Miniaturization of homogeneous assays using fluorescence polarization

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Homogeneous fluorescence-based assays are a significant development in HTS. Fluorescence polarization (FP) is emerging as an important HTS technology that reduces costs while greatly increasing throughput. Inherently suitable for miniaturization, FP has been demonstrated to run in as little as 4 μl total assay volume.

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▼ There have been enormous changes in drug discovery over the past five years. It is now taken for granted that screening rates of >100,000 data points-per-day per workstation are required for success. In turn, this has refocused efforts to focus on the potential of alternative technologies such as fluorescence, which has major theoretical advantages over other techniques of analysis. Fluorescence methods are capable of fulfilling several important requirements of today's ultra-HTS enterprise. Fluorescence can be used with homogeneous formats, which is important because separation and washing steps are too cumbersome, expensive and slow to be considered competitive. High sensitivity is also required to be able to detect biological binding at sub-nanomolar concentrations, and fluorescence delivers this capability. Flexibility is of key importance because these assays must be able to focus on several different targets. Finally, it is ever-increasingly important to miniaturize assay formats into volumes that just five years ago were considered to be unusual (1–5 μl), but which today are achievable[1].

In the move towards a wider adoption of fluorescence technologies, there is the added benefit of abandoning radioactive labels, which are increasingly becoming liabilities because of their cost, safety profile, and other concerns. However, from a strictly mechanical point of view, radioactivity is a liability because of the time it takes to quantify it. For example, 10 fmol of ^{3}H produces approximately 5 counts-per-second (cps) in a standard scintillation detector. However, the same mass of fluorescein can deliver approximately 3 million cps in an optimized instrument. Thus, to collect 10,000 counts (to reduce relative counting error to 1%), it takes approximately 25 min for radioactivity versus <50 ms with fluorescence. While this million-fold advantage does not, of course, directly translate into a linear increase in sensitivity (because the background of radioactive detectors is so low), this factor does highlight one clear advantage of fluorescence methods: they are capable of very rapid read times.

Current fluorescence technologies

Several fluorescence technologies are currently in use[2,3]. Simple fluorescence intensity can be used when the biological reaction generates an increase or decrease in fluorescence, as can occur in certain binding assays or in any assay where hydrolysis releases a quenched fluorescent species. However, intensity-based assays are usually heterogeneous in nature, or lack the sensitivity required for their broad application. Also, intensity assays are relatively sensitive to interferences from library compounds (from quenching caused by absorbance – the 'inner filter' effect) and variability in reagent quantities. Fluorescence resonance energy transfer (FRET) between two visible-wavelength dyes, such as fluorescein and rhodamine, was one of the first homogeneous fluorescence assays to be used in screening[4]. However, interferences and distance constraints still limit the utility of this 'prompt' form of FRET (Ref. 5).

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Reduction of interferences from assay and compound backgrounds has been a major objective for those who want to take advantage of the high speed and sensitivity of fluorescence. The technique of time-resolved fluorescence (TRF) was developed almost 20 years ago, initially as a diagnostic application. This technology, based on the long-lived fluorescence of lanthanide chelates, effectively discriminates against short-lived or prompt fluorescent interferences. However, TRF has been of limited application in HTS because it is still a heterogeneous assay technique requiring evacuation and wash steps followed by a final reagent addition to measure the fluorescence. More recently, a homogeneous format for TRF has become available. This technique, which is variously known as homogeneous TRF (HTRF) or time-resolved fluorescence resonance energy transfer (TR-FRET or simply TRET), quantifies molecular binding by fluorescence energy transfer between a lanthanide chelate donor and an acceptor molecule such as the fluorescent protein allophycocyanin (APC). Another technology, fluorescence correlation spectroscopy (FCS), offers a homogeneous assay format suitable for microvolume methods[5].

