Light on high-throughput screening: fluorescence-based assay technologies

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Fluorescence-based assay technologies can offer equivalent, if not greater, sensitivity to isotopic detection systems but have the added advantages of greater versatility, stability, safe handling and ease of disposal. Emerging technologies for high-throughput applications can be divided into four categories: time-resolved fluorescence, fluorescence resonance energy transfer, fluorescence polarization and fluorescence correlation spectroscopy. Novel fluorescence detection equipment has also been developed specifically for cellular systems. This report describes some of the key technologies that are now commercially available.

Many fluorescence-based generic technologies are being developed for both *in vitro* and cell-based high-throughput screening (HTS). These technologies can offer equivalent, if not greater, sensitivity to isotopic detection systems and have the added advantages of greater versatility and stability, safe handling and ease of disposal. Moreover, some fluorescence-based methodologies are being developed specifically for ultra-HTS using nanoscale technologies[1,2].

There are unique problems associated with the detection of fluorescence signals, which have compromised many potential fluorescence-based HTS assays in the past. The problems are threefold, firstly the signal can be subject to quench by compounds, plastics or media. Secondly, fluorescence emissions can be scattered by particulates. Lastly, the signal can be masked by autofluorescence (from proteins or compounds) or by high background fluorescence from unbound labelled probes. More recently, new hardware for fluorescence detection has become available that has quench-correction adjustment features built into its data-analysis systems.

Emerging fluorescence-based technologies for HTS fall into four categories, which are time-resolved fluorescence (TRF), fluorescence resonance energy transfer (FRET), fluorescence polarization (FP) and fluorescence correlation spectroscopy (FCS). In addition, novel fluorescence detection equipment has been developed specifically for cellular systems and is mentioned at the end of this review.

Time-resolved fluorescence intensity and resonance energy transfer assay technology for HTS

This area is dominated by two major companies, Canberra-Packard (Meriden, CT, USA) and Wallac (Turku, Finland). Both companies have developed their own unique time-resolved fluorescence technologies based on fluorescent lanthanide ions, and both report the development of homogeneous methodologies. The technologies marketed by the two companies are different and have their own selective advantages. This provides assay designers with a powerful set of highly sensitive (i.e. homogeneous TRF has an analytical sensitivity value of 8.8×10^{-13} M, see below), non-isotopic tools to build fluorescence-based HTS assays with.

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Canberra-Packard's homogeneous time-resolved fluorescence (HTRF) technology

CIS bio International (Bagnols, France) and Canberra-Packard have co-developed this impressive technology primarily for HTS, although it is also well suited to lower-throughput drug-screening assays. Because the technology is solution-phase, weak interactions that would be perturbed by association with a solid support can be studied. HTRF uses a europium (III) ion caged in a proprietary macropolycyclic ligand, containing 2,2′-bipyridines as light absorbers (Eu-cryptate). Energy is nonradiatively transferred from Eu-cryptate excited at 337 nm to a fluorescence acceptor molecule, a proprietary chemically modified allophycocyanin, termed XL665. In the presence of pulsed laser light, energy is transferred from the Eu-cryptate to the XL665 resulting in emission of light at 665 nm over a prolonged timescale (microseconds). This signal can, therefore, be distinguished from natural fluorescence and from the short-lived emission from XL665 in the absence of Eu-cryptate (nanoseconds). There is a 75% energy-transfer efficiency between Eu-crypate and XL665 over 7.5 nm. This rapidly diminishes over greater distances. Thus, free molecules in solution are unlikely to emit a signal because they are rarely in close enough proximity over the required timescale[3].

Eu-cryptate has a broad fluorescence emission spectrum, but does not emit in the range close to that of XL665 (665 nm). One of the europium emission peaks (620 nm) has been used as an internal control, as the signal at 620 nm is proportional to the concentration of free Eu-cryptate. Thus, the ratio of fluorescent signal from XL665 (665 nm) to that of Eu-cryptate (620 nm) defines the concentration of biological complexes formed that are producing FRET signals. Canberra-Packard point out that the inclusion of this internal control has been the first successful solution to the problem of inner filter effects and turbidity so often encountered using assay technologies based on fluorescence intensity.

