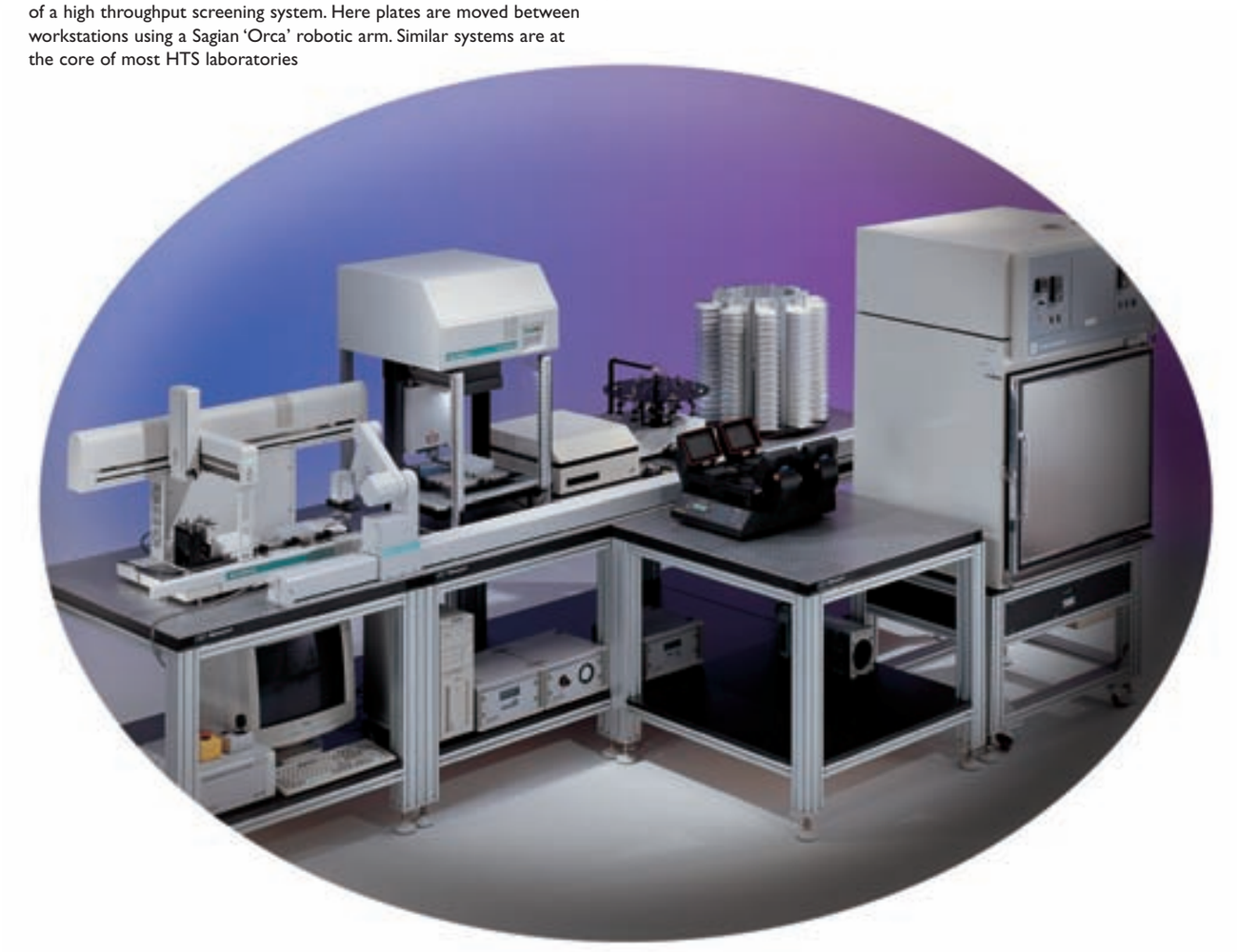
Incubators, plate stackers, 96 well pipetters and serial diluters are key components of a high throughput screening system. Here plates are moved between workstations using a Sagian 'Orca' robotic arm. Similar systems are at the core of most HTS laboratories



HTS
a strategy for drug discovery

By Dr AR Knight, Cerebrus Ltd

Whichever technologies are implemented, high throughput screening is set to become one of the cornerstones of drug discovery, however deciding which strategy to implement will provide many headaches. This article concentrates on screens identifying the interaction of small molecules with protein targets rather than target identification screens.

