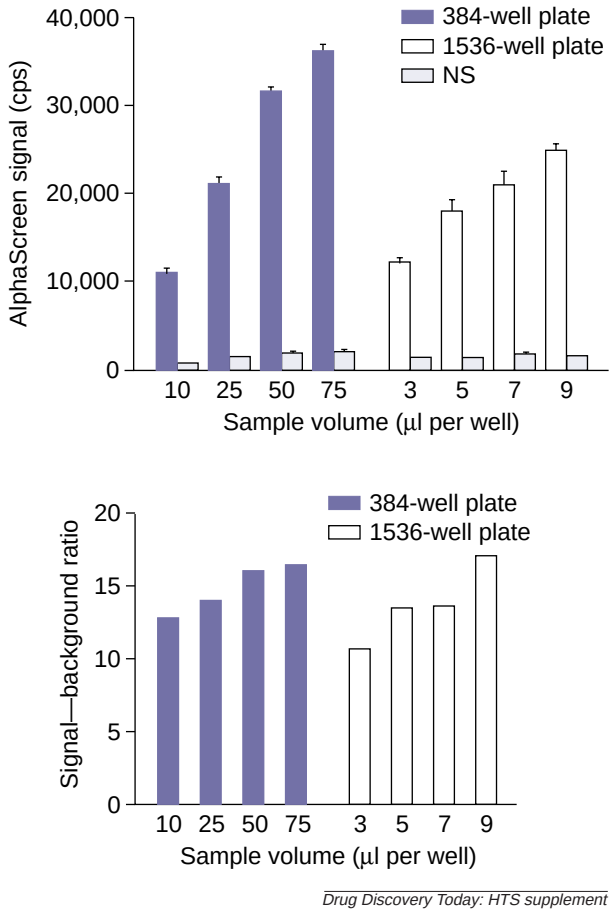

Figure 1. Binding assay between TNFα and the soluble portion of the TNFα receptor using AlphaScreen technology (Packard Instrument Company, Meriden, CT, USA). Signal and background (top) and signal–background ratio (bottom) are shown for a range of volumes in either a standard 384-well plate (Corning, Acton, MA, USA) or a standard 1536-well plate (Greiner, Frickenhausen, Germany) (data unpublished).

High-density readers

As screeners were first contemplating the merits and pitfalls of micro-volume screening, they tested not only the available assay formats, but also the possibilities for different plate densities. Most screeners looked at multiples of 96 such as 384, 864,

