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Challenges and solutions to ultra-high-throughput screening assay miniaturization: submicroliter fluid handling

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The miniaturization of HTS assays is an important objective in the pharmaceutical industry. The ability to perform primary screening assays in high-density micro-well plates at volumes of 1–2 μl will accelerate the early stages of drug discovery and reduce costs. Ultra-HTS (uHTS) assays require an accurate and reliable means of fluid handling in the submicroliter volume range. This relates to the design of instrumentation for dispensing fluids, as well as assay plates. Fluid handling has been a major obstacle to the full implementation of miniaturized assays. This report focusses on current approaches to submicroliter fluid handling in high-density multi-well plates.

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▼ The need to discover new pharmaceuticals rapidly and at a lower cost continues to change the way drug discovery is practiced within the pharmaceutical industry. Increasingly, drug developers are confronting the need to streamline their processes, improve the robustness of their screening operations and enhance the quality of early development candidates. HTS of large collections of compounds against therapeutic targets is increasingly employed as part of an early-stage strategy for identifying active chemotypes, which can eventually be developed into marketable drugs[1,2]. The number of compounds screened in HTS laboratories runs into the millions[3]. As the number of therapeutic targets increase, so will demand for HTS, creating pressure to improve efficiency. Ultra-HTS (uHTS), in which more compounds are screened at lower cost and in less time, has been a major goal[4–6]. The expansion of the role of uHTS in the drug development process creates the need for new technologies.

Technology that enables the miniaturization of screening assays has been one route towards accomplishing uHTS (Refs 7,8). Miniaturized assays consume less reagents and compounds, and reduce the cost of screening. Miniaturized assays can also be performed faster and therefore reduce the time required to complete primary screens.

Assay miniaturization has followed an evolutionary process, starting with the movement of tests away from milliliter volumes in test tubes, and towards microliter volumes in standard 96-well micro-plate format[9]. This evolution has continued with the increased utilization of 384-well plates, which enable assays to be performed in the range of 10–20 μl. The next logical step is the development of assays in the submicroliter volume range. Assays performed in 1536-well plates at volumes of <2 μl would significantly reduce the cost and time of screening[10]. The greatest impact would be for screens performed with very large compound collections.

Specific challenges to the implementation of a miniaturized screening platform based on the use of 1536-well plates include the development of instrumentation for detection and fluid handling. Miniaturized assays performed at volumes of 2 μl or less require fluid handling equipment capable of operating reliably in the submicroliter volume range. Assay plates also need to accommodate these volumes. Many of these challenges are being actively pursued by the suppliers of screening instruments and commodities. Detectors

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capable of reading 1536-well plates have been on the market for more than a year[11]. Although dispensers capable of working in the submicroliter volume range are beginning to become available[12], fluid handling remains the greatest barrier to full implementation of miniaturized uHTS.

This article describes the problems and challenges associated with fluid handling for miniaturized uHTS. The focus will be on approaches that have been undertaken to develop instruments for fluid handling in the submicroliter volume range. We will also discuss the impact of plate design for assay miniaturization in regard to fluid handling at these volumes.

Design considerations

Many factors and requirements have an impact on the design and development of devices intended for fluid handling in miniaturized uHTS assays. The selection of any design should be driven by how well it can meet the specific needs of its intended use[13]. Fluid handling is required in two aspects of the uHTS process. First, test compounds must be arrayed in assay plates. This process usually requires the reformatting of compounds from storage plates into the plates in which the assays will be run. The reformatting process requires a system that can transfer liquid samples from storage containers into test plates, in which the maximum volume of the wells is 5 μl or less. Often, test compounds are stored in DMSO or solubilized in organic solvents such as alcohols that have fluid properties that can be problematic for many standard liquid dispensers.

The other step in fluid handling is the process of transferring assay reagents into test plates. This step, performed during the assay run, has different requirements. In reagent dispensing, volumetric precision between wells is crucial in establishing reliable and robust assay performance. Furthermore, because most assays require multiple reagent additions, total assay volumes of 2 μl or less require reagent additions in the nanoliter volume range. The handling of most assay reagents is generally straightforward because they do not usually contain volatile organic solvents. However, reagents that contain high concentrations of protein or detergent can create challenges. Assays that utilize cells or microparticles can be especially challenging because reliable well-to-well metering of these components is important. With cells and microparticles, the problem of clogging can be especially challenging.