High throughput screening arose in the 1990s as 96 well microtitre plates took over from test tubes as the receptacle of choice for biological assays. In combination with combinatorial chemistry it resulted in a paradigm shift from knowledge-based sequential synthesis and testing to parallel processing of multiple compounds. High throughput screening has frequently been defined by the rate at which compounds can be screened: high throughput being defined as 100 to 1,000 data points per day. More recently assays capable of producing 100,000 data points per day have been described, for which the phrase 'ultra high throughput screening' has been used[1,2]. While these numbers are impressive enough, to define high throughput screening as purely in terms of numbers, undersells HTS, as it is not a tool with specifications such as a microscope or a pipetting robot, it is a strategy for drug discovery.

High throughput screening has evolved as part of the three-cornered strategy involving HTS, combinatorial chemistry and the information technology associated with computational chemistry. The throughput of the assay has simply to deal with the output of the compound source, whether that is a standing compound, *de novo* synthesis or a database search. The system must return data rapidly to allow further iterations of synthesis or searching. It must return data in a useable format: data processing whether for quality control or to discover structure activity relationships is dramatically faster if presented in simple, intuitive colours, symbols and pictures[3]. It must also deal effectively with the number and nature of new targets investigated by the sponsoring company. If genomics and proteomics produce vast numbers of targets, an equivalent number of assays need to be developed and run. A company focusing, for instance, in classical GPCRs might run fewer assays but try harder to identify the optimal compound. Above all it must complete these processes in a way in which the sponsoring company believes gives it a competitive edge over other companies. The throughput of the screen is only one way that this might be defined. The rapidity with which multiple rounds of testing takes place, the quality of the SAR deduced from the assay, the novelty of the assay technology (giving access to unique targets), the sophistication of the data analysis and the ability to mine data from previous screens are all areas where companies can gain a competitive edge. Compound selection is also key – screening a small focused library is obviously faster than screening a vast, diverse library, so perhaps the key question in HTS is: what is the minimum number of compounds that can be tested to identify a lead? When these fundamentals are understood, HTS can be placed effectively in a company, whether as a core technology, around which projects are arranged or as an extra weapon in the armoury of a big pharmaceutical company.

The most common targets in HTS laboratories today are G-protein coupled receptors (GPCRs), ion channels, kinases, proteases, immune system modulators and hormone receptors[4]. HTS technology appropriate for one target might be inappropriate for other targets. From the point of view of an assay technologist this is frustrating since the optimal assay might not always be used for a particular target, and from a management point of view this is frustrating as a large investment in time and money might actually limit the choice of targets. To some extent it is the available technology, which is driving the direction of drug discovery, so in the following paragraphs I have reviewed the biological assays most prevalent in HTS.

Assays for HTS

Most HTS laboratories are involved in identifying the interaction of small molecules with a target molecule, usually a protein. Assays should be configured to have the smallest number of steps or reagent additions to maximise throughput, they must also provide a reli-

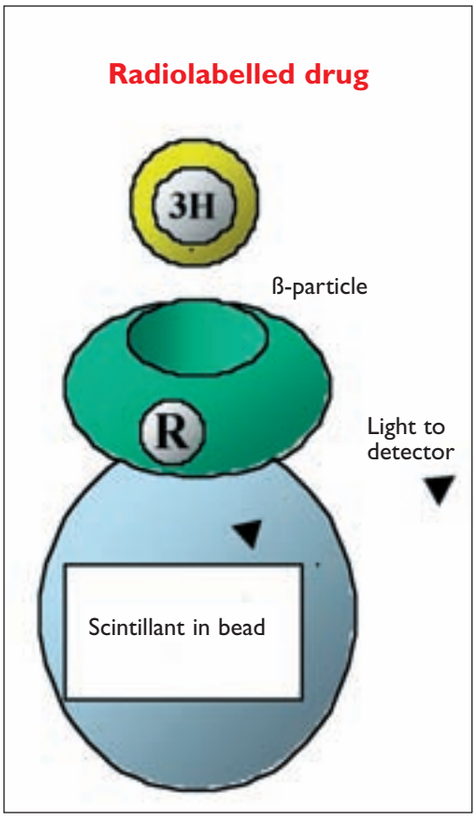


Figure 1
The receptor or protein target is captured on to a resin bead containing scintillant. Radiolabelled drug-like molecules bind to the target protein. Only ß-particles derived from bound molecules are sufficiently close to the bead to activate the scintillant

