Miniaturized FRET assays and microfluidics: key components for ultra-high-throughput screening

Lora Mere, Todd Bennett, Peter Coassin, Paul England, Brian Hamman, Timothy Rink, Susan Zimmerman and Paul Negulescu

Assay miniaturization applicable across a wide range of target classes, along with automation and process integration, are well-recognized goals for ultra-high-throughput screening on an industrial scale. This report summarizes the implementation of fluorescence resonance energy transfer (FRET)-based biochemical and cell-based assays in 3456-well NanoWell™ assay plates using key components of Aurora's ultra-high-throughput screening system.

The desirability of miniaturization and increased throughput has been addressed in several recent reviews[1,2]. Reduction of assay volume from 50–200 μl per well to 1–2 μl per well requires a comprehensive approach based on both biology and instrumentation. Key aspects for successful implementation of miniaturization are accurate and precise microfluidics and sensitive detection technology. Equally important, however, is a portfolio of robust, and preferably addition-only, assay technologies. Therefore, an integrated approach has been taken towards miniaturization and automation to address these requirements. Figure 1 shows an overview of the integrated Ultra-High Throughput Screening System (UHTSS™) platform, which is expected to be operational by the end of this year. This platform integrates several microfluidic dispensing devices, which have been developed to deliver nanoliter to microliter volumes at high speed into the appropriate wells of the 3456-well NanoWell™ assay plates (Aurora Biosciences, San Diego, CA, USA). The activity of compounds dispensed into the assay wells is quantified via a highly sensitive dual-wavelength emission fluorescence detector. The screening system is integrated with an automated compound storage and retrieval system linking existing chemistry information databases with master compound-store inventories through a computer control system. One primary purpose of this system is the automated screening of over 100,000 compounds and 2400 re-tests per day in a miniaturized assay format. It will also provide an automated plate replication process for 96- and 384-well screening plates. Key design features of the UHTSS platform have been previously reviewed[3]. The system is modular in that other dispensing and detection devices can be integrated to increase versatility. Moreover, as in the experiments detailed in this review, the individual components can be deployed as stand-alone instruments, combined into subsystems for a more 'work-station' approach to miniaturization, or used to focus on the compound library management and plate replication functions.

Lora Mere, Aurora Biosciences Corporation, 11010 Torreyana Road, San Diego, CA 92121, USA. tel: +1 858 404 6600, fax: +1 619 404 6727, e-mail: MereL@aurorabio.com

