Fluorescence correlation spectroscopy: lead discovery by miniaturized HTS

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Miniaturized high-throughput screening offers great promise for increasing the productivity of the pharmaceutical drug discovery process. By monitoring interactions of single molecules in femtoliter volumes, fluorescence correlation spectroscopy (FCS) offers the highest potential as the detection technique in the nanoscale. The authors (Box 1) summarize the current status of practical experiences with FCS assays for HTS and explore the scope for further developments.

Box 1. Contributing research teams

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Three new scientific disciplines show the highest promise of fulfilling the need for increasing the predictability and for lowering the overall attrition rate of the drug discovery process.

- Functional genomics was invented to generate new innovative molecular targets
- Combinatorial chemistry provides increasingly efficient ways to generate molecular diversity with which to probe the targets
- High-throughput screening (HTS) platforms provide efficiency and quality in finding potential lead compounds

HTS within most pharmaceutical companies currently involves performing several million assays per year. Meanwhile, HTS has become a discrete discipline assimilating biochemistry, biophysics and cell/molecular biology combined with detection/liquid-handling technologies and automation processes. Testing a vast number of synthetic compounds and natural products against a target of therapeutic interest has imposed an ever increasing demand for short assay turnaround times, fast and high quality lead validation, immediate profiling of potential lead compounds, use of very diverse sample collections (e.g. discrete compounds, microbial extracts and combinatorial libraries), and saving of compounds, biological tools, reagents and

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waste (especially radioactive). In order to fulfil these requirements a screening concept based on homogeneous, automated assays in a miniaturized format that provides the highest possible mechanistic information (kinetic and thermodynamic) seems most appropriate.

Fluorescence spectroscopy

The only detection technique available that combines homogeneous mixtures of reagents, high sensitivity, true equilibrium in complexation reactions and a wide range of solution conditions is fluorescence spectroscopy. The picosecond to microsecond timescale of the emission of photons corresponds to the timescale of many dynamic events, including macromolecular rotational diffusion, solvent reorientation, energy transfer or motion of domains. By choosing the appropriate fluorescence label and strategy, fluorescence can provide information on size, distances, ligation state, conformational rearrangements and sample heterogeneity. Many pharmaceutical companies are increasingly developing and operating fluorescence-based HTS screens, typically using time-resolved or continuous wave fluorescence resonance energy transfer, fluorigenic enzyme substrates or fluorescence anisotropy, prior to and in preparation for the ultra-HTS screening era.

All macroscopic fluorescence methods, whether based on intensity, anisotropy or lifetime detection, average the emission signal from all excited molecules in the cuvette or well (ensemble averaging techniques). With these techniques the fluorescent signal typically decreases and the assay variability (coefficient of variation) increases as assays are conducted in 1–10 μl assay volumes (c.f. 50–200 μl volumes for conventional microtiter plate assays). Therefore, the current limit for the miniaturization of many types of assays is ~10–20 μl using commercially available readers. The only technical possibility to overcome the finite resolution dictated by the level of background noise in average intensity measurements is to record signals from single molecules. Such a measurement is feasible with a detection volume that is small enough to host, on average, only one or a few particles. The time-dependent fluctuations of (fluorescent) particles with different molecular properties, like molecular weight, translational and rotational diffusion time, colour and fluorescence lifetime, potentially provide all the kinetic and thermodynamic information required to study complex molecular interactions.

Fluorescence correlation spectroscopy

With the technical possibility to focus a laser beam to femtoliter volumes, such a new fluorescence technique, fluorescence correlation spectroscopy (FCS), finally became a practical possibility after it had been established in principle about 20 years ago[1,2]. Although the fundamental principles behind FCS are described elsewhere[3–9], a very brief summary of the basic technology is included here.

Conventional FCS takes advantage of differences in the translational diffusion of large versus small molecules. Each molecule that diffuses through the illuminated confocal focus gives rise to bursts of fluorescent light quanta during the entire course of its journey, with each individual burst being registered. The length of each photon burst corresponds to the time the molecule spends in the confocal focus. The photons emitted in each burst are recorded in a time-resolved manner by a highly sensitive single-photon detection device. This detection method achieves single-molecule sensitivity, but the fact that diffusion is a random process requires that the diffusion events for a minimum ensemble of molecules must be averaged to achieve statistically reliable information. The detection of diffusion events enables a diffusion coefficient to be determined. This diffusion coefficient serves as a parameter to distinguish between different fluorescent species in solution; for example, between free or bound ligand. In screening, the diffusion coefficient can be used to determine such factors as concentration or degree of binding. In addition, confocal optics eliminate any interference from background signals and allow homogeneous assays to be performed. FCS measurements are conducted in seconds making the technology ideally suited for high-throughput applications.