Fluorescence polarization

At approximately the same time that TR-FRET was being found to be useful for HTS applications, an older technique, fluorescence polarization (FP), began to be reapplied in the field of drug discovery research. FP has been known for over 70 years, and is widely used in basic biophysical research. In the early 1980s, the technique was successfully applied to clinical diagnostic immunoassays for therapeutic drugs and drugs of abuse[3]. The widespread adoption of this technique in clinical assays has been because of its convenient homogeneous format, high sensitivity and resistance to interferences encountered in biological matrices. However, the lack of a high-performance instrument that could accommodate microplate formats has delayed the acceptance of FP in the growing field of HTS. As will be discussed, the potential of FP in HTS only began to be realized following the recent introduction of new instruments with subnanomolar capabilities in polarization.

FP can detect changes in molecule sizes as well as quantify the binding of small molecules to large molecules. Polarization of fluorescence occurs when a fluorescent molecule is illuminated with plane-polarized light, providing that the molecule does not move during the course of the fluorescent lifetime (as would be the case, for example, if the molecule were entrapped in a gel or a glass). For typical fluorescent molecules such as fluorescein, this polarization is not observed if the molecule is rotating rapidly in solution under conditions typically used in biological assays (such as in aqueous buffers and at room temperature). Thus, if the polarization of fluorescence of a fluorescein-labeled ligand is measured, a low polarization will be observed when the ligand is free in solution, and a high polarization will be observed when the ligand is bound to a macromolecule such as a specific receptor or antibody. This is because in the latter case, the fluorescent species will rotate at the much slower rate of the macromolecule.

FP is technically a rather simple technology, requiring little more than a filter fluorometer equipped with polarizing filters. The intensity of fluorescent emission is then quantified through a polarizing filter, which is initially set parallel, and then set perpendicular, to the direction of an excitation polarizer. These two measurements are referred to as the parallel and perpendicular intensity measurements, I_p and I_s, respectively. Polarization is then calculated as the difference between these values divided by their sum (Equation 1).

$$\text{Polarization (mP)} = 1000 \times \frac{(I_P - I_S)}{(I_P + I_S)} \quad (1)$$

Two important points should be mentioned here. Firstly, from Equation 1, it is clear that polarization is a ratiometric measurement, which is an advantage as these types of measurements can self-correct for variations caused by fluctuations in lamp intensity or interferences caused by quenching of the fluorescence. A second point is the theoretical boundaries of polarization measurements. No biological system can show polarization below 0 mP or greater than 500 mP. If a value outside this range occurs, it is a clear sign that there is an error, either in the instrument or, more likely, in the elements comprising the assay sample (perhaps the presence of a highly fluorescent or scattering compound). Thus, FP automatically checks assay validity. A second check of validity is provided by the intensity parameter, which is calculated from the same data used for polarization, as shown in Equation 2.

$$\text{Intensity} = I_P + 2I_S \quad (2)$$

This intensity value can be thought of as the intensity that would be measured if there were no polarizers in place (i.e. the intensity of a conventional fluorescence measurement). As a constant quantity of fluorescent tracer is added to every sample in most FP assays, this intensity value should not be outside a certain statistical range. If it is outside this range, the presence of a fluorescent or quenching compound might be implicated, and a spurious result would be flagged.

Changing to microplates and increasing sensitivity

The first examples of the application of FP to pharmacological screening targets were in the early 1990s. Michael Tota and coworkers at Merck (Rahway, NJ, USA) demonstrated the potential of FP for measuring binding of ligands, such as exendin and glucagon[6,7], to G protein-coupled receptors (GPCRs), a class of targets that requires sensitivity measurements of ≈1 nM. This

work was accomplished using a custom-built instrument, whose high performance was not yet extended to include rapid reading of large numbers of samples. At approximately the same time (1994), the first FP plate-reading instrument appeared, namely the Jolley Consulting and Research (Grays Lake, IL, USA) FPM-2P. This instrument could read levels down to 10 nM fluorescein in a 96-well microplate at a precision of 2.5 mP or better. While this performance was adequate for many applications, the subnanomolar sensitivity required for many classes of assays was not achieved. This instrument was shortly followed by the Polarstar (BMG Labtechnologies GmbH, Hamburg, Germany) that could read 96- and 384-well plates, but still only in the range of >1 nM.