 1359-6446/99/$ – see front matter PII: S1359-6446(99)01377-X

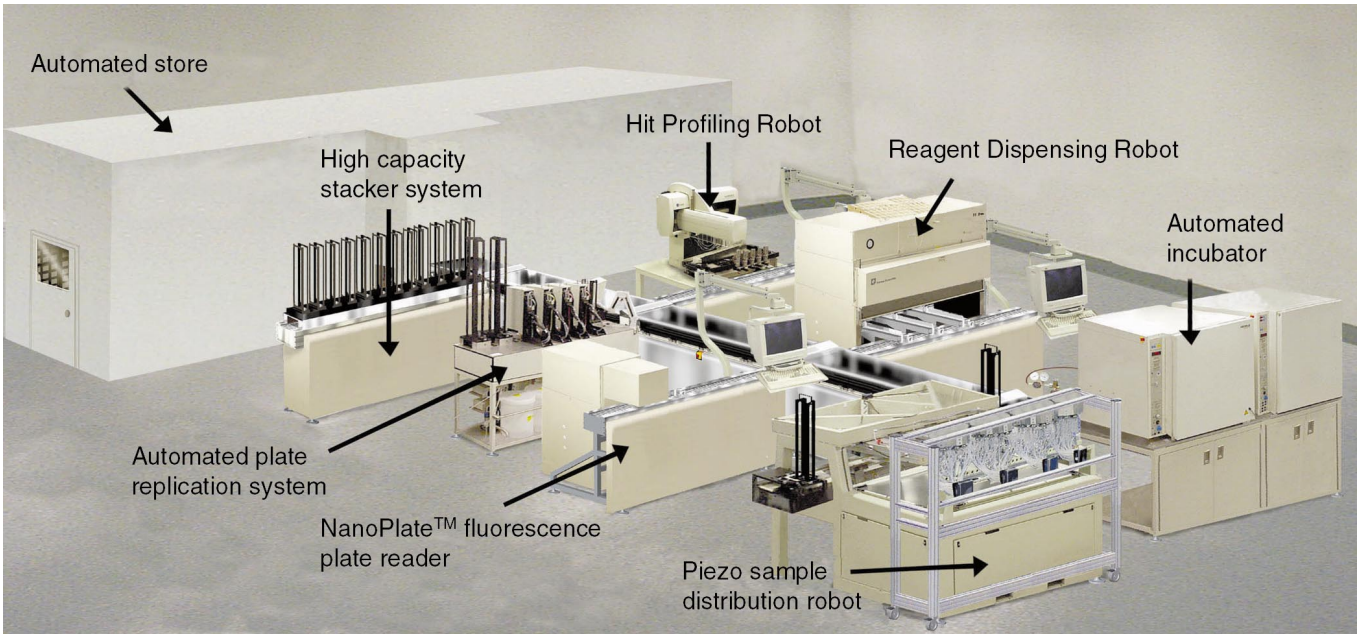

Figure 1. *Computer-generated visualization of the UHTSS™ configuration (Aurora Biosciences, San Diego, CA, USA). The system is designed to integrate a working compound solution store, 96- or 384-well plate replicating system, components for automated distribution of compounds and assay reagents into high density 3456-well NanoWell™ assay plates (Aurora Biosciences), automated incubators and a high-performance fluorescence plate-reader. The Hit Profiling Robot provides automated re-formatting of selected compounds for re-testing.*

FRET-based assays

Fluorescence resonance energy transfer (FRET) can occur between two fluorophores in close proximity with suitably overlapping spectra (Fig. 2). Excitation of a 'donor' fluorophore can result in the transfer of energy from the donor to an 'acceptor' that has a longer wavelength emission. An event causing an increase in distance between these two fluorophores can be measured by monitoring disruption of FRET through re-establishment of the fluorescence emission from the donor. Many biological responses result in, or can be engineered to cause, a change in distance between molecules, and therefore, this approach can be used to configure cell-based or biochemical assays. In high-throughput screening (HTS) assays, the two fluorophores can be brought together by a linker moiety, which can be a chemical entity[4] or a peptide, or by being confined to the cell's lipid bilayer, as can be used for voltage-sensitive dyes[5,6]. A key advantage of FRET assays is that the results produced are in the format of a ratio between the two wavelengths, thereby greatly reducing the many potential artifacts and signal variability, such as variation in cell number, probe concentration, optical paths or light fluctuations[6]. Together with the inherent sensitivity of fluorescence, these features are well suited for assays that work in current HTS and can migrate to miniaturized, high-density formats.

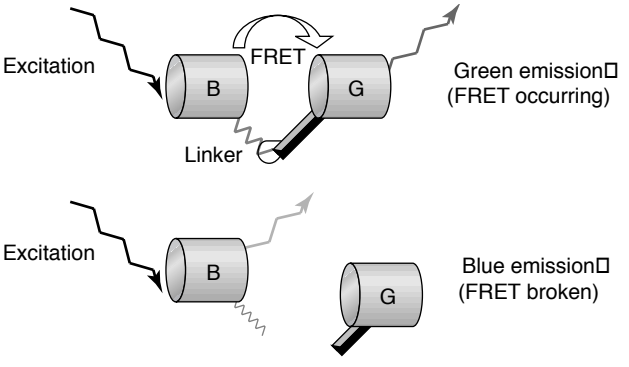

Figure 2. *A diagrammatic representation of fluorescence resonance energy transfer (FRET), which can be used to configure a wide range of biochemical and cell-based assays. A key advantage of FRET is the ratiometric readout, which reduces or eliminates many common assay artifacts.*

The β-lactamase gene-reporter system has been previously used both with the membrane-permeable, fluorogenic substrate ester (CCF2/AM) for the real-time, high-sensitivity measurement of gene expression in single living mammalian cells, and as a screening assay in 96-well microtiter plates[4,6,7]. This report describes the use of this reporter system both in miniaturized assays and as a useful indicator of cell viability.