Canberra-Packard have developed a dedicated time-resolved fluorimeter called the Discovery, which reads 96-well proprietary black plates, and a 384-well version of the Discovery is being developed. The Discovery contains a low wattage nitrogen laser with a fixed wavelength of 337 nm and delivers a beam of light at 20 pulses per second with a delay of 50–100 μs between pulses. After each pulse natural/autofluorescence from compounds or proteins, plastics and media decays rapidly, but the signal emitted from the XL665 has a much longer lifetime. The Discovery measures fluorescence intensity at two different wavelengths (620 and 665 nm), 50 μs after the light pulse from the laser, and averages the readings following 20 laser pulses. To obtain a value for the specific signal, the Discovery calculates the 665/620 ratio and normalizes the data against the signal from a blank containing only Eu-cryptate. The Discovery takes one second to complete 20 measurements per well and approximately 90 seconds to read an entire 96-well plate. The instrument is provided with a 40-plate stacker and has been equipped with a robotics interface and bar code reader for automated HTS screening systems. With this in mind the Discovery could achieve a throughput in the order of 50,000 wells per day.

HTRF has only recently become commercially available but has received much interest and has already been used for screening[4]. The technology is most amenable to *in vitro* biochemical HTS assays[5], although it may be possible to develop cell-based assays using this technology. In addition, development of the HTRF technology for 384-well plates is under way, and information received on a comparative immunofluorometric assay of prolactin in 96- and 384-well plates showed very comparable 665/620 ratios.

Wallac's lanthanide chelate excitation (LANCE) technology

Wallac has both solid-phase and solution-phase methodologies and their technology is based around lanthanide chelates, not cryptates[6]. Wallac have described five different homogeneous formats comprising the LANCE system. These include fluorescence resonance energy transfer (FRET), quenched fluorescence energy transfer (QFRET), environmentally sensitive chelate (ESC), spin label quenching (SLQ) and a non-separation fluorescent bead assay (NSFBA). All systems use a lanthanide (europium or terbium) as the fluorescence energy donor and in the FRET (distance dependence <10 nm), QFRET (distance dependence >10 nm to get signal quench) and NSFBA systems a second label, such as rhodamine or a cyanin dye, is present to act as an energy acceptor and light emitter. In the ESC system the signal is quenched by interaction with receptor protein and in the SLQ technique the signal acceptor is a spin label (distance dependence <3 nm). With these versatile tools a range of different HTS assays are possible. The technology has already been used for receptor-binding interactions, enzyme analysis, protein–cell interaction, competitive and noncompetitive immunoassays, protein–protein and protein–DNA binding interactions.

Coupled with this technology, Wallac have developed a multidisciplinary plate reader, the Victor, which can read both 96- and 384-well plate formats in the following label technologies: time-resolved fluorescence, fluorescence, photometry and luminescence. The Victor uses a xenon flash tube lamp for TRF measurements and is capable of detecting <10 amol/well of europium. Wallac have installed a proprietary quench adjustment on the Victor based on a combination of photometric adjustment, software and multidecay measurements to correct for all quench events (takes one second per well). The HTS-Victor is modified with appropriate changes in hardware (including plate stackers and a bar code reader) and software to utilize these corrections and runs on a Windows 95 format.

Fluorophores for ratiometric fluorescence resonance energy transfer analysis of reporter gene transcription

Several smaller companies, most notably Aurora Biosciences (San Diego, CA, USA) are developing cellular assays that utilize fluorescence-based technology. Aurora's fluorescence technology is based on the work of Dr Roger Tsien and includes β-lactam fluorophores for ratiometric analysis of β-lactamase reporter gene transcription. Bristol-Myers Squibb and Eli Lilly & Co. have recently signed major agreements with Aurora to develop fluorescence cell-based screening technologies and ultra-HTS systems designed to screen up to 100,000 samples per day[1].

Fluorescence polarization (FP) and its application to HTS

The fundamental principles of fluorescence polarization were developed as long ago as 1926 but were not applied to measurement of biological interactions until some 40 years later. Jolley and coworkers have developed FP into a commercial system for monitoring pharmacokinetics in body fluids and have recently adapted the technology for HTS of compounds. FP is a sensitive technology for the determination of molecular size and microviscosity (although less so than the Canberra-Packard and Wallac technologies). The limit of sensitivity of FP, as is true for any of the other technologies mentioned here, is dependent on the affinity and quantity of the target in the assay. It is possible to measure the FP of as little as 1 nM fluorescein in 100 µl, going down to 0.3 nM with special instrumentation.

Fluorophores emit light when excited by plane-polarized light and the polarization of the emitted light depends on how far the fluorophore rotates during the lifetime of its excited state. FP, therefore, depends on the rotational characteristics of the fluorophore in solution: the smaller the molecule, the faster it rotates, and the smaller its FP will be. Binding of a fluorescence-labelled ligand to its receptor in solution or on the surface of a living cell will result in slower rotation and an increase in FP.