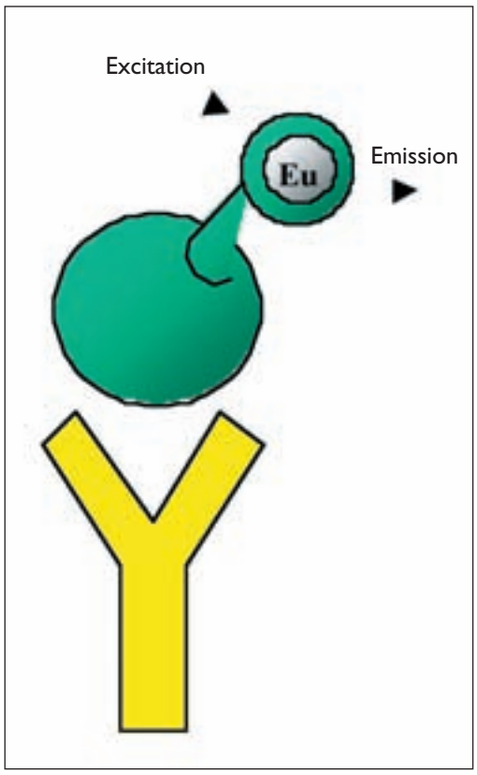


Figure 2
Large molecules can be tagged with fluorescent lanthanides such as Europium. Immuno-assays such as the one illustrated here require the immobilisation of one of the reagents and a washing step to remove background

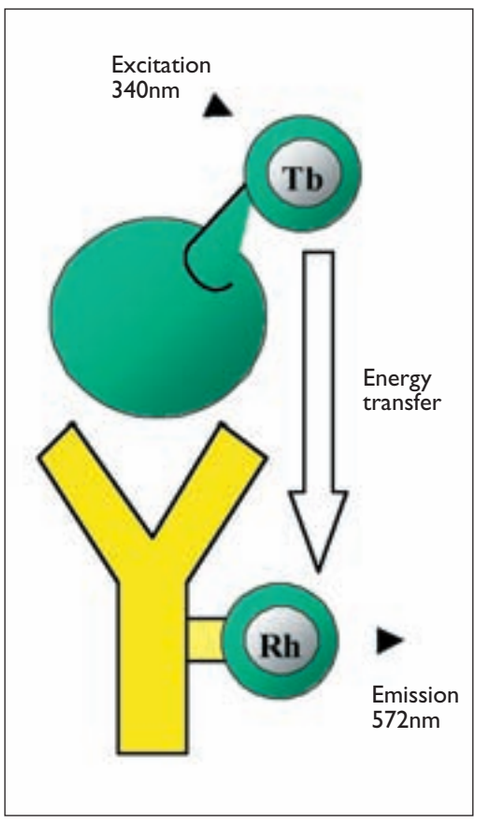


Figure 3
Fluorescence resonance energy transfer assays rely on two different fluorescent atoms. Upon excitation of one atom (in this example Terbium), energy is transferred to the second atom by resonance. The second atom (in this example Rhodamine), emits light of a characteristic wavelength, indicating that binding has occurred

able readout in a 96 well or higher density plate reader. Good HTS assays are 'homogeneous'. Homogeneous assays are mix and read assays, that is to say that all reagents are in the same phase (usually aqueous solution) and the reaction is not stopped, Assay parameters are measured when the assay system has reached a steady state (or before to allow real time kinetic measurements). Many traditional assays are heterogeneous, that is to say they require a phase separation of 'free' from 'bound', such assays are more difficult to automate. A number of assays have proved especially amenable to HTS. These are detailed below.