Reagent dispensing into 3456-well assay plates

The 3456-well assay plate is designed for miniaturized UHTSS platform-based assays using the standard 96-well plate rectangular footprint. The 3456-well assay plates contain 48 rows and 72 columns of wells at 1.5 mm spacing, making the well-density 36-fold higher than for a conventional 96-well plate, whilst lowering operating working volumes to 1–2.2 μl. The overall external dimensions of the 3456-well plate are consistent with industry-standard microtiter plates and the plate is constructed of a black polymer with a low fluorescence clear bottom. The plate also has a tight-fitting, specially designed lid, which forms an important part of evaporation control. These disposable plates are designed for measurement of epifluorescence from below the plate, with a low fluorescence background and good light transmission in the region of the ultraviolet to red wavelengths.

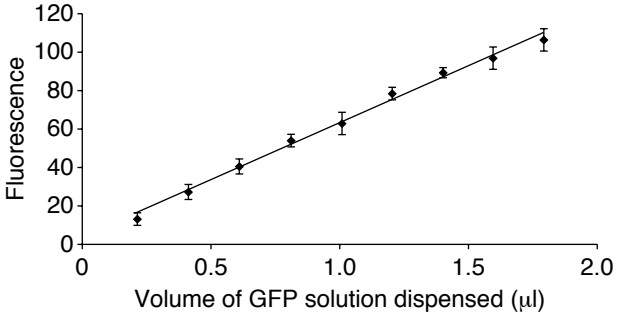

Figure 3. *The graph illustrates the linearity and reproducibility of the dispensation of a green fluorescent protein (GFP) solution into a segment of a 3456-well NanoWell™ plate (Aurora Biosciences, San Diego, CA, USA). Each point shows the mean and standard deviation of dispensation from eight tips.*

The Reagent Dispensing Robot (RDR) is designed for precise, high-speed dispensing of assay reagents, including cell suspensions, into high-density plates. Each RDR has a total of six dispensers, each capable of dispensing a different reagent across a 3456-well plate in less than 30 s. Each dispenser has 48 tips that can simultaneously deliver aliquots to all the wells in a column. If desired, independent control of each tip allows the programming of different volumes to be dispensed into each well. Figure 3 shows the dispensing of a solution of a green fluorescence protein (GFP)–fusion protein into a segment of a 3456-well assay plate, indicating the linearity and precision over the volume range of 0.2 to 1.8 μl.

FRET-based protease assay

As a representative biochemical assay, chymotrypsin cleavage of a FRET-peptide was performed in the 3456-well assay plate, using the RDR to add the reagents and the NanoPlate Fluorescence Plate Reader™ (NPR, Aurora Biosciences) for dual wavelength measurement. The NPR measures fluorescence from individual wells of the 3456-well plate using a xenon lamp excitation source with an appropriate interference filter. The emission fluorescence is collected by two separate light guides that lead via independent emission filters to two photomultipliers. The resultant ratio of the two measurements is then used to quantify the readings. A complete set of data can be obtained from all the wells in less than 90 s, giving a potential throughput of over 50,000 assay points per hour. At this speed, the sensitivity is sufficient that fluorescein, at concentrations of less than 1 nM, can be accurately measured in two microliter volumes. As shown in Figs 4 and 5, the system has sufficient sensitivity for robust biochemical and cell-based assays.

The peptide used as a substrate for this protease assay contains terminal donor and acceptor fluorophores and therefore cleavage of the peptide causes disruption of FRET – under the conditions of this assay, complete cleavage resulted in a four-fold increase in the FRET ratio. In this experiment, the peptide substrate was dispensed into all 3456 wells via one dispenser of the RDR. Using other RDR dispensers, aliquots of buffer (rows 1–16), chymotrypsin (rows 17–32) or chymotrypsin plus an inhibitory concentration of phenylmethylsulfonyl fluoride (PMSF) (rows 33–48) were dispensed. The assay plate was incubated for the required time and subsequently read on the NPR. The resultant ratios of donor to acceptance fluorescence detected are shown in Fig. 4 (for clarity the data with the inhibited enzyme has not been plotted, as they overlie the buffer control results). These results illustrate the reliability and performance of this assay, and similar results can be obtained by these assays using two different GFPs for FRET (Ref. 6).