With all liquid dispensing steps, speed is an important factor. Many screening assays are time sensitive. Assays that utilize enzymes or measure equilibrium binding will vary according to the time at which reagents are added to test wells. Evaporation of reagents as they sit in the plates can lead to variable assay performance. Dispensers that require 30 min or more to transfer reagents to a test plate will create timing problems for many assays that utilize volatile reagents. Rapid transfer (within minutes or seconds) of reagents to test plates is an important requirement for any fluid-handling system.

The design of reformatters, dispensers and microwell plates must address all of these issues. These problems become especially difficult when working in the submicroliter volume range. Challenges such as these require technological solutions. In some cases, improvements in existing technology is sufficient. In other cases, more innovative solutions are required.

Approaches to miniaturized dispensing

Fast, precise, accurate and controllable dispensing is crucial for the performance of uHTS assays. Current approaches to submicroliter fluid dispensing will now be described.

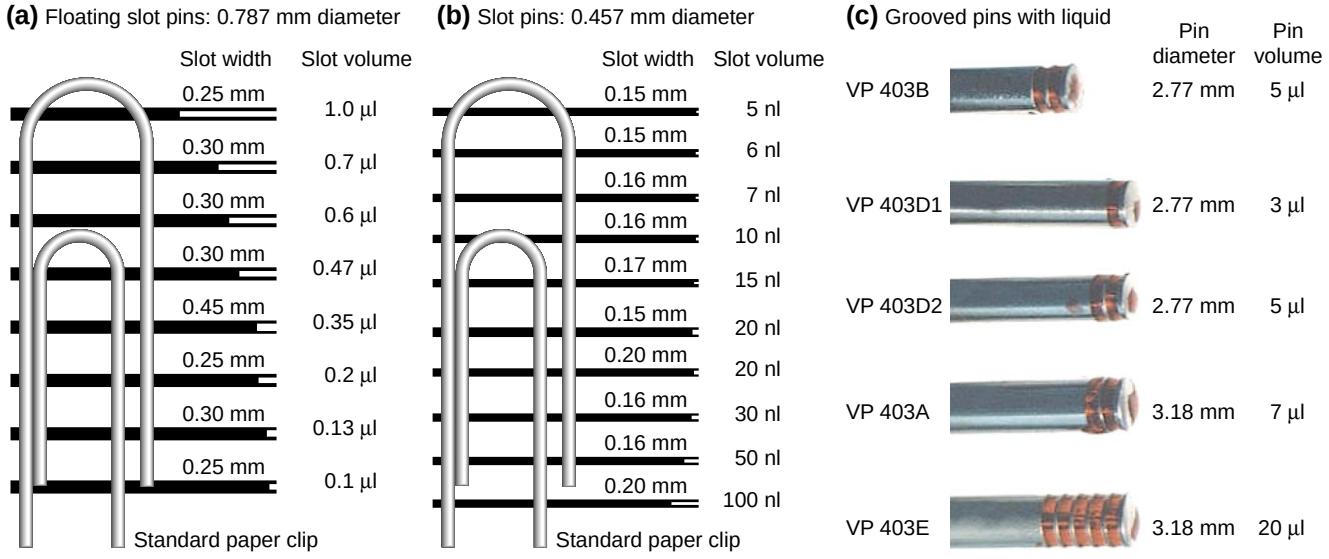
Contact dispensing

Contact dispensing is one of the earliest and most fully developed methods of transferring very small volumes of liquid. Originally developed for other applications including clinical testing, this method has found its way into the uHTS field owing to its simplicity and reliability. Contact dispensing utilizes various types of pins and/or needles to pick-up, transfer and dispense liquids by surface contact. Transfer from one surface to another is facilitated by the difference in adhesion between them.

However, there are problems and limitations associated with contact dispensing including its inability to handle a variety of fluids. Moreover, fluid parameters such as viscosity and vapor pressure can have an effect on accuracy and precision. Fluids containing particles or cells can be particularly difficult to transfer. In addition, contact dispensing relies heavily on precise, three-axis positioning of the dispensing device. Washing or replacement of the transfer tips is required in cases where it is necessary to minimize the cross-transfer of fluids. Because of these requirements, contact dispensing can be very slow.