ASSAYS FOR INDICATING MOLECULAR INTERACTIONS

Radioligand binding

This is perhaps the oldest of the screening technologies, it was originally developed in the 1970s as a way of defining the interaction of small molecules with membrane bound proteins through displacement assays. Traditional binding experiments are terminated via a filtration step and thus cannot be considered mix and read. However, scintillation proximity assays (SPA) have eliminated the necessity for a filtration step. Amersham Plc played a key part in developing these assays in the 1980s[5]. While not a true homogeneous assay as some of the reagents are in a resin bead (solid phase), the assay system is now definitely mix and read. Briefly, ß-emitting radioisotopes are detected by means of a scintillant which converts the energy of the ß-particle into light. In this assay system the target protein is captured onto the bead (by wheat germ agglutinin or biotinylation). The short path length of ß-radiation in water ensures that radiolabelled drug molecules bound to the target protein activate the scintillant, while those in free solution are too distant to do so (**Figure 1**). Scintillation proximity assays have also been developed where the scintillant is incorporated into the plastic of the microtitre plate itself. SPA assays run well in 96 and 384 well format, however further miniaturisation may be difficult as smaller assay volumes produce fewer radioactive disintegrations per minute and the accuracy of the assay is limited. Radioactive detection methods are very reliable and are still the method of choice for determining the interaction of small molecules with a protein binding site. However, the technique can only be used where a high affinity ligand for the site has already been identified.

Fluorescence technologies

Fluorescence is used to demonstrate the interaction of two molecules in a variety of different ways, these include time-resolved fluorescence, fluorescence resonance energy transfer and fluorescence polarisation.

Time-resolved fluorescence

This technique utilises the unique fluorescent properties of lanthanide atoms. These elements, particularly europium and terbium accept excitation energy from their surroundings and re-emit light of a characteristic wavelength over many milliseconds[6]. The long lifetime of the fluorescence allows a delay to be built into the plate reader between excitation and recording. This delay is a very effective way of eliminating background fluorescence derived from the reagents and the plastic of the plate. Elimination of background is also eliminated by the large Stokes shift of these elements. The lanthanide ions are incorporated into assay reagents using a larger ion chelating tag. This makes them inappropriate for the labelling of small molecules but very effective in large biomolecular interactions. A typical assay might be constructed in a similar format to an ELISA (**Figure 2**).

Fluorescence resonance energy transfer

This is a true homogeneous assay, the principle was first described by Foster[7], and the first assay was an immuno-assay described by Ulman[8]. Fluorescence resonance energy transfer relies on not one, but two different fluorescent labels in close proximity (<10nM). The two fluorescent labels are sometimes located at opposite ends of a single molecule. In such a system one fluorophore is excited by light of a particular wavelength. This energy is transferred by resonance to the second fluorophore, which then emits light of a characteristic wavelength. Such a system would be sensitive to enzymatic cleavage between the two labels, and is used as an enzyme assay in this format. Alternatively the two fluorescent moieties might be brought together in a binding reaction (**Figure 3**). This technique also utilises the unique fluorescent properties of lanthanide chelates so it is difficult to label small molecules without effecting their pharmacological properties. For a review of FRET and time resolved fluorescence see Hemmila and Webb[9].

Fluorescence polarisation

This technique originally arose from analytical chemistry, where it was used to assess the size of labelled molecules. Its use as an assay technology has recently been championed by Jolley[10]. The technology is based on the observation that immobilised fluorophores, which are excited with polarised light, re-emit polarised light. However, small molecules in solution move and rotate. This movement introduces a degree of depolarisation, small molecules move more rapidly than larger molecules, introducing a greater degree of depolarisation

The assay technology is based on a small, labelled, molecule binding to a larger molecule, upon binding , the small molecule is no longer able to rotate freely and the degree of depolarisation introduced falls dramatically. Many good fluorescent tags are available from Molecular Probes, Inc, however the technique requires relatively large quantities of target protein. The technique has yet to gain widespread acceptance.