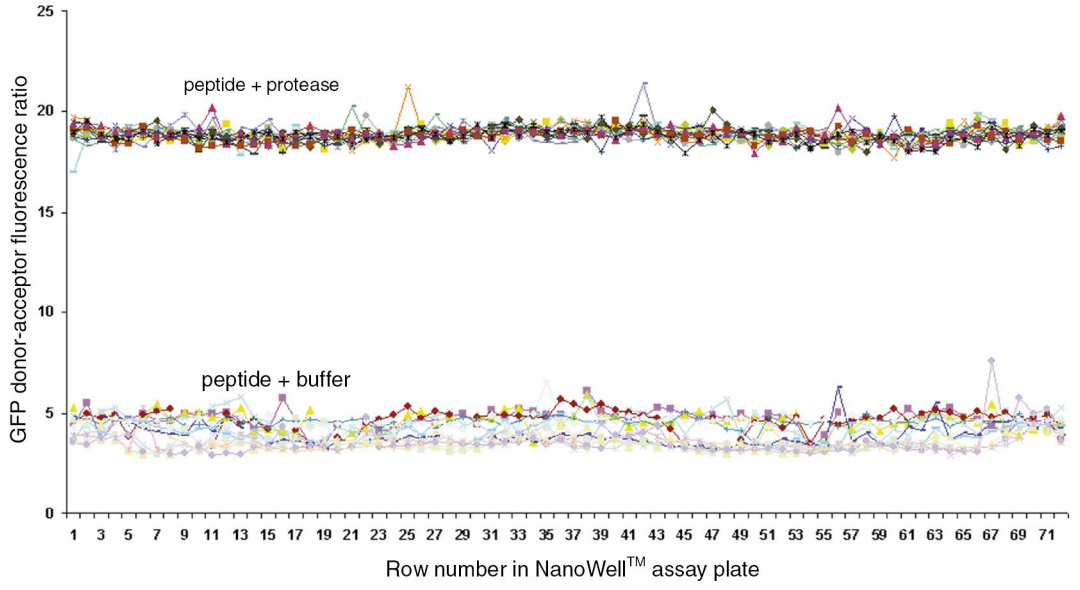

Figure 4. *Consistency of a biochemical protease assay across 32 rows and 72 columns of a NanoWell™ plate (Aurora Biosciences, San Diego, CA, USA). Fluorescence resonance energy transfer (FRET)-peptide substrate (100 nM) was dispensed using the Reagent Dispensing Robot into all the wells of a 3456-well assay plate. Buffer solution was added to all 72 columns of rows 1–16, 0.1 μM chymotrypsin was added to rows 16–32 [and 0.1 μM chymotrypsin with 0.5% phenylmethylsulfonyl fluoride (PMSF) inhibitor to rows 33–48; data not shown]. Following proteolysis of the peptide, the fluorescence ratio was measured on the NanoPlate Fluorescent Plate Reader™ (Aurora Biosciences). In this assay, a four-fold change in fluorescence ratio reflects complete cleavage of the peptide.*

Using cells in 3456-well assay plates

Cell-based assays are increasingly used in HTS (Ref. 1), partly because of the development of new, highly sensitive fluorescence reporters, such as the β-lactamase system[4], which were originally introduced to provide quantitative data from single living cells. However, various concerns have been raised over the feasibility of conducting such cell-based screening assays in highly miniaturized formats, such as risks of evaporation, cell compatibility with the materials used to make high-density plates, and potential harmful effects of microfluidic dispensing of cell suspensions. However, it was found here that a variety of mammalian cell lines can be successfully dispensed from the RDR and appear to thrive and replicate in 3456-well plates.

In the experiment shown in Fig. 6, Chinese Hamster Ovary (CHO) cells were dispensed at low density into 3456-well plates (approximately 250 cells per well) using the RDR, and the plates incubated at 37°C in the UHTSS incubator. Cell viability was monitored by loading the cell samples with CCF2/AM. Although this dye serves principally as a reporter for β-lactamase expression, it is also useful as a live cell stain, as only intact viable cells will take up and retain the dye showing green fluoresce. By contrast, in the absence of β-lactamase expression, the CCF2 molecule remains uncleaved and FRET stays intact. Quantification of the green fluorescence in the 3456-well plates following incubation then correlates with the absolute cell number per well. The replication-doubling time calculated for this CHO cell line was similar to that observed for cells plated in conventional 96-well black Costar clear-bottom plates (data not shown).