Pin tools

Devices that utilize pins for contact dispensing are simple and reliable. However, the volume of liquid that is retained on the pin and transferred to the receiving surface depends greatly on the consistency of the physical characteristics of the transfer surfaces and liquids being transferred. The liquid-carrying capability of pin devices varies from nano- to microliters and can be controlled by using pins of different shape, material and surface treatment. Capillary, 'split capillary' or 'slot pins' (Fig. 1a,b) as well as mushroom shaped or 'grooved' pins (Fig. 1c) can transfer volumes up to 1 μl or more if required (V&P Scientific, San Diego, CA, USA). Under controlled conditions, the volumes dispensed with these devices can be accurate and repeatable (Fig. 1d). However, standard unmodified pin devices are susceptible to volumetric error owing to variation



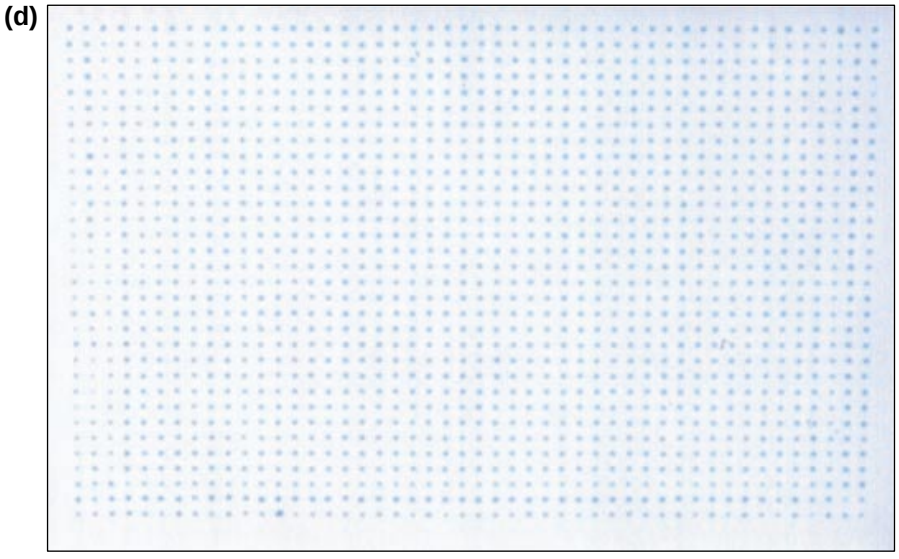


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Figure 1. Examples of **(a, b)** slotted and **(c)** grooved-pin-tool devices, showing the transfer volume for each pin of different diameter. Also shown **(d)** is the accuracy and precision achieved when a 1536-solid-pin tool was used to spot a membrane with dye. Images courtesy of P. Cleveland, V&P Scientific (San Diego, CA, USA).

in surface parameters as well as the depth of immersion during pick-up and transfer. This can result in coefficients of variation (CV) of >10% (Ref. 14).

Unlike syringe (needle) devices, pin transfer systems usually require a complete 'refill' between transfers, which necessitates washing and drying between dispenses. More elaborate pin devices (enhanced for larger volume handling) require, by definition, additional washing. Capillary pins are non-washable and should be considered disposable, which impedes their use in automated systems. The Pin and Ring™ arraying mechanism, which has been implemented in the Affymetrix 417 Arrayer (Affymetrix, Santa Clara, CA, USA), partially avoids this problem by using a pin traveling through a liquid-retaining ring.

Contact dispensing can be accomplished by transfer to a liquid or to a dry surface. Normally, the entire array of pins or needles is lowered into the corresponding wells, followed by a horizontal 'touch off' motion towards the side wall. Transfer by liquid–liquid contact does not require highly precise positioning. By contrast, contact dispensing to a dry, flat surface is very

demanding and requires a high degree of positional accuracy. Here, the entire array of pins must align precisely with the receiving surface. Such a process is used in the preparation of MALDI-TOF plates in instruments such as the Voyager-DE (Applied Biosystems, Foster City, CA, USA). These positioning requirements have triggered the development of new tools. Many of these new tools have evolved from the multi-point testing systems widely used in the electronics industry and are commonly known as the 'bed of nails'. V&P Scientific has developed a device that uses floating pins that enable better contact with a rigid surface. In this device, individual pins are free to slide or 'float' up and down as they contact the receiving surface, while the device maintains X–Y positioning.