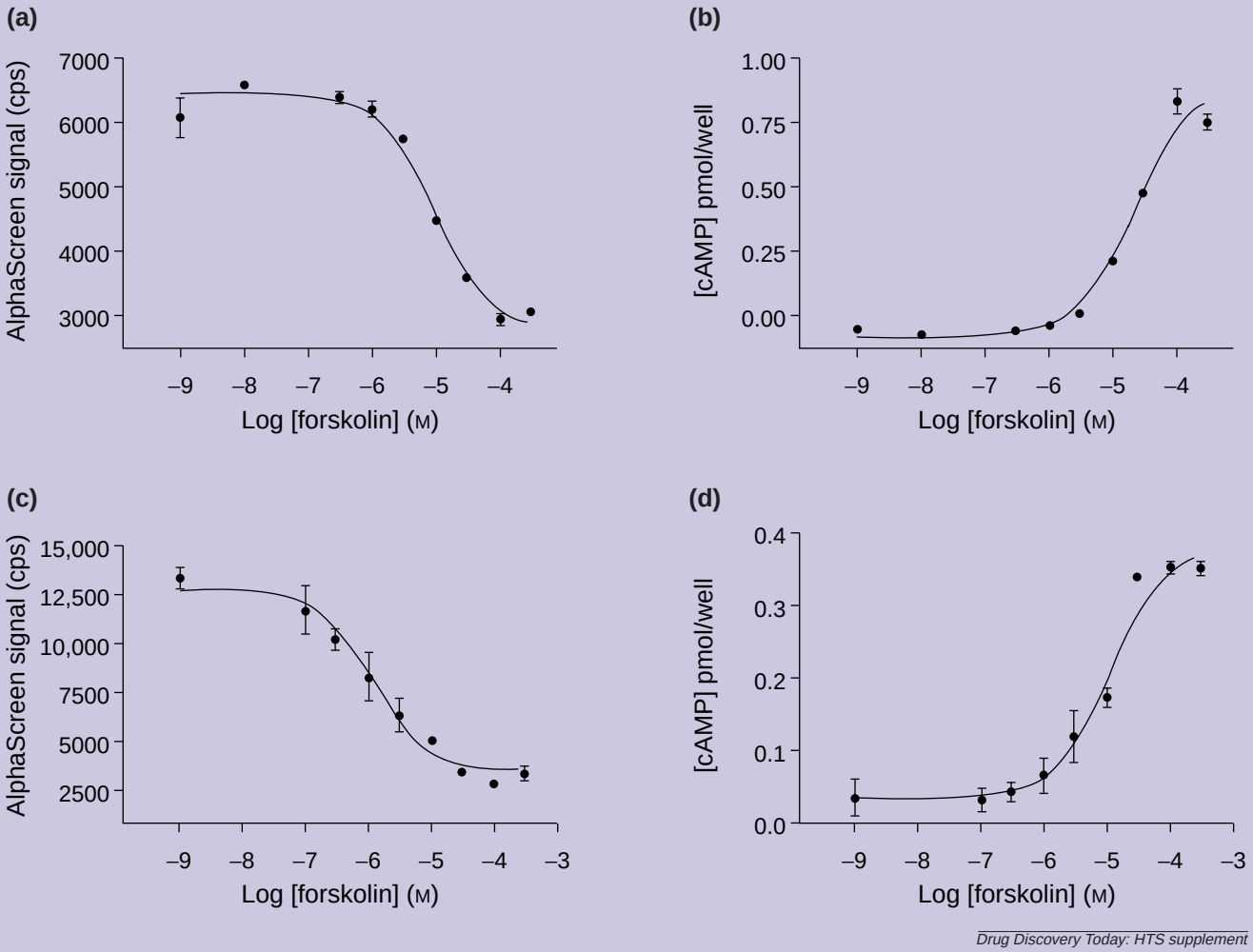

Figure 2. Functional assay in CHO cells for response of cAMP levels to forskolin. The assay was performed (a) with 10,000 cells in a 22.5 μl final volume using 384-well OptiPlate microplates (Packard Instrument Company, Meriden, CT, USA) or (b) with 3000 cells in 6.6 μl final volume using 1536-well plates (Greiner, Frickenhausen, Germany). Using a standard curve generated with a known quantity of cAMP, raw values were converted to picomoles-per-well cAMP for the (b) 384- and (d) 1536-well assays (data unpublished).

1536, 3456 and 9600-well plates to take advantage of existing instrumentation, compound formatting and software. To accommodate the pervading experimental atmosphere, many instrument manufacturers offered open-format systems, typically based on CCD cameras, or multi-format systems with discrete detectors coupled to plate positioners driven by accurate stepping motors. Although both approaches offer substantial flexibility, most screeners have settled for 384-well plates and, when the screening technology allows, on 1536-well plates. The 1536-well plate format has become so accepted as the near future of screening that many customers are not interested in purchasing new instruments or technologies if they do not promise to function on this scale. This has, in turn, ensured that instrument manufacturers will treat 1536-well capability as a required specification for new instrument development.

Until a few years ago, most detectors were based on photomultiplier tube technology. This approach offers high sensitivity, low background levels, and low cost of production. The main failing of such systems is their relatively slow speed. This can be compensated for, however, by using multiple detectors per system, making them just as fast as the latest CCD camera detectors. CCD cameras offer simultaneous readout of an entire plate, usually resulting in a rapid read time. However, they have several disadvantages that make them less than optimal for all applications. The price of such systems starts at over $100,000, because of the high cost of the camera with its expensive components including a high-quality 'megapixel' CCD, camera controller and cooling system. Costs are even higher for the new generation of ultra-low-temperature cameras (CCDs cooled to −100°C). These cameras are designed to avoid another drawback

of most camera systems, that being high noise levels. Even for cameras cooled to −20°C or −30°C, noise levels can be significant and this seriously affects signal-to-background ratios at low-signal intensities. CCD cameras also typically rely on large lenses to collect light from the entire plate. These add to the cost, lose a significant portion of the light, and make uniform illumination of the plate difficult for those applications requiring an excitation light source.

A variety of fluorescence detectors are available in the 384-well plate format. For non-radioactive applications, the Victor (Perkin Elmer Life Sciences, Gaithersburg, MD, USA) and Analyst (LJL BioSystems) can handle fluorescence, time-resolved fluorescence and luminescence. The Analyst is also capable of fluorescence polarization. For scintillation and luminescence applications, the TopCount can also handle 384-well plates, and has been shown to be effective for the detection of radioactivity with scintillation cocktail, SPA beads, and FlashPlate microplates as well as for luminescence[15]. The TopCount and related instruments, however, cannot handle 1536-well plates.

For radioactive applications, only the LEADseeker instrument, with its CCD camera-based detection[16], can handle the higher-density plates, but this is limited to SPA. For luminescence in 1536-well plates, several detectors are available, including the LEADseeker. One that is particularly interesting is the NorthStar by Tropix (Bedford, MA, USA), with its CCD camera detector, 16 independent injectors and filter wheel. Other instruments for use with 1536-well plates include the Acquest for fluorescence polarization (multipurpose reader) and the AlphaQuest microplate analyser. Both are photomultiplier tube based and can rapidly read a plate and produce screening-quality data. An instrument recently introduced with capabilities similar to the Acquest, but using a CCD-based detector, is the ViewLux from Perkin Elmer Life Sciences.

Conclusions

We are still on the learning curve for low-volume screening. In a survey two years ago[3], it was determined that 7% of HTS screens were being performed in 384-well plates. Those same laboratories predicted that over 50% of their screens would still be run in 384-well plates today. No one was using 1536-well plates, but many thought that 10% of their screens would be run in that format today. Based on this survey and other very informal surveys taken in the industry, it is clear that the progress to high-density format screening is occurring much more slowly than expected. This is mainly because of the fact that much of the infrastructure necessary for high-density screening is only now being created, including assay technologies, liquid handling and detectors.

Now that there is finally access to this infrastructure, the trend towards using low volumes should begin to accelerate. The main hurdle for this progress is the much higher budget often required to acquire the necessary instrumentation, as well as the time required to evaluate, set up and validate the new technologies. However, the time and money will be well-spent for the productivity gains that can be made by the transition. Given the continued acceleration in the size of compound collections and the number of screening targets, there is little choice but to incorporate the 1536-well plate screening format as the near term solution.

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