The viability of another adherent cell line, the human endothelial cell line (ECV), was also assessed with similar results (data not shown). This experiment clearly showed viability of the cells for at least 48 h after dispensing into the assay plates. Visual inspection of the CHO and ECV cells in the assay plates confirmed a similar morphology to that observed both in conventional HTS plates and in general tissue culturing conditions such as in flasks. These results clearly indicate that the 3456-well plate will support the viability and proliferation of cultured cells. Although in short-term assays, cell growth and replication may not be required, these factors might be important for miniaturization of certain functional cell-based assays in which several days may elapse from initiation to analysis.

One important advantage of miniaturization is conservation of reagents, including the ability to use only small quantities of cultured cells. The number of cells per well required for an acceptable assay will clearly depend on several factors, including the quantity of fluorophore per cell, the wavelengths used, the system background and detector sensitivity. It was found that approximately 1000 cells per well gives reliable results with both the UHTSS components

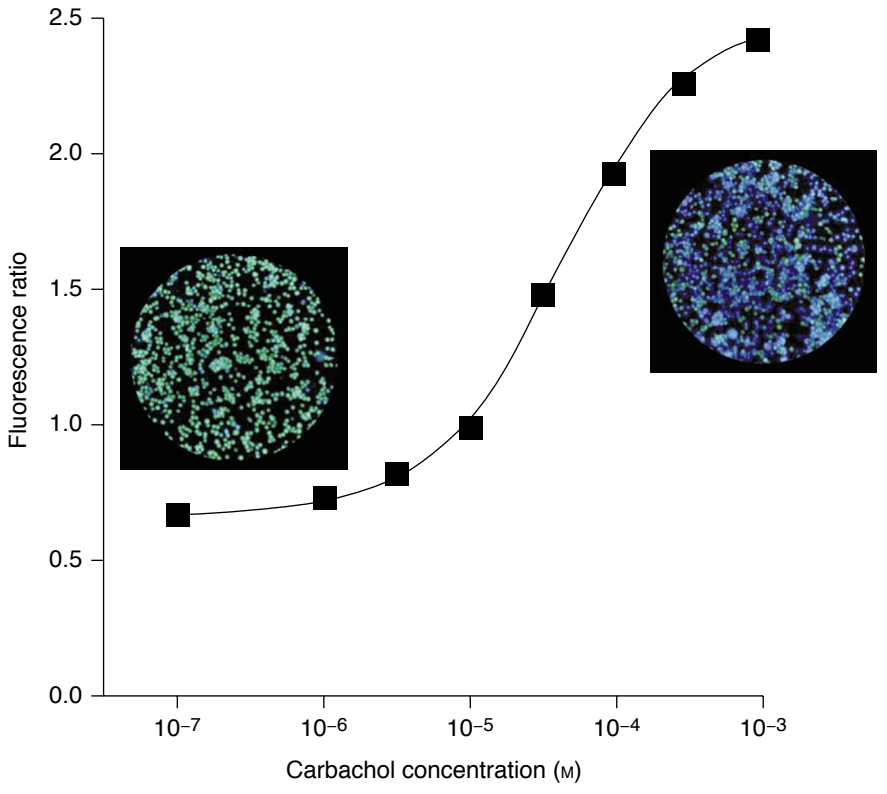

Figure 5. *Cell-based, G-protein-coupled receptor (GPCR) assay using the β-lactamase reporter gene system, in a 3456-well NanoWell™ assay plate (Aurora Biosciences, San Diego, CA, USA). Jurkat cells (human T-cell line) were stably-transfected with the muscarinic M_1 receptor and nuclear factor of activated T-cell (NFAT)–β-lactamase reporter gene construct. Using the Reagent Dispensing Robot (RDR), cells with various concentrations of carbachol in the media were dispensed. After 4 h of incubation in the ultra-high-throughput screening system (UHTSS) incubator, cells were loaded with the β-lactamase substrate, CCF2/AM (via the RDR) for 1 h at room temperature. Fluorescence was measured via the NanoPlate Fluorescent Plate Reader™ (Aurora Biosciences). The graph shows an eight-point carbachol concentration-response curve, with one well per point.*