Syringes

Utilization of syringes and syringe needles as a contact tool enables multiple dispensing from a single 'fill'. Generally, syringe dispensing has a higher degree of accuracy and repeatability compared with pin dispensing tools. Syringe-based systems, such as the 'Tango' developed by Robbins Scientific (Sunnyvale, CA, USA), are designed to handle nanoliter volumes for liquid-to-liquid transfers[15]. Table 1 shows that CVs of $<10\%$ are possible with these devices when dispensing in the 10–50 nl volume range. Robbins Scientific has also introduced flexible and unbreakable titanium needles that further enhance the capability of dispensing into small dry wells or onto hard surfaces. Not surprisingly, the price on these systems exceeds the pin devices by two orders of magnitude.

At Pharmacopeia, we have developed both semi- and fully-automated reformatting systems equipped with Robbins 96-syringe heads and multi-axis, custom-made motion platforms for the precise positioning of plates. Such systems are in use for reformatting compounds from 96-well source-plates into Corning (Acton, MA, USA) 1536-well destination-plates at 1 μl per well.

Another instrument developed at Pharmacopeia that utilizes syringe-based contact dispensing is the Single Channel Reformatter. This device is equipped with a unique 'push–pull' dual syringe-washing mechanism activated by the same Z-axis drive that actuates the main dispensing syringe (patent pending). This arrangement enables very fast and precise single channel liquid handling. The Single Channel Reformatter is used for arraying controls onto test plates, as well as for setting up experiments for assay development.

The advantages of syringe devices for contact dispensing are accompanied by a higher initial cost and substantial maintenance expenses (diagnostics, cleaning, replacement parts and labor, realignment and calibration). The 96-syringe-heads currently used in Pharmacopoeia's reformatting equipment must be serviced every three to four months.

Table 1. Precision of syringe dispensing by liquid–liquid transfer using the Robbins 'Tango'*

	Median % CV		
	10 nl	20–25 nl	50 nl
Aqueous buffer	6	5	3
DMSO	8	6	5

*Data courtesy of Robbins Scientific (Sunnyvale, CA, USA).

Non-contact dispensing

Non-contact dispensing can be defined as any method capable of generating directionally controlled droplets by applying an external force. The primary challenge is to balance the competing forces of gravity and surface adhesion (tension). There are many advantages associated with this method of dispensing, including speed, minimal cross contamination, reduced liquid exposure and evaporation, and the elimination of the need for washing between transfers. An additional benefit is the agitation that results from the impact of the drop into the liquid within the receiving well.

As already mentioned, the basic challenge of generating controlled, small droplets is to overcome surface adhesion, which impedes the separation of the droplet from the dispensing tip. Analogous to capillary action[16], as the liquid droplets approach submicroliter volumes, surface adhesion becomes the dominant force that retains the drop at the delivering orifice. Gravity is not sufficient to separate the fluid from the delivering orifice without the use of additional force. This can be especially problematic for fluids with low specific gravity or high surface tension. Surface modification can help to overcome the problem of surface adhesion. A variety of methods for surface modification such as silanization or teflon coating can be considered.

Many approaches are available to achieve controlled release of submicroliter droplets. One common approach, borrowed from the ink-jet printing industry, employs piezo or magnetostrictive actuators to modulate the flow of fluid from the dispensing orifice[17–19]. Companies such as TECAN (Durham, NC, USA), CCS Packard (Torrence, CA, USA) and EVOTEC Biosystems (Hamburg, Germany) currently offer dispensers that utilize this approach. However, accurate and precise volumetric metering at very small volumes is still challenging with these systems owing to their sensitivity to fluid viscosity and dependence on temperature[20].