FP assays are reported to be easier to construct than assays based on fluorescence intensity, because the tracers do not have to respond on binding by intensity changes. However, the tracer must be constructed to give maximum polarization change on binding to its receptor and the link between fluorophore and ligand in the tracer must be short and as rigid as possible (to avoid the 'propeller effect'). Moreover, the fluorophore used must have a high quantum yield and extinction coefficient. Fluorescein has been the fluorophore of choice, although recently BODIPY (Molecular Probes, Inc., Eugene, OR, USA) has been successfully used by Jolley and coworkers. BODIPY is both pH-independent and emits in the red portion of the spectrum away from potential interference by fluorescent organic compounds, so is more applicable to HTS[7].

FP is independent of fluorescence intensity and is more tolerant of fluorescence quenching and light scattering. For example, as long as a tracer signal is not quenched by more than 80% you can still derive accurate FP values, although sensitivity will be compromised. FP signals can be difficult to analyse if natural/autofluorescence emission from organic compounds or proteins accounts for more than 20% of the tracer signal. Some chemical libraries may, therefore, be difficult to screen using this technology.

The rapid speed of FP assays is possible because the technology is homogeneous, without the need for separation of free from bound fluorescent probes. FP is easy to perform and the kinetics are fast, taking seconds to minutes to reach equilibrium. The technology has been widely applied to *in vitro* biochemical assays, such as antibody–antigen interactions, enzyme inhibitors and inhibitors of DNA–protein interaction, but has only more recently been applied to HTS[8].

Recently, Jolley Consulting and Research, Inc. (Grayslake, IL, USA) in partnership with Dynatech Laboratories (Chantilly, VA, USA) have developed a 96-well plate fluorescence polarization reader called the Fluorolite FPM-2. The FPM-2 instrument has been designed to allow control by a central computer linking it with a robotic interface and it is anticipated that this will allow screening of up to 25,000 samples per day.

Dr J.R. Sportsman from Terrapin Technologies, Inc. (South San Francisco, CA, USA) mentioned that it is possible to achieve a throughput of 1,700 determinations per hour (a single 96-well plate every 3.25 minutes). At Terrapin Technologies, FP has been used to generate an affinity database for organic compounds, which may be a useful approach to primary screening of compounds against several targets, to select promising chemical classes for secondary assays. The process allows the identification of functional similarities in the binding sites of proteins that are structurally unrelated, and so nonspecific compounds that bind proteins other than the specific target of interest can be excluded from further analysis[9].

Fluorescence correlation spectroscopy and nano-ultra fluorescence-based HTS technology

EvoTec BioSystems (Hamburg, Germany) has developed, and is further improving, a miniaturized ultra-HTS system based on confocal nano-fluorescence read-out techniques, such as fluorescence correlation spectroscopy (FCS). FCS is an extremely sensitive technique for the measurement of fluorescent ligand concentration, and for the quantification of ligand–receptor binding in homogeneous assays. In FCS single molecules are measured as they diffuse through the extremely small measurement volume of 1 fl (the size of an *E.coli* cell). Free ligands diffuse more rapidly than ligand–receptor complexes because of the latter's greater molecular mass. Statistics associated with these diffusion events are recorded and automatically processed in real time during an FCS measurement. The entire task of measurement and data processing takes only a few seconds.

EvoTec have used their technology for the analysis of molecular binding events such as ligand–receptor binding, for enzymatic processes such as substrate oligomerization, degradation and modification, and for DNA binding. Cell-based assays are currently in development. In addition, EvoTec has recently developed confocal nano-fluorescence read-out techniques that are independent of mass differences between free and bound ligand, which is a prerequisite for FCS in its original form. This greater versatility in the measurement technology allows simultaneous evaluation of a variety of fluorescence parameters such as fluorescence depolarization, molecular brightness and fluorescence lifetime. Thus, rapid assay prototyping is achievable for assay systems using fluorescence read-out. In addition, new types of assays such as cellular uptake of compounds, protein oligomerization, and nuclear receptor response in primary cellular systems, for which HTS assays have been difficult or impossible to develop, are made possible.

The extremely small measurement volume associated with confocal read-out techniques lends itself ideally to miniaturization. When assay volumes are reduced, other methodologies often suffer from the increased contribution of surface effects and from drastically reduced signal to noise ratios. In contrast, confocal nano-fluorescence and FCS-based assays show equally good performance in miniaturized formats compared with conventional microtitre formats.