and the β-lactamase reporter gene system. Figure 7 shows results with different quantities of cells dispensed into different wells. Only a few hundred cells per well were required to generate a signal above background levels at both the green (535 nm) and blue (460 nm) wavelengths. In a similar experiment using cells constitutively expressing the β-lactamase gene and thus emitting blue fluorescence after loading with CCF2/AM, a greater signal-to-background ratio was recorded at both emission wavelengths (data not shown).

A miniaturized cell-based G-protein-coupled receptor assay

A key goal for the UHTSS platform has been the rapid development and performance of tractable cell-based screening assays for major target classes, such as the G-protein-coupled receptors (GPCR). As previously discussed[4,6], the β-lactamase system permits rapid assay development and should be well suited to miniaturized screening. Figure 5 shows an example of the quality of data that can be generated with this assay technology in 3456-well plates. The RDR dispensed Jurkat T-lymphocyte cells expressing the muscarinic M_1 receptor, in tandem with a NFAT (nuclear factors of activated T-cell)–β-lactamase reporter gene into cell culture medium in an assay plate, which had been pre-formatted with various concentrations of the muscarinic agonist, carbachol. Following incubation in the UHTSS incubator, aliquots of CCF2/AM were also dispensed into the assay wells by the RDR. Ratiometric fluorescence signals were subsequently measured using the NPR. The results obtained showed a robust activation profile in response to carbachol. Both the 'assay window' and the stimated EC_{50} for carbachol were consistent with screening data obtained from conventional 96-well formats. This assay, which required four independent reagent addition steps via the RDR, yielded low inter-well variability (<10% coefficient of variation). A concentration-response curve of the fluorescence ratios from single wells against the concentration of carbachol is shown in Fig. 5. These results indicate that, as with screening this type of assay in 96-well plates, the precision and reliability of these miniaturized assays may permit reliable primary screening as single determinations.

Compound reformatting into high-density plates

The majority of cells and many enzymes will only tolerate small quantities of solvent, usually dimethylsulfoxide (DMSO), for dissolving working stores of compounds. In