Another approach, used by Cartesian Technologies (Irvine, CA, USA), Lee Co. (Westbrook, CT, USA) and others, is to modulate the flow of the pre-pressurized fluid through fast-operating miniature valves to form droplets of a desired volume[21]. The

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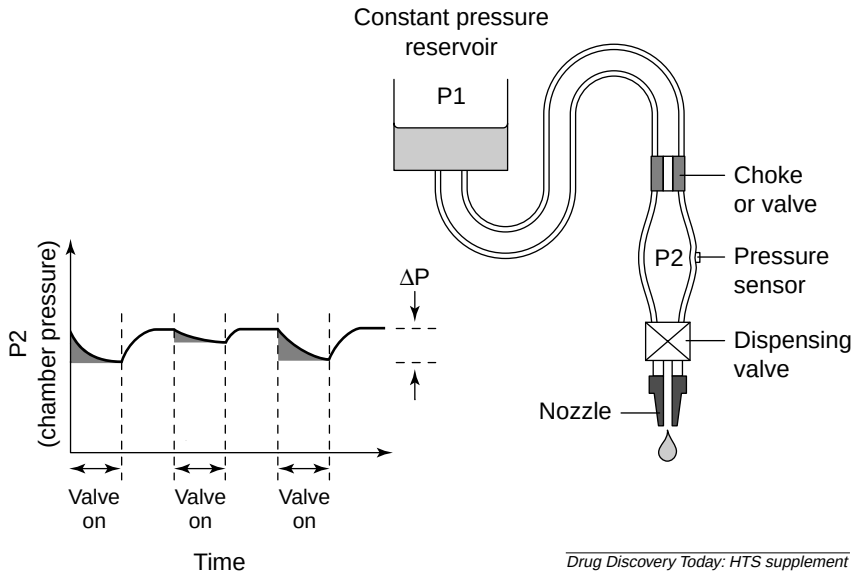

Figure 2. Schematic diagram of a non-contact liquid dispenser utilizing a feedback loop to adjust pressure or valve on-time. Liquid is delivered into the resilient pre-dispensing chamber (P2) under a pre-set pressure (P1). The chamber input is restricted by a calibrated orifice or by a second (incoming) valve. Control of the dispensing valve defines the size of the droplet. The pressure drop inside the flexible chamber is proportional to the volume dispensed. Both incoming pressure and valve timing can serve as volume adjustment parameters.

PixSys synQUAD™ offered by Cartesian Technologies uses liquid pressurization from a syringe pump to produce a displacement of a desired volume correlated with the opening of the modulating valve. This positive displacement method produces CVs of <10% when employed under stable conditions.

A substantial improvement in the accuracy and reliability of modulated flow dispensing can be achieved by using a feedback loop, which adjusts pressure or valve opening times to compensate for disturbances in fluid lines, minor occlusions or variation in the properties of the fluid being dispensed. A custom dispenser that utilizes this approach, named BlueBiRD™, has been developed at Pharmacopeia. The principle lies in measuring pressure at the point of discharge as shown in Fig. 2. If measured before, during and after the discharge, the pressure drop is proportional to the volume of liquid lost during the discharging pulse (i.e. the flow or the size of the droplet per unit time). This approach has been shown to provide reproducible and highly precise dispensing in the submicroliter volume range (Fig. 3). Figure 4 shows the results from the BlueBiRD™ when used to dispense a variety of fluids at 500 nl per well into 1536-well plates over a 20-plate run. Full-plate CVs of consistently <6% can be achieved. This system is currently being used for miniaturized uHTS screening at Pharmacopeia[22].

Another positive displacement method utilizes pumps capable of generating discrete pressurized liquid pulses. Companies such as FMI (Syosset, NY, USA) and IVEC (North Springfield, VT, USA) offer custom dispensers based on this approach. This method uses fast reciprocal piston motions to generate liquid pulses that are adjustable down to a fraction of a microliter. Although widely used for dispensing small volumes, this approach has certain volumetric and speed limitations that limit its use in screening.

Plates for miniaturized uHTS

1536-well plates

Any miniaturized screening platform operating in the submicroliter volume range requires the design of devices such as multi-well plates that can handle assay volumes in this range. There are several design features of multi-well plates that have a direct impact on fluid handling. These include plate flatness, well density, spacing, depth, volume and shape.

The density of wells within the plate will determine the ultimate throughput capability of the system that is used for screening. Well-density and spacing will have an impact on the design of dispensing fixtures such as syringe heads, pin tools or valve blocks. A compromise must be reached between selecting a plate design that enables the desired throughput, savings in reagent and compound cost, but that does not require an unreasonable investment in the design and development of new fluid-handling equipment. Corning (Acton, MA, USA), Greiner (Frickenhausen, Germany), Nunc (Rochester, NY, USA) and MatriCal (Chadds Ford, PA, USA) currently offer multi-well plates at densities of 1536 wells per plate. BD Biosciences (Franklin Lakes, NJ, USA) will be offering such plates in the near future. Table 2 summarizes some of the design features of 1536-well plates currently on the market.