Box 1. Fluorescence polarization specifications of the ANALYST

Detection limit (fluorescein, 40 μl) intensity mode, no polarizers	3 pM
Precision of polarization	≤10 mP SD at 100 pM fluorescein
Read time, 384-well plate	<2.5 min
[a]Throughput (samples-per-day)	>70,000

[a]Using a 40-plate stacker for eight hours-a-day plus one off-shift cassette (~11 h total), (unpublished data).

New higher-efficiency FP technologies

The implementation of FP into a highly sensitive instrument suitable for HTS was accomplished in 1998 with the introduction of the Analyst (from LJL BioSystems, which can be used with 96- and 384-well plates), followed shortly by the Acquest (LJL BioSystems; for up to 1536-well plates). This instrument enabled a new range of FP assays to be carried out. Box 1 shows the sensitivity that this instrument can achieve (unpublished results).

It is worth noting that these instruments accurately measure the polarization of fluorescein at concentrations below 100 pM with a precision of better than 7 mP. As most biological binding assays involve a change of >100 mP, assay signal-to-noise ratios of ten or greater are routinely produced at these concentration ranges. Furthermore, the confocal design and high-intensity continuous Xe arc lamp in this design focuses the illumination into a very small volume. Assays that are run in volumes of 5 μl or less show a virtually identical performance to those run in more conventional volumes of 40–100 μl. This improvement in assay performance is accompanied by a full realization of the earlier-mentioned theoretical speed advantages of fluorescence. Because of these improvements in FP, we have termed the technologies high-efficiency fluorescence polarization (HEFP).

The successful implementation of HEFP has stimulated activity among instrument makers for the HTS market. At present, the Ultra from Tecan Austria (Salzburg, Austria) is the only other instrument that is available for 1536-well plates using FP. Some comparative information is provided in Table 1.

HEFP in HTS assays

Since its introduction in 1997, HEFP has found its way into the screening laboratories of numerous biotechnology and pharmaceutical companies. HEFP's application to a variety of important targets in molecular binding and catalysis is now well established and growing rapidly. This section will

Table 1. Fluorescence polarization instruments currently available

Manufacturer (instrument)	Well-plate formats	Recommended volume (μl)	Polarization precision (mP SD)
LJL BioSystems	384	40	[a]3 mP
(Analyst, Acquest)	1536	5	7
Tecan	384	50	[b]5
(Ultra)	1536	10	7
BMG LabTechnologies (Polarstar Galaxy)	384	50	NA

[a]Determined with 1nM fluorescein.

[b]Concentration not reported.

Abbreviation: NA, information not available.