CELL-BASED ASSAYS

Fluorescence imaging assays

One of the most frequently used technologies for fluorescence detection in HTS labs is Fluorescence Imaging Plate Reader (FLIPR®, Molecular Devices). FLIPR® utilises an argon laser to illuminate all wells of a 96 well plate simultaneously, and a CCD camera for detection. It can be used with a variety of fluorescent dyes to measure intracellular responses to receptor activation. The most commonly used dyes are associated with calcium mobilisation and membrane potential. Where cell surface receptors do not couple naturally to calcium mobilisation, it is frequently possible to link them to this system via 'promiscuous' G-proteins.

An alternative fluorescent technology has been built up around green fluorescent protein. This protein, originally isolated from luminous jellyfish, is also sensitive to calcium, but can also be used as a reporter gene to assess gene transcription[11]. In addition it has also been used to tag endogenous proteins, to study the movements of endogenous proteins in a high through put system. Arrayscan™ (Cellomics) is probably the most advanced imaging system for studying protein trafficking.

Luciferase

Another frequently used light-based technology is luciferase. Which is used to detect the production of cAMP by cells, and also as a reporter gene to detect gene transcription. Cell-based assays usually utilise transformed cells, often with multiple transfections of receptor and reporter gene. A detailed discussion of the optimisation of such assays is beyond the scope of this article.

Automation

Another angle on high throughput screening which needs to be carefully considered is the level of automation that is appropriate for a particular application. There is little doubt that large core systems and fully integrated robots such as those constructed at Glaxo Welcome[12] complete large screening runs with optimal efficiency. However, such machinery may bring its own set of problems which need to be solved. These

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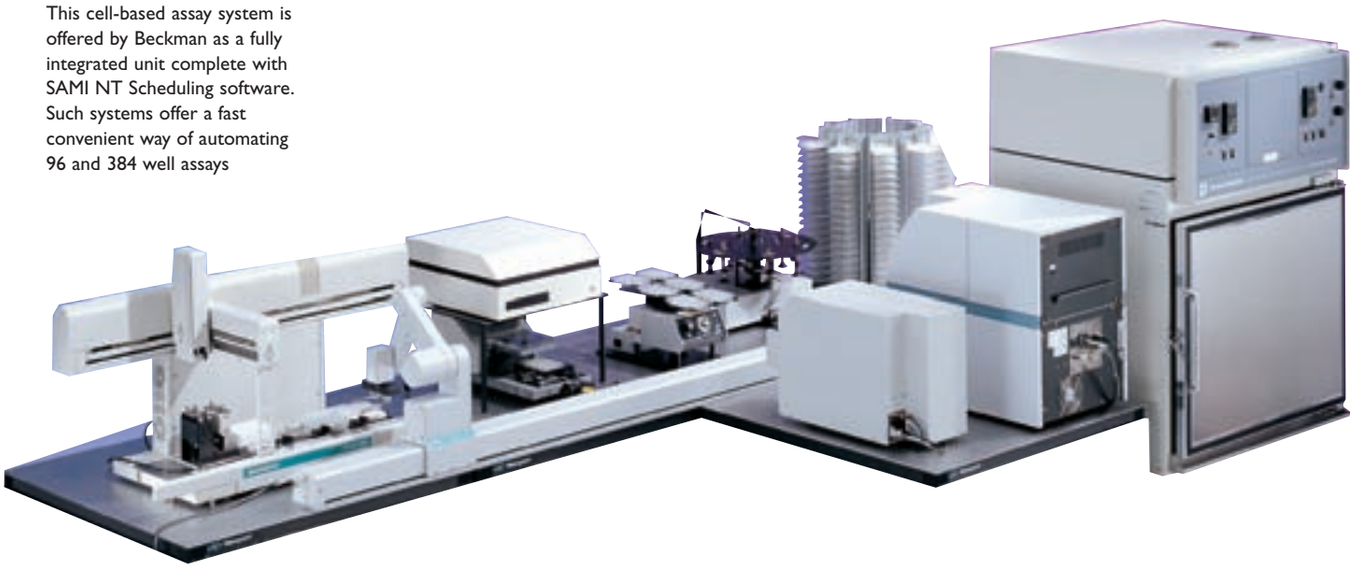