Standard dispensing fixtures can be used for plates with well densities in multiples of 96 with little or minor modification. This enables correct addressing of each well in higher density plates with smaller center-to-center well spacing. Plates that contain wells in multiples of 96 also enable convenient and straightforward transfer of fluids between standard plates such as 96- and 384-well plates, during operations such as compound reformatting. Maintaining center-to-center distances between wells in miniaturized plates, which enable addressing with standard dispensing fixtures, can be accomplished with plates containing as many as 1536 wells within the standard dimensions of a 96-well plate. An increase in well density also increases the chance that plate dimension, well volume, or

center-to-center distance must be changed to avoid the need for the design of custom fluid-handling equipment.

The depth and volume of wells also have a direct impact on fluid handling. To maximize the efficiencies gained by miniaturization, plates designed to hold sample volumes of <2 μl offer the greatest advantage and provide the impetus to move away from well densities of 96 and 384 (Ref. 10). Sample volumes of 2 μl, however, require a fluidics system that can operate reliably in the submicroliter volume range (50–500 nl per dispense) because most assays require multiple reagent additions. However, precise volumetric metering in this volume range exceeds the working range of most standard fluid-handling systems and therefore requires the development of new tools.

Plates with deep wells are advantageous because they can minimize potential evaporative loss of fluid during incubations. The challenge of deep wells, however, is to design dispensers that can deliver a small volume of fluid to the bottom of the well reliably without some of the fluid being captured on the wall of the well. Wells with sloped walls, such as the pyramidal shaped wells of the MatriPlate™ (MatriCal, Chadds Ford, PA, USA)[24], enable less precise location of the dispensing fixture because the sloped walls direct the fluid to the bottom and center of the well. This reduces the risk of air-lock or bubble formation, which can occur in wells with steep walls. Shallow, sloped wells such as those found in the Corning 1536-well plate (Corning, Acton, MA, USA) also reduce the risk of liquid capture on the walls, although care must be taken to control the rate of liquid evaporation from the shallow wells.