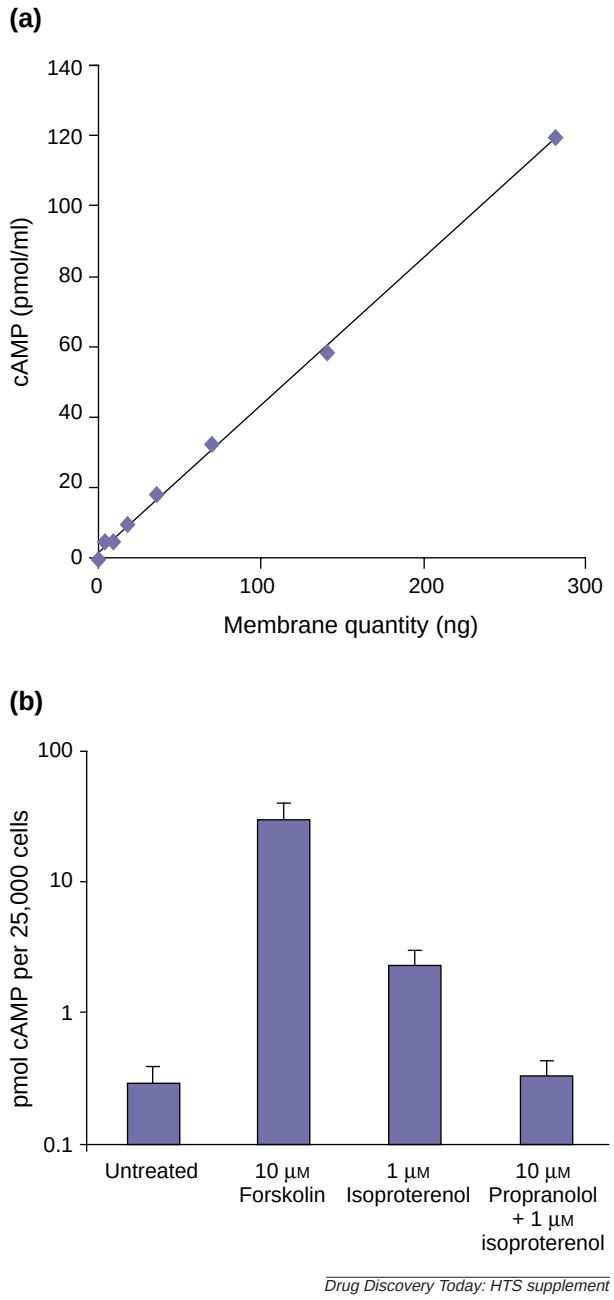

Figure 1. Applications of the high-efficiency fluorescence polarization (HEFP) assay for cAMP in biochemical and cell-based assays. (a) Assay of adenylyl cyclase (AC) in membrane preparations. Recombinant AC expressed and partially purified from *Escherichia coli* was incubated with ATP for 0.5 h and the products analyzed by HEFP. (b) Assay of cAMP responses in cultured cells. Adherent T47D cells were washed and incubated in Krebs-Ringer buffer with the treatments shown for 15 min, followed by lysis and analysis using the HEFP assay. The AC activator, forskolin, produced a large increase in cAMP content, as might be expected. The β-adrenoceptor agonist, isoproterenol, also stimulated cAMP production, and this effect was abrogated by the antagonist propranolol, demonstrating receptor-mediated stimulation of cAMP. (Courtesy of LJL BioSystems.)

examine some examples of where HEFP has been shown to be an important technology for drug discovery.

There are several examples of assays run in FP formats for a number of important receptor-binding targets. As already mentioned, early work carried out by Tota and coworkers using custom-designed instruments showed that FP was a competitive technology for GPCR binding assays, this being even more true with the advent of HEFP (Ref. 8). FP has been used for several types of enzyme assays, particularly of protein kinases[9], which comprise a large part of the screening agenda of numerous companies. Several important intracellular signals are generated by enzymes, and these are also well studied using FP. For example, Fig. 1 shows the HEFP assay for cAMP to detect products of adenylyl cyclase, both *in vitro* and in cell culture assays. The high linearity of the response seen in Fig. 1 suggests that FP might have advantages over solid-phase technologies. As FP can determine cAMP production in whole cells, HEFP can be used in high-throughput cell-based assays in which the readout of several important GPCRs can be coupled to cAMP.

Realities

As with any assay technology, there are several practical considerations regarding the use of FP. Polarization (or anisotropy) data quantify molecular binding in a less intuitive manner than data such as radioactivity or fluorescence intensity that is directly proportional to mass. However, if the relationship is understood, the advantages of ratiometric correction from polarization are appreciated.

As FP is a fluorescence technique, it is affected by quenching, caused primarily by compounds that absorb in the visible regions of the electromagnetic spectrum. To a certain extent, this interference is corrected by the ratiometric nature of FP, as absorbance will affect both intensity values equally (see Equation 1) and will usually cancel out. However, as intensity values drop too low, the noise of the measurement predominates and the precision is lost. Of course in addition, excessive background fluorescence or fluorescence caused by compounds will compromise the fidelity of the polarization value. This can often be minimized by determining background fluorescence intensity values, I_P and I_S, for the sample prior to addition of the fluorescent tracer. This background level can be subtracted from the final measurements and, hence, the true polarization determined. Although analysis time would double, this background level could be measured for every compound tested in the screen. As in the case of quenching, there is a limit to how much interference can be removed in this way.