We emphasize five points relevant to miniaturized HTS:

- FCS is effectively insensitive to miniaturization because of the small size of the confocal detection volume. With less than a femtoliter (10^{-15} liter) as detection volume FCS is the ideal method for nanoscreening.
- By detecting the temporal fluorescence intensity fluctuations caused by the diffusion of single molecules in and out of a laser focus, FCS measurements contain all the spectroscopic information required to monitor changes in the molecular state and number of fluorescent molecules. In addition to the increase in translational diffusion time of a molecule by binding to a substrate or the decrease of translational diffusion time by cleavage reactions interpreted from the FCS signal, detection techniques

Box 2. The EVOscreen™ platform

The FCS and FCS-based assays described above were developed on an extended, FCS$^+$plus capable reader based on the Zeiss-Evotec Confocor™ and run on separate pipetting, dispensing, storage and FCS detection and data evaluation software modules. In the meantime all individual components have been combined to form the EVOscreen™ system, a unique platform integrating highly sensitive detection technology and high-precision liquid handling systems in a modular system which will be capable of 100,000 assays per day. The core elements of the modular system are:

- A high performance confocal fluorescence detection unit usable in either a single channel or multi-channel mode
- Proprietary signal processing protocols based on FCS and related single molecule-based confocal fluorescence methodologies
- A miniaturized, automated liquid handling system for nano- to low-microliter volumes (including pipetting, dispensing and compound retrieval)
- A rapid, miniaturized multi-replica compound repository providing the link between traditional single compound library formats and EVOscreen™
- A micro-separation device (HPLC) coupled to the detection system
- A scanner and picker device for the analysis of combinatorial libraries and for functional genome analysis

monitoring intensity, particle number, anisotropy, cross correlation (energy transfer) and lifetime are technically feasible. At a mature state of development, FCS may therefore be expected to cover all traditional fluorescence techniques in one measurement with single-molecule resolution. In HTS the high information content also improves well-to-well and assay-to-assay quality control.

- FCS, as a diffusion method, is the only technique currently known that determines the concentration of interaction partners free of artefacts.
- The combination of 'true' concentration measurements with the detection of up to five different molecular parameters from one experiment allows molecular interactions to be mechanistically characterized during or immediately after the HTS run (see CD45 example below).
- Improvements in FCS optics have reduced read times to one or a few seconds per well for many types of assay, such that FCS can deliver the throughputs necessary for HTS screening (>20,000 assays day^{-1}).

Evotec Biosystems in Hamburg was founded in 1993 to exploit the possibilities of FCS in biology and medicine. Since 1996, in a milestone-driven collaboration, at first with Novartis Pharma and, since 1997, also with SmithKline Beecham Pharmaceuticals, the activities at Evotec have been concentrated on lead discovery. The EVOscreen™ platform – a modular, miniaturized ultra-HTS system based on FCS and Evotec's proprietary FCS-related single-molecule detection technology (FCS$^+$plus) – is operational as of April 1998. In this review, Novartis, SmithKline Beecham and Evotec jointly report on a selected series of FCS-based assays that were developed to run on the EVOscreen™ system (Box 2).

Examples of FCS-based assays

Between the three companies, over 50 FCS-based assays including examples from many classes of target proteins typically encountered in the pharmaceutical industry were developed during the past two years. Outlines of some of these are given below. In addition, for one example (CD45 phosphatase), we describe in detail the steps from assay development through to the operation of a miniaturized prototype HTS screen.