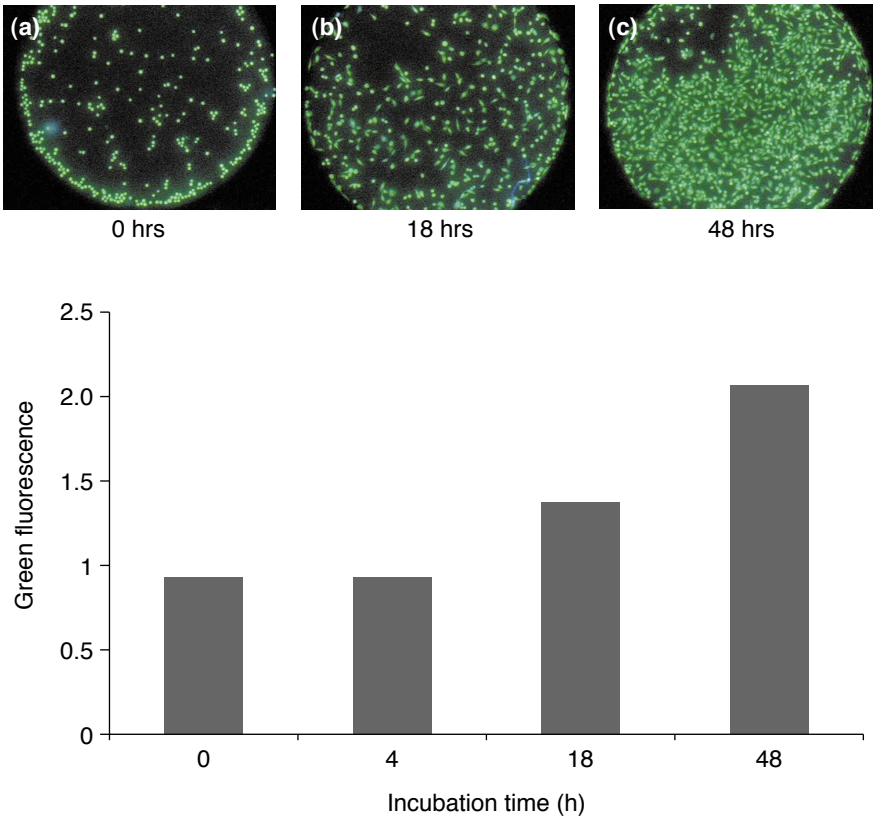

Figure 6. *Viability and replication of Chinese Hamster Ovary (CHO) cells in 3456-well NanoWell™ (Aurora Biosciences, San Diego, CA, USA) plates. Cells were dispensed into all of the 3456-well plate using the Reagent Dispensing Robot. Assay plates were incubated in the ultra-high-throughput screening system (UHTSS) incubator at 37 °C, 5% CO_2 for 0, 4, 18 and 48 h. Subsequent to incubation, samples were loaded with the β-lactamase substrate, CCF2/AM, to assess the number of viable cells in each well. Green fluorescence was measured via the NanoPlate Fluorescent Plate Reader™ (Aurora Biosciences). The histogram shows the increase in average well fluorescence across the whole 3456-well plate (corresponding to cell number). The photographs show representative wells at (a) 0 h, (b) 18 h and (c) 48 h.*

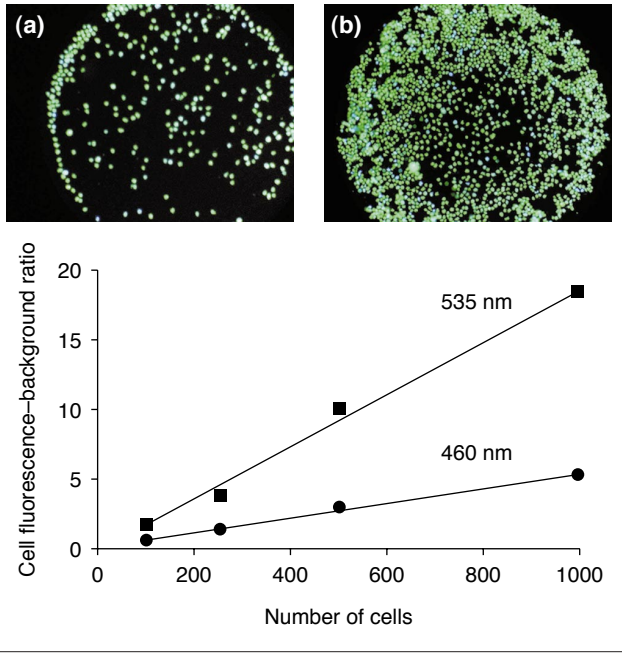

Figure 7. *Number of cells per well required for cell-based assays using β-lactamase reporter gene. Jurkat cells (human T-cell line) were dispensed using the Reagent Dispensing Robot at varying densities in 3456-well NanoWells™ (Aurora Biosciences, San Diego, CA, USA), loaded with the β-lactamase substrate, CCF2/AM. The graph shows fluorescence intensity of cells, relative to background, measured simultaneously at 460 nm and 535 nm, with 400 nm excitation, using the NanoPlate Fluorescent Plate Reader™ (Aurora Biosciences). The photographs show representative wells with (a) 250 cells and (b) 1000 cells.*

both conventional HTS and in miniaturized assay formats, these compounds must be delivered in a way that minimizes the final concentration of solvent in the assay. Most drug discovery laboratories store their compound libraries in DMSO solutions in 96- or 384-well storage plates. This therefore requires the reformatting of compounds into higher-density plates and this is recognized as one of the more challenging aspects of miniaturized screening[1].