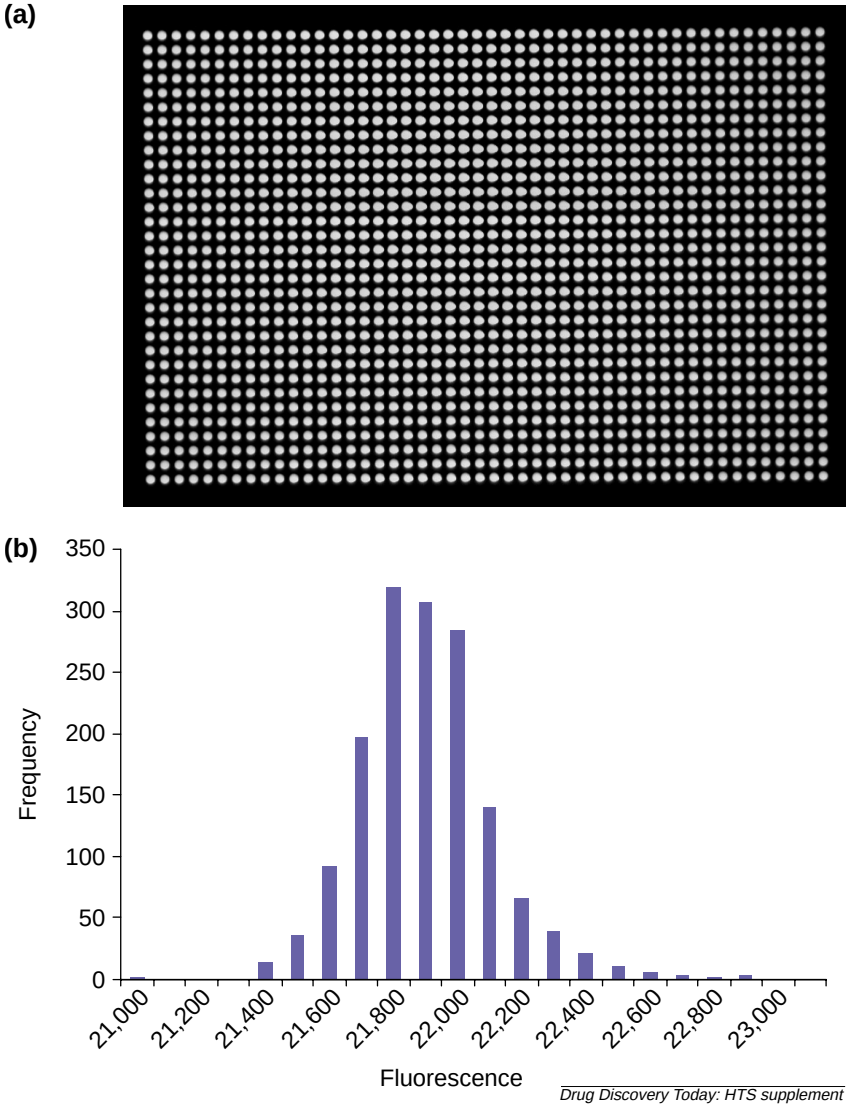

Figure 3. (a) Shows a charge-coupled device (CCD) image using ViewLux (PerkinElmer Life Sciences, Turku, Finland) of a 1536 Corning (Acton, MA, USA) plate containing 1.5 μl per well of fluorescein at a concentration of 1.3 μM, dispensed using BlueBiRD™ (Pharmacopeia, Princeton, NJ, USA). The image visually shows the accuracy and precision that can be achieved. **(b)** A corresponding histogram of (a) depicting fluorescence per well (CV of 0.95%).

The shape of the wells within the plate further influences the handling of fluids at very small volumes. When fluids with a low surface tension, such as alcohols and volatile organics, are dispensed into small-volume wells with a high surface area, they have a tendency to adhere to and climb the walls of the well. This phenomenon, often referred to as 'wicking', can be a problem when control of cross-transfer of fluids between wells is important, for example during the arraying of test compounds within plates. This can lead to cross-contamination between wells and loss of compound on the sides of wells. Wicking is especially problematic in wells that are shaped in a way such as to provide a channel for the fluid to follow, for example in wells that are square. Fluids dispensed in round wells, or wells with rounded corners, exhibit significantly less wicking (Kneibel, G. Ultra-high-throughput screening with 1536 wells: where is the physical limit in well size? *International Society of Laboratory Automation and Robotics*, 13–16 October 1997, Boston, MA, USA).

The Society of Biomolecular Screening (SBS) has been working towards the development of a recommended specification for