Topoisomerase–DNA binding

Topoisomerases catalyse changes in the topology of supercoiled DNA during its replication, transcription and recombination. Inhibitors of human topoisomerases have been shown to be useful anticancer agents. Here, we developed an FCS assay to screen for inhibitors of DNA binding to *E. coli* topoisomerase I (Topo-I) and thus identify potential novel antibacterial agents. Topo-I is capable of binding relatively short fluorescent, single-stranded DNA oligonucleotides (e.g. TMR-22mer, M_r = 8 kDa). This results in a 13-fold increase in the molecular mass of the DNA–Topo-I binary complex (Topo-I, M_r = 97 kDa) relative to free TMR-22mer, and a corresponding increase in diffusion time measured by FCS (t_{DNA} = 237 μs, $t_{DNA–Topo\text{-}I}$ = 480 μs; Fig. 1a). The K_d for DNA binding determined by equilibrium titration (25 nM, Fig. 1b) is consistent with that determined by fluorescence anisotropy (35 nM; data not shown). Furthermore, the K_d determined for fluorescein-labelled DNA (13 nM) using assay volumes of 20 μl and 1 μl are identical (Fig. 1c) and consistent with that determined by non-FCS techniques (10–25 nM). These data exemplify the inherent insensitivity of FCS to miniaturization in the 1 μl range.

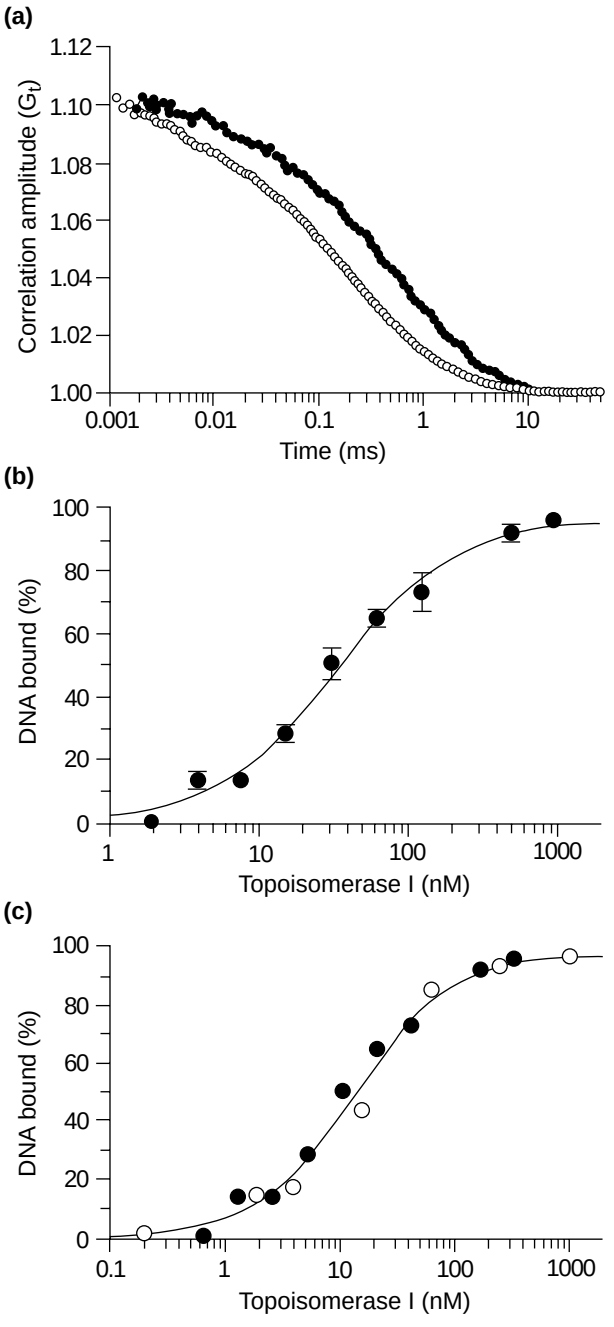

Figure 1. *FCS assay for DNA binding to* Escherichia coli *topoisomerase I (Topo-I). (a) Correlation curves of free (white circles) and bound (black circles) TMR–DNA. (b) TMR–DNA (10 nM) binding monitored by FCS in 20 μl assay volume* (K_d *= 25 ± 5 nM). (c) Fluorescein-DNA binding to Topo-I monitored by FCS in assay volumes of 1 μl (white circles) and 20 μl (black circles)* (K_d *= 13 ± 3 nM). Error bars show SD.*

Src-SH2–phosphotyrosine-peptide binding

Src is a membrane-associated protein tyrosine kinase containing an SH2 domain which recognizes and binds specific phosphotyrosine-containing proteins and peptides. We configured an FCS-based binding assay for this interaction using the soluble src-SH2 domain (M_r = 14 kDa) engineered to contain an N-terminal epitope tag for a high-affinity non-neutralizing antibody. Using a fluorescently-labelled phosphopeptide specifically recognized by src-SH2 (TMR-Ahx_2-pY; M_r = 2.5 kDa), we observed a 3.7-fold increase in diffusion time upon formation of the src-SH2–TMR-Ahx_2-pY binary complex (t_{free} = 120 μs; t_{bound} = 440 μs; M_r = 16.5 kDa) by FCS. This increase in diffusion time was amplified further ($t_{complex}$ = 550 μs) when the mass of the fluorescent src-SH2–TMR-Ahx_2-pY complex was enlarged by complexing SH2 with the non-neutralizing monoclonal antibody (mAb) that recognizes its epitope tag (M_r = 160 kDa, Fig. 2a). The K_d for TMR-Ahx_2-pY binding to either src-SH2–mAb (215 ± 18 nM, Fig. 2b) or src-SH2 alone (240 nM) is comparable with that obtained from competition studies in a conventional ELISA assay (200 nM) and also, using the biotinylated peptide, in competition FCS assays (K_i = 420 ± 45 nM, Fig. 2c) and in radioligand binding/ELISA assays (470 nM). The FCS assay volume was successfully reduced to 1.2 μl with no loss in performance (standard deviations = 5%, 1.6 s read times) and yielded identical K_d values to those obtained in 200 μl. Dose–response curves of unlabelled peptide and known small-molecule inhibitors randomized within 40 assay test plates were successfully identified in a test screen performed at Evotec Biosystems.

Peptide cleavage-based protease assay

Proteases are implicated in a wide range of pathologies and, as such, are relatively common targets for pharmaceutical intervention. We chose the serine protease essential for human cytomegalovirus replication (CMV-protease) to exemplify the development of FCS-based assays for proteolytic activity. A peptide substrate was prepared with biotin and Rhodamine Green (RhGn) on either side of the known CMV-protease cleavage site. Using this substrate, proteolysis could be readily monitored by FCS following addition of avidin, as the diffusion time of the substrate–avidin complex (t_b = 240 μs) differs from that of the RhGn-labelled cleavage product (t_f = 92 μs). Comparable k_{cat}/K_m values for cleavage of this substrate by CMV-protease were obtained using FCS (k_{cat}/K_m = 320 $M^{-1}\,s^{-1}$),

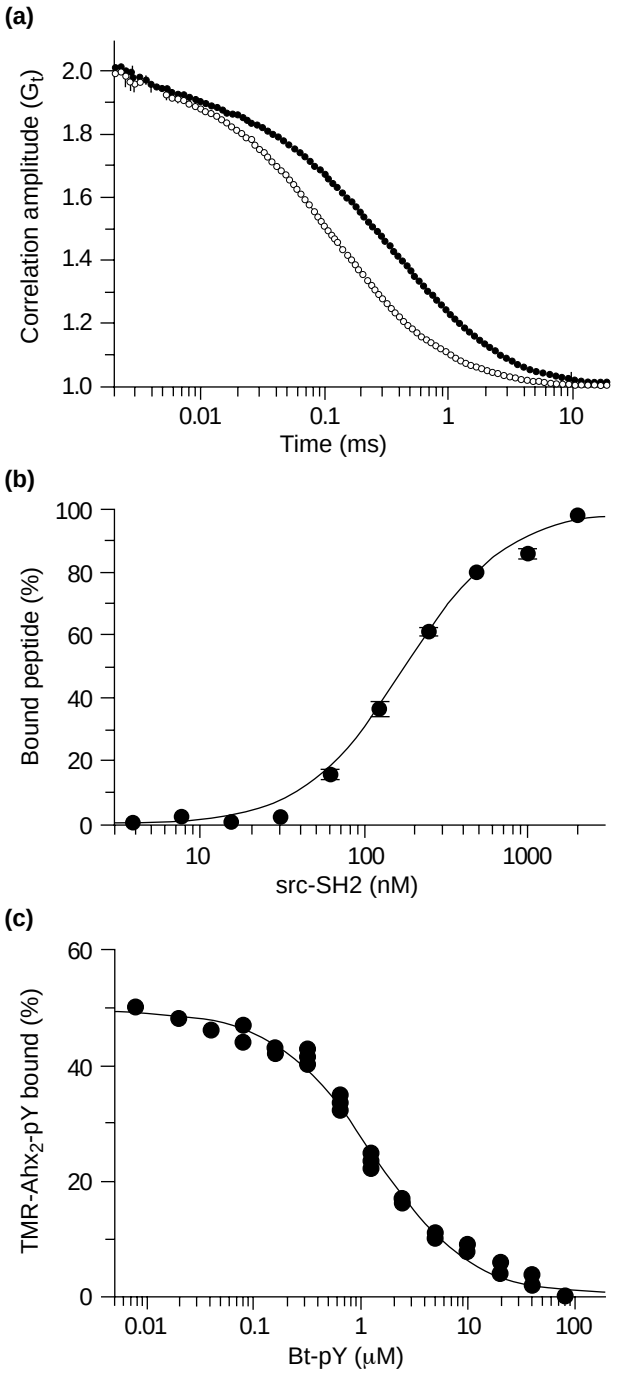

Figure 2. *FCS assay for peptide binding to src-SH2 domain: (a) correlation curves of free (white circle) and bound (black circles) fluorescently-labelled phosphopeptide, TMR-Ahx$_2$-pY, (b) TMR-Ahx$_2$-pY binding isotherm to src-SH2.* K_d *= 215 nM. (c) Displacement of TMR-Ahx$_2$-pY with biotinylated phosphopeptide, Bt-pY (*K_i *= 420 ± 45 nM). Error bars show* SD.

fluorescence anisotropy (k_{cat}/K_m = 300 $M^{-1}s^{-1}$) and time-resolved fluorescence (k_{cat}/K_m = 290 $M^{-1}s^{-1}$). In fact, from other examples of human, viral and bacterial proteases, near identical k_{cat}/K_m values were always obtained using FCS, anisotropy and HTRF approaches similar to that outlined above for CMV-protease. However, both anisotropy and HTRF assays required assay volumes of >10 μl to generate useful signals for test compound screening, compared to 1 μl or less for FCS.

FCS-based 7-TM receptor binding assays

G-protein-coupled (or 7-transmembrane; 7-TM) receptors represent possibly the single most important class of targets for current and prospective drug therapies. To date, few successful examples of the development of miniaturized homogeneous assays for ligand binding to these receptors have been reported. In this regard, FCS may provide a more-or-less unique solution. We have successfully configured membrane vesicle ligand-binding assays for all of the 7-TM receptors investigated to date (at expression levels of <100,000 receptors $cell^{-1}$). In one case, a peptide ligand 7-TM receptor, the binding and displacement of a TMR-labelled ligand could be monitored by FCS with K_d values (3 nM) comparable with those of the radiolabelled peptide (1.5 nM) and with <10% nonspecific binding. The binding of a TMR-labelled chemokine to its receptor also yielded K_d values (1.1 ± 0.3 nM) consistent with those obtained by scintillation proximity assay (0.8 ± 0.5 nM) and, again, with <10% nonspecific binding (Fig. 3). We have successfully measured specific ligand binding and displacement in this system with receptor expression levels <10,000 receptors per cell or in membrane preparations with a B_{max} ~0.2 pmol mg^{-1} measured by radioligand binding.

Binding of the human type-2 high-affinity somatostatin (SMS) receptor (sst_2) to its 7-TM receptor represents a further example of a successful vesicle-based FCS assay. Binding of a tetramethyl-rhodamine-labelled somatostatin-14 conjugate to small membrane vesicles prepared from CCL39 hsst2 cells overexpressing the receptor and competition with a non-labelled somatostatin-14 resulted in a K_d of ~1.6 nM for the natural ligand. With the application of specific assay conditions minimizing the surface adhesion and nonspecific binding of the labelled SMS, the miniaturization from the microscale to the nanoscale was possible without further optimization. Figure 4 shows a typical competition curve for a synthetic SMS analogue at fixed

nanomolar concentrations of 5TMR-SMS14 and sst_2-receptor-expressing vesicles. Together, these represent the first examples of the routine development of homogeneous fluorescent ligand-binding assays for this class of target in a miniaturized (<10 μl) form suitable for HTS screening, and as such, represent an important advance.

CD45 phosphatase – from assay development to prototype HTS screen

CD45 phosphatase, a transmembrane protein tyrosine phosphatase, is expressed in haematopoietic cells. It is one of the most abundant leukocyte cell-surface glycoproteins (Leukocyte Common Antigen, LCA). CD45 plays a pivotal role in antigen-stimulated proliferation of T cells. Two Src-family protein tyrosine kinases (PTKs), Lck and Fyn have been implicated as physiological substrates of CD45. The enzyme dephosphorylates the negative regulatory tyrosine residue of Lck, thereby serving as an obligate positive regulator.

The development of low molecular weight compounds that inhibit protein tyrosine phosphatase (PTP) activity of CD45 would potentially lead to novel drugs for suppression of immune and inflammatory reactions. The cytosolic part of the CD45 receptor consists of two tandem domains PTP-D1 and -D2. The catalytic activity is present in D1. With recombinant CD45 D1–D2, an ELISA assay had been established in the conventional 96-well plate format using biotinylated phosphotyrosine peptide substrates with different motifs for a specificity control-based *in vitro* screening program. After the enzymatic (inhibition) reaction was performed in homogeneous solution, the N-terminally biotinylated peptide recognition motifs were captured on a streptavidin microtiter plate to enable the detection reaction by a mouse-anti-phosphotyrosine/anti-mouse horseradish peroxidase-antibody cascade. As shown in Fig. 5, this solution ELISA assay format enabled a very quick adaptation to FCS and also to fluorescence anisotropy.

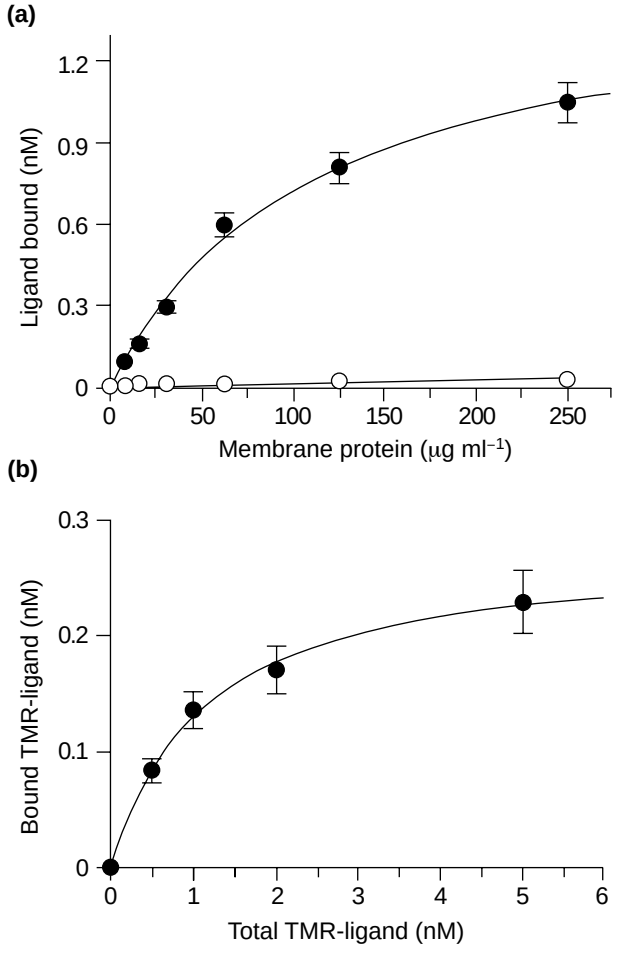

Figure 3. *Binding of TMR-labelled chemokine to membrane vesicles prepared from cells overexpressing the chemokine receptor. (a) Titration curve at fixed TMR–ligand concentration (1.5 nM) ± 1 μM unlabelled chemokine. Black circles, total binding, white circles, nonspecific binding. (b) Titration curve of TMR–ligand at 25 ng μl⁻¹ membrane protein, K_d = 1.1 ± 0.3 nM. Error bars show SD.*

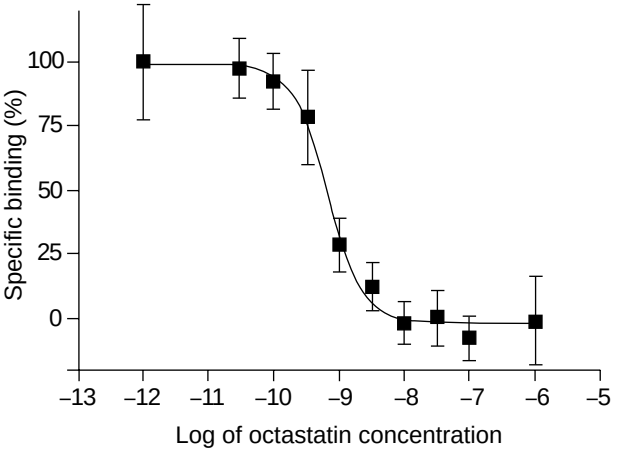

Figure 4. *SMS competition assay showing the dose–response curve for octastatin. Error bars show SD.*

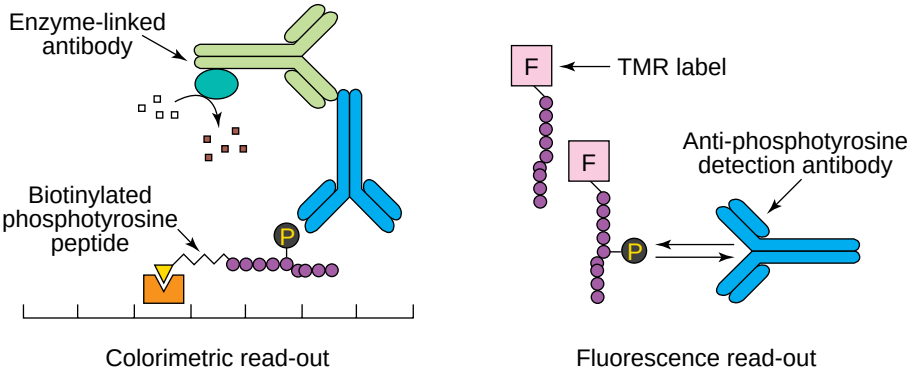

Figure 5. *CD45 phosphatase assay schemes. Left: solution mixing ELISA format. Right: homogeneous solution assay for fluorescence anisotropy and for translational diffusion measurements in FCS. F, fluorescent label; P, phosphate.*

Adaptation to FCS

The N-terminal-$(CH_2)_6$-linked biotin in the phosphotyrosine-containing peptide substrate was replaced by a TMR fluorescence label. The labelled substrate allowed for a very simple assay principle based on fluorescence anisotropy or FCS. Thus, in the absence of CD45 phosphatase activity or in the presence of an inhibitor the anti-phosphotyrosine detection antibody on binding to the peptide leads to a significant increase in rotational or translational diffusion time of the complex. If full cleavage of the phosphate occurs the detection antibody cannot bind and the anisotropy or FCS signal remains unaltered. Figure 6a shows a typical antibody equilibrium binding curve (K_d = 123 ± 15 nM) from a fluorescence anisotropy titration. The fluorescence label in the anisotropy experiment shown was EDANS. Figure 6b shows typical normalized autocorrelation curves of the free and Ab-bound TMR-(PO_3^{2-})–Y-peptide; it demonstrates the shift in translational diffusion time in relation to the data quality achieved in the measurement at 5 nM substrate concentration.

Systematic evaluation of the assay conditions in micro-format with specific emphasis on the following: ease of handling, robustness, sensitivity, reproducibility (different degrees of Ab binding and phosphatase reaction), order of addition of assay components, stop reagents, impact of organic solvent, incubation time and measurement time. The nano-format HTS run was then performed as follows:

Some 4000 pure organic substances and 4000 natural broths were screened by transferring 105 nl of each into prototype 200-well nanotiter plates. From a four-channel piezo-driven nanodispenser the assay components were transferred into the nanowells to yield a final volume of 1.3 µl per well. After preincubation of the CD45-LCA enzyme (150 nM) with the potential inhibitor to establish