The extent of interference in HEFP assays will vary depending on the nature of the compounds being screened. However, recent reports suggest that typically 1–2% of compounds in

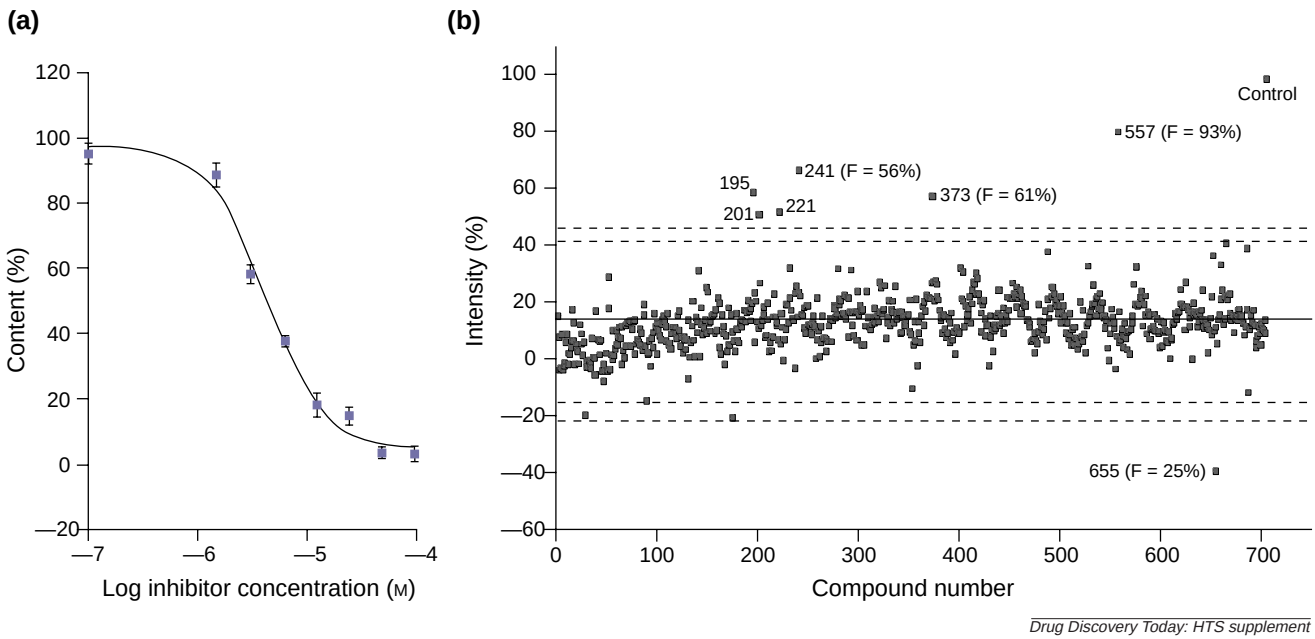

Figure 2. Assay miniaturization examples using high-efficiency fluorescence polarization (HEFP). (a) Adenine transferase assay. The reaction volume was 7 μl in the 1536-well plate format, and the signal was detected by Acquest system from LJL BioSystems (courtesy of Zhuyin Li, Aventis Pharmaceuticals, Bridgewater, NJ, USA). (b) A HEFP assay for CDK2E binding to a cognate peptide. This is representative data from 700 compounds using a total assay volume of 4 μl. Data points 241, 373, 557 and 655 were false-positives identified by aberrant fluorescence intensity values that are indicated in parentheses as the percentage of expected tracer intensity. Compounds 195, 201 and 221 were true 'hits' that had normal intensity values. The data were acquired using the LJL Acquest (courtesy of Kevin Oldenburg, DuPont Pharmaceuticals, Wilmington, DE, USA).

a chemical library can be expected to be difficult to assay at fluorescein wavelengths under normal screening conditions. Moving assays from the wavelength regions of fluorescein into redder regions of the spectrum are expected to reduce this problem considerably[8,10].

There is also an upper limit of the MW that can be examined by FP using conventional fluors. Generally, ligands of MW <5000 Da are optimal, although higher MWs can sometimes be measured. New fluors with longer lifetimes are expected to widen the range of assayable MWs. The synthesis of active fluorescent tracers is a requirement of FP that involves additional design considerations. As these tracers are usually small molecules and peptides, their derivatization using fluors is technically more complex than the simple labeling of macromolecules. However, numerous examples of success, such as those cited here, demonstrate that this is not an insurmountable problem.