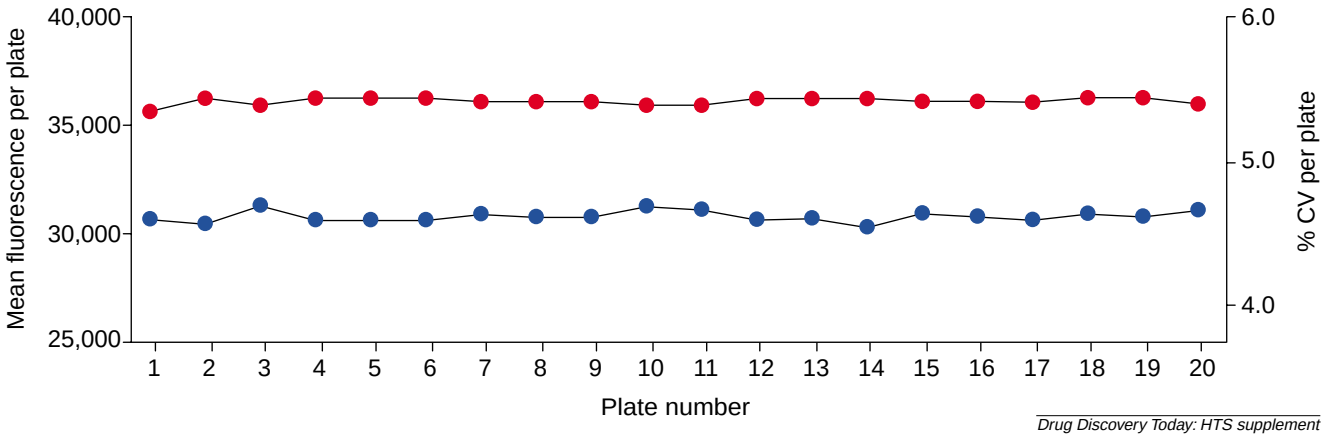

Figure 4. Mean fluorescence (red circles) and % CV (blue circles) for each plate in a 20-plate run using the BlueBiRD™ dispenser (Pharmacopeia, Princeton, NJ, USA). Each plate was dispensed first with 500 nl per well of 100 mM TRIS at pH 8, then 500 nl of Dulbeccos Modified Eagles Media (DMEM), and finally, 500 ml 80:20 dimethyl sulfoxide water. Each solution contained 100 μM fluorescein.

microwell plates. Their objective is to provide a guide for plate and instrument developers and to assure the marketing of quality plates. Much of the specification has focussed on the design features of the plate that relate directly to laboratory robotics, such as plate height, length and width. Dimensions such as the location and center-to-center spacing of wells relate directly to the design of fluid-handling equipment. The focus of the SBS's recommendations has been on 96-well plates, with the intent that this will also guide the design of plates with higher well densities. However, although all of the 1536-well plates that are currently on the market are guided by the SBS recommendations, their capabilities differ considerably when viewed from the submicroliter fluid handling standpoint.

Other plates

Other plates and devices are being developed that address directly some of the fluid-handling challenges already discussed. In some cases, they introduce a completely new approach to performing assays and handling fluids. These include the micro-fluidic devices currently being developed by companies such as ACLARA BioSciences (Mountain View, CA, USA)[25] and Caliper (Mountain View, CA, USA). Although many of the micro-fluidic approaches are beyond the scope of this review, some of the more standard, yet new approaches, will now be described.

Aurora Biosciences (San Diego, CA, USA) has developed and utilized a 3456-well plate called the Nano-plate™ (Ref. 26). This plate contains wells with a working volume of 2 μl. Aurora has also designed and developed fluid-handling equipment for these plates to accommodate for the higher density of wells.

Merck (Rahway, NJ, USA) has developed a unique solution to submicroliter fluid handling that uses specially designed 'well-less' plates[27]. These plates are made of glass that is

Table 2. Design features of 1536-well plates

Manufacturer	Well shape	Well depth (mm)	Working volume (μl)	Well spacing (mm)	Well diameter or width at top (mm)	Refs
Corning	Round with sloped walls	1.5	2	2.25	1.50	23
Greiner	Square with rounded corners and perpendicular walls	5.0	10	2.25	1.70	*
Nunc	Square with rounded corners and perpendicular walls	5.0	10	2.25	1.70	Nalge/Nunc 2000 Product Catalogue
MatriCal	Pyramidal	3.6	6	2.25	1.75	24

*Kneibel, G. Ultra-high-throughput screening with 1536 wells: where is the physical limit in well size? *International Society of Laboratory Automation and Robotics,* 13–16 October 1997, Boston, MA, USA.

patterned with 1536 hydrophilic zones within a hydrophobic matrix. Aqueous reagents spontaneously locate themselves within the hydrophilic zones of these plates. When two plates are sandwiched together they yield an assay plate with 1536 'virtual wells' in which the reagents are confined. The design of these plates simplifies fluid handling by reducing the need for precise location of fluids during the dispensing process. Reagents can be added to the plates in bulk and locate themselves within the virtual wells. Typical assay volumes of 2.5 μl are used with these plates.

Conclusions

As the components for assembling a miniaturized uHTS laboratory are becoming available, screening of large compound collections in assays using volumes of 1–5 μl are becoming practical. Crucial components include fluid-handling instrumentation and assay plates. Technology for dispensing fluids at volumes of >50 nl is being developed. Instrument manufacturers are beginning to offer fluid-handling instruments capable of operating in this range. In addition, manufacturers are marketing 1536-well plates that can be used for uHTS assays at such volumes. At Pharmacopeia, we have successfully utilized technology based on both contact and non-contact fluid transfer in our uHTS laboratory.

As these tools continue to be developed and improved, miniaturized uHTS will become regular practice in screening laboratories. The benefits that will be achieved include reduced cost of primary screening and time required to identify lead compounds. These will be important factors as more new therapeutic targets are identified and the demand for uHTS becomes greater.

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