This cell-based assay system is offered by Beckman as a fully integrated unit complete with SAMI NT Scheduling software. Such systems offer a fast convenient way of automating 96 and 384 well assays

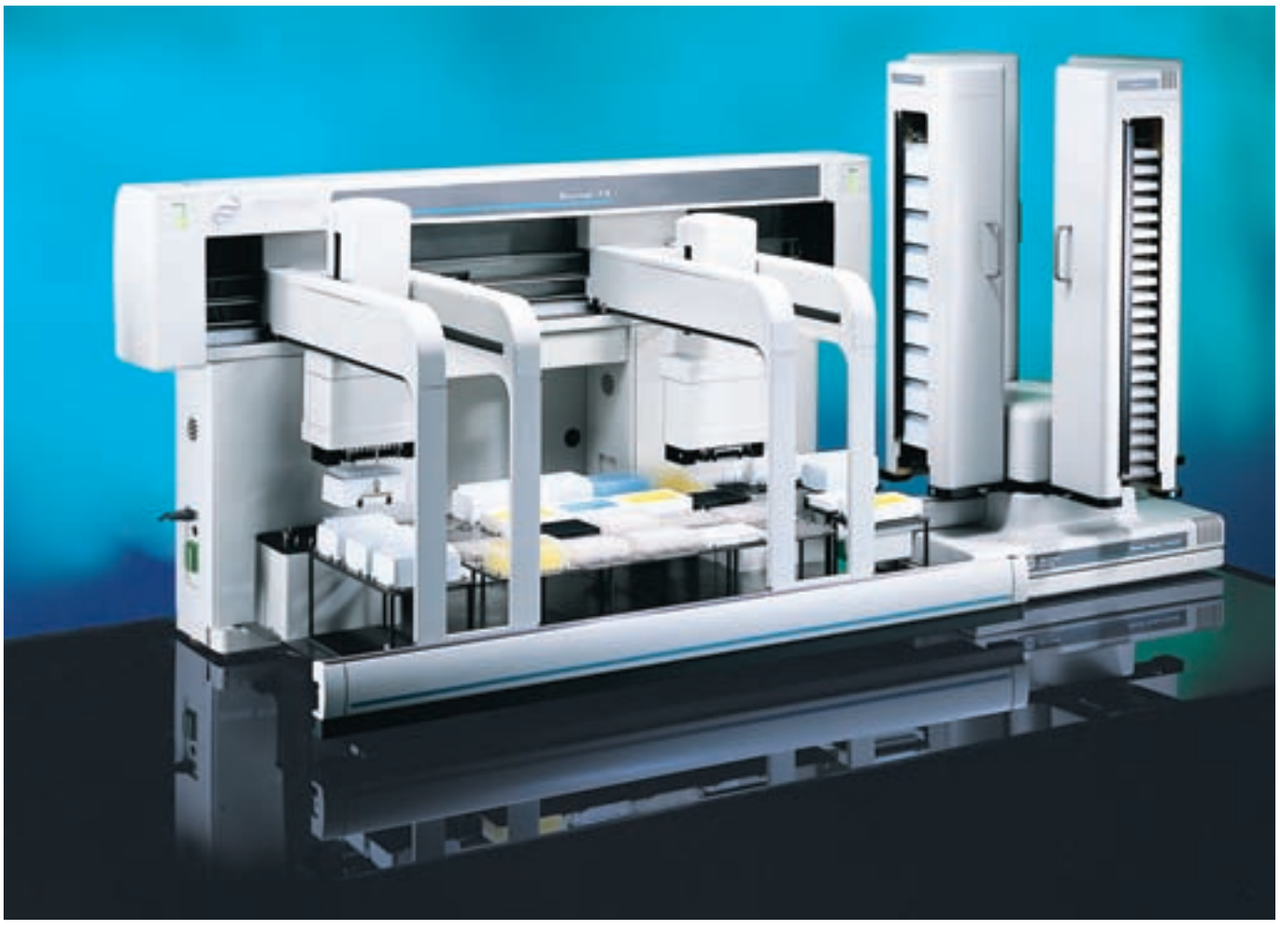
include the necessity to provide specialist support such for maintenance, programming and running the robot. Initially many large pharmaceutical companies created specialist HTS departments. Such departments demanded 'robot ready' assay protocols to be devised by research biologists. Unfortunately, it was often found that ostensibly robust assays had to be completely revamped by the HTS group. In addition, the lack of specialist biological knowledge in the HTS group can lead to costly mistakes, a technician charged with feeding the robot with reagent might inadvertently select the wrong splice variant of a GPCR, or an inappropriate heteromer of a multi-subunit ion channel. Such issues reveal a necessity to instigate a very high level of integration of HTS groups with other departments.