The Piezo Sample Distribution Robot (PSDR™, Aurora Biosciences) has been designed to perform this task and uses high-precision robotics to accurately position storage plates and 3456-well plates. There are also four 96-piezo-electric tip-dispenser arrays to aspirate the compound samples from the storage plates and then dispense the

requisite low-nanoliter volume samples into the 3456-well assay plates at rates of up to several hundred drops per second. The number of drops per tip selected determines the volume of each dispensation, and typically, tips aspirate 1–2 μl sample volumes from source plates and then deliver the required number of drops to the 3456-well assay plates. There are many design and engineering challenges in the production of an instrument with the requirements of the PSDR, including the development and manufacture of the 96-piezo-electric tip-dispense head. Thus far, piezo-electric devices appear to be the only type of projectile dispensers capable of delivering rapidly defined volumes of DMSO in the sub- to low-nanoliter range. Figure 8 shows data from a test-run for a prototype UHTSS piezo-dispense head showing the drop size of all 96 tips simultaneously dispensing a DMSO solution of fluorescein.

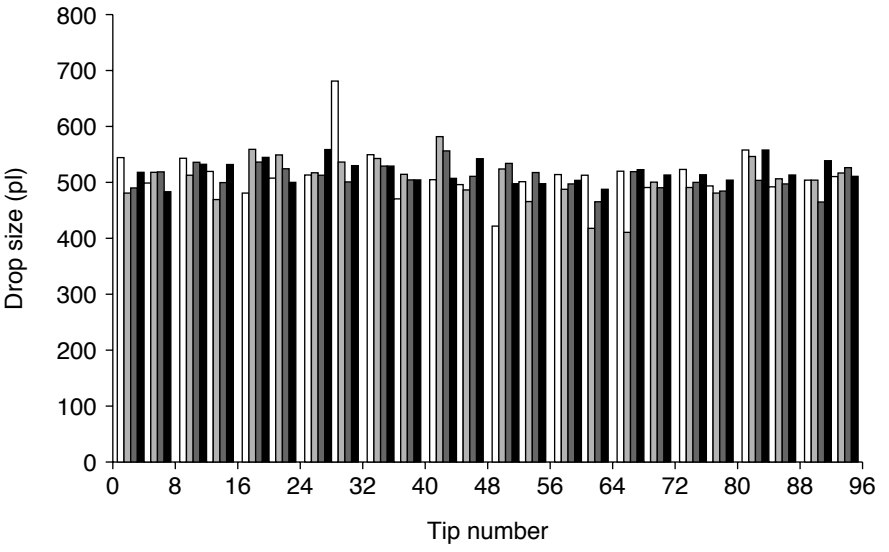

Figure 8. *The histogram shows the performance of a prototype head, dispensing sub-nanoliter drops of a dimethylsulfoxide (DMSO) solution of fluorescein. The average drop size in a burst of 32 drops, calculated from the fluorescein fluorescence, is shown for each individual tip.*

For creating primary screening plates, each PSDR dispenser head will normally dispense simultaneously from all 96 tips, thus supplying a different compound to each well in one array of the 36 arrays of 96 wells that comprise the 3456-well plate. The dispensers can be used to produce concentration series for IC_{50} or EC_{50} determinations by adding different numbers of drops of the same compound to different wells of the assay plate. The PSDR is designed to reformat at least 100,000 different compounds into high-density screening plates each day. If the system is used to produce batches of high-density screening plates from the same storage plates, then the output can be many-fold greater. This is because once the tips have aspirated a given set of compounds from a storage plate, many high-density plates can be dispensed with that set of compounds without the system needing to go through the rigorous wash cycle required between aspiration from different storage plates, which is necessary to avoid cross-contamination.

Conclusions

This review has demonstrated the capabilities of miniaturized FRET-based biochemical and cell-based screening assays with automation-compatible instrumentation. A key success factor has been the integration of innovations in assay chemistry and biology, instrumentation, software and process design. The authors believe that these technologies will allow ultra-high-throughput screening in dedicated centralized facilities to generate primary and secondary screening data to populate very large structure–activity databases. In addition, the assay technologies and instrumentation should be useful for significant enhancement of distributed, research laboratory-based screening programs.

Acknowledgements

The authors would like to thank the many scientists and engineers at Aurora Biosciences who made contributions to the implementation of UHTSS. They would particularly like to acknowledge the contributions made by Dylan Vu, Jeffrey Ho, Andrew Pham, Glenn Sasaki, Walter Niles, Tony Torres, David Heiner and Minh Vuong. They thank Eunice Spanburgh for her administrative assistance.

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