Miniaturization

The benefits of assay miniaturization are now coming into focus because of the reported cost advantages of changing assay volumes from 100 μl down to 5 μl. Numerous investigators are currently developing and running screening assays in this format using technology such as the Acquest or Ultra. Figure 2 shows the performance of two assays in miniaturized format where the assay volume is <10 μl. Here, the activity of a transferase enzyme, and the binding protein cyclin-dependent kinase 2/E (CDK2/E), are both analyzed in the 1536-well format. In the first case, the IC_{50} for a known inhibitor, determined by HEFP (see Fig. 2a), was 4.6 μM, which was identical to the value determined in a radioactive assay. This transferase assay was used to screen library compounds at considerable cost savings[11]. In the second example (Fig. 2b), an assay with a total volume of 4 μl was used to screen for inhibitors of CDK2/E binding[12]. This example also illustrates the use of intensity as an independent check on the validity of polarization data.

Miniaturization is clearly an inherent capability of FP. Now that HEFP and other fluorescence methods can deliver assay performance in volumes of <10 μl, systems that can fully integrate this ability into the assay process are essential. This is particularly important with respect to liquid handling capabilities. Effective use of this level of miniaturization is not merely a question of automation but rather of the integration of microplates, submicroliter pipetting and reagent systems. Available commercial pipetting systems can adequately deliver volumes down to approximately 1 μl, including systems from Zymark

Box 2. ScreenStation for ultra-HTS assay assembly and detection

[a]The ScreenStation (LJL BioSystems) is capable of:

- Integrating homogeneous assay assembly, incubation and detection in 96-, 384- and 1536-well formats into one unit
- 0.5–5.0 μl non-contact dispensing with up to four separate reagents
- Integrated high-speed 40-plate stacker
- Multi-mode ultra-high sensitivity detection.

[a]Unpublished data.

(Hopkington, MA, USA), Robbins Scientific (Sunnyvale, CA, USA) and Tecan. A few systems can now deal with the ultra-high-density microplate formats that run in HEFP including those from Cartesian (Irvine, CA, USA), Robbins Scientific and Cybio (Jena, Germany). Several technological solutions exist for the task of distributing compounds into microplates in a manner suitable for screening. However, there is still a lack of integration in the field of assay assembly, that is, the task of adding one or more assay reagents in submicroliter volumes to a high-density microplate on a timeframe that is compatible with the high speeds now possible by homogeneous assay technologies such as HEFP.

One approach to this problem is to optimize fluidics as part of the assay assembly process such that crucial aspects of assay performance in a homogeneous format are specifically addressed. The ScreenStation (Box 2; LJL BioSystems) is one concept that enables the non-contact addition of up to four assay reagents at volumes of 0.5–5 μl, integrating assay protocols that could involve several steps but whose format and final readout is a homogeneous one.

Future of HEFP

The future of HEFP technology will proceed with technological advances. There is a high potential in the chemistry of fluorescent tracers to expand the range of MWs that can be analyzed by HEFP as well as to improve the robustness of the assays that incorporate this technology. Although HEFP has largely been limited to the analysis of low-MW ligands (<5000 Da), this is a function of the fluorescent lifetime of the fluorescent tracers used. Fluorescent tracers with lifetimes in the order of tens to hundreds of nanoseconds are now being developed. These new fluors enable protein–protein binding assays to be measured by HEFP, extending the MW range to several hundred thousand daltons[13].

In terms of instrumentation, future developments will probably consist of improving the speed and sensitivity of FP technology. Imaging technologies for fluorescence have been discussed. It remains to be seen whether the cost-to-benefit ratio of imaging systems will justify their wide acceptance over existing point reading systems. Even so, interesting data has recently been presented to show that the advances in system designs, targeting the theoretical possibilities of fluorescence-based methods, will continue to advance the capabilities of FP (Hoyt, C.C. *et al.* Novel high-accuracy fluorescence polarization plate reader. *5th Annual Conference of the Society for Biomolecular Screening*, 13–16 September 1999, Edinburgh, UK).

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