Future developments

Faster, cheaper, better are key words identified by Fox et al[4]. How can these be achieved? Having said that speaking of high throughput screening purely in terms of numbers undersells the activity, there is little doubt that one of the key responsibilities of assay technologists is to increase throughput. It has been estimated that up to 45% of drug development costs are used in the screening operation[2] and HTS labs consume some of the largest departmental budgets. Not-with-standing that automation systems such as the Allegro™ robot (Zymark) have been claimed to handle a thousand 96 well microtitre plates per 24-hour period[13], quantum leaps in throughput are unlikely to be achieved through 'more of the same'. The key to faster/cheaper has rightly been identified as miniaturisation. While most HTS assays are currently run in 96 well format, it has been predicted that most assays will run 384 well format at some point in 2001[4]. Enzyme assays have already been reported in 1536 format[14], and Aurora Biosciences have reported assays based on a 3456 plate[15]. Some companies are working with even higher densities such as 6500[16], and 9600 assays per plate[17]. It is difficult to predict what assay formats will be at the cutting edge five or even three years from now, or indeed if there are physical limits on assay volume. Piezoelectric pipetting devices are available that can pipette nano and pico litre volumes, and EvoTec have readout technology (fluorescent correlation spectroscopy) which is sensitive enough to measure the diffusion of a single fluorescent molecule into and out of a femto litre volume.

In addition there has been much recent talk of annealing biological molecules to a small metal plate, or 'chip'. This Technology, such as Seldi™ (Ciphergen), has already been used for target identification, and may well find an application in screening small molecule libraries. These technologies, associated with a move from channel based detection systems to CCD imaging devices may eliminate the concept of a plate completely.

In the future there is likely to be a prevalence of fluorescent and cell-based assay systems, but it is unclear which technologies will be integrated into systems for screening, and which cellular processes (eg uptake, gene transcription, membrane potential) will be easily probed. It is possible that large phar-



The Beckman Biomek FX dual arm robot and plate stacker is a stand-alone workstation. Workstations are used to increase the throughput of plate-based assay systems, while retaining day-to-day flexibility in assay parameters

maceutical companies will lead the process by investment in untested technology but it is also possible that smaller companies will take the lead, each championing a proprietary assay technology. While many HTS technologies are available to big pharmaceutical companies by purchase, or licensing agreement, it is possible that small biotechnology companies will usurp this aspect of drug discovery from big companies, protecting their investment with patents and trade secrets.

The key to better drug discovery has to be an increase in the approval rate of compounds. It is still accepted, as it was 30 years ago, that 90% of compounds submitted as investigational new drugs will not be approved[18]. One way of achieving this is to develop assays associated with developmental issues and run these as part of the primary screening cascade. Thus ADME and toxicology may enter the loop as a fully integrated part of the HTS-combinatorial chemistry-computational chemistry system. Whichever technologies are implemented, HTS is set to become one of the corner stones of drug discovery, however, deciding which strategy to implement will provide many headaches. DDW

Dr Tony Knight *has 12 years' experience in neuropharmacological research. His first degree was from the University of Bristol. He subsequently worked at Merck Sharp and Dohme in Harlow, UK where he was intimately involved in the development of novel benzodiazepines, and the development of the NMDA antagonist MK-801. After obtaining a PhD with Professor Norman Bowery, working on GABA receptors at London University, he continued his studies with GABA receptors at Neurogen Corporation (Connecticut, USA). At Neurogen he also became responsible for the development of novel assays for high throughput screening. He joined Cerebrus in 1997 where he is head of the Receptor-Pharmacology Group.*