A highly sophisticated, miniaturized liquid-handling and sample-carrier technology is required to take full advantage of nano-fluorescence read-out technology. These hardware components must be carefully adapted in a systems approach. EvoTec has configured and applied dispensing and pipetting instrumentation capable of nanolitre resolution (a submicrolitre assay can be dispensed with a dynamic range of 1000:1). Assay miniaturization to submicrolitre levels with technology provided by companies like EvoTec will provide very significant reductions in reagent costs for HTS compared with the standard microtitre plate format. Savings of this kind should make this technology a highly desirable acquisition by HTS programmes.

A completely automated ultra-HTS system (EVOscreen), including the proprietary miniaturized liquid handling and storage system, will be available by 1998 to EvoTec's industrial partners contributing to its development. A throughput of 100,000 samples per day is envisaged.

Fluorescence imaging hardware for cell-based screening

Two systems are noteworthy. Firstly, the Fluorescence Imaging Plate Reader (FLIPR), which uses a 96-well plate format, allows the simultaneous addition of reagents and measurement of kinetic events in all wells [this is now being developed by Molecular Devices, Inc. (Sunnyvale, CA, USA)]. The FLIPR uses its powerful argon laser (488 nm) to simultaneously illuminate all wells on a 96-well plate. The system comprises a cooled charged coupled device (CCD) camera for fluorescence detection, an automated pipettor head for addition of reagents and a temperature-controlled plate chamber. The instrument can run confocally and at single or dual wavelength emission modes. Assays can be carried out in this instrument under precise temperature control to measure cellular responses with fluorescent intracellular ion indicators, membrane potential and vital dyes. The

FLIPR has a throughput of approximately 20 plates (1,980 determinations) per day without automation.

Secondly, the ArrayScan (BioDx, Pittsburgh, PA, USA) is a spectroscopic scanning system that is also developed on the 96-well plate format. The ArrayScan obtains 'real time' measurements of fluorescence signals at the single cell/sub-cellular level, sequentially for all 96 wells. The system uses a CCD camera coupled to a Zeiss microscope. Several measurements can be taken per well and the data integrated and analysed by the software provided. The ArrayScan has a throughput of 1–2 96-well plates per hour.

Conclusion

There have been many exciting developments in fluorescence technology and the future for high-throughput assays is clearly one in which homogeneous (without the need to separate bound from free ligand/probe) formats with light-based read-outs will play an important role in drug screening programmes. Several companies are developing fluorescence-based technologies and methodologies, which will provide a versatile set of assay design tools and detection systems. The technologies being developed will be applicable to both *in vitro* biochemical and cell-based screening assays alike and will, in time, largely replace the existing isotopic detection systems. In the future it may be possible using fluorescence-based technologies, such as that being developed by EvoTec, to miniaturize to nanoscale and achieve throughputs that are well beyond our current screen capacities at a fraction of the cost. A revolution in HTS technology is around the corner.

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In short...

Bristol-Myers Squibb recently received FDA clearance to market VAGISTAT®-1 (active ingredient: tioconazole), a broad-spectrum antifungal agent, as an over-the-counter treatment for vaginal yeast infections. The product is the first one-dose medication for vaginal yeast to be approved for use without a prescription.

Biostratum (Lake Forest, IL, USA) has completed initial preclinical studies of its anticancer drug candidates in several animal models of metastasis. Several candidates were found to inhibit the development of metastatic tumours significantly. Biostratum has developed a series of compounds called COLLAMERS™, which are stable peptides that mimic regions of the type IV collagen involved in tumour binding, the type IV collagen involved in basal lamina assembly, and monoclonal antibodies directed against specific basal lamina components involved in tumour migration. Several of these peptides were found to block basal-lamina-mediated tumour invasion and migration, and some were also found to inhibit angiogenesis in model systems.

The AIDS Clinical Trials Group, a cooperative group supported by the National Institute of Allergy and Infectious Diseases, has begun a Phase II study of 141W94/VX478, a second generation HIV protease inhibitor developed jointly by **Glaxo Wellcome**, **Kissei Pharmaceutical Company** and **Vertex Pharmaceuticals**. The 24-week, double-blind study will assess the antiviral efficacy, tolerability and pharmacokinetics of the drug as a single agent versus a combination therapy of 141W94/VX478 plus AZT plus 3TC. Various other Phase II trials of 141W94/VX478 are in progress. Earlier this year, Glaxo Wellcome reported preliminary results suggesting that 141W94/VX478 is well tolerated and displays potent antiviral activity as a single agent and in combination with Glaxo Wellcome's reverse transcriptase inhibitor 1592U89. The company is planning to start Phase III trials of 141W94/VX478 early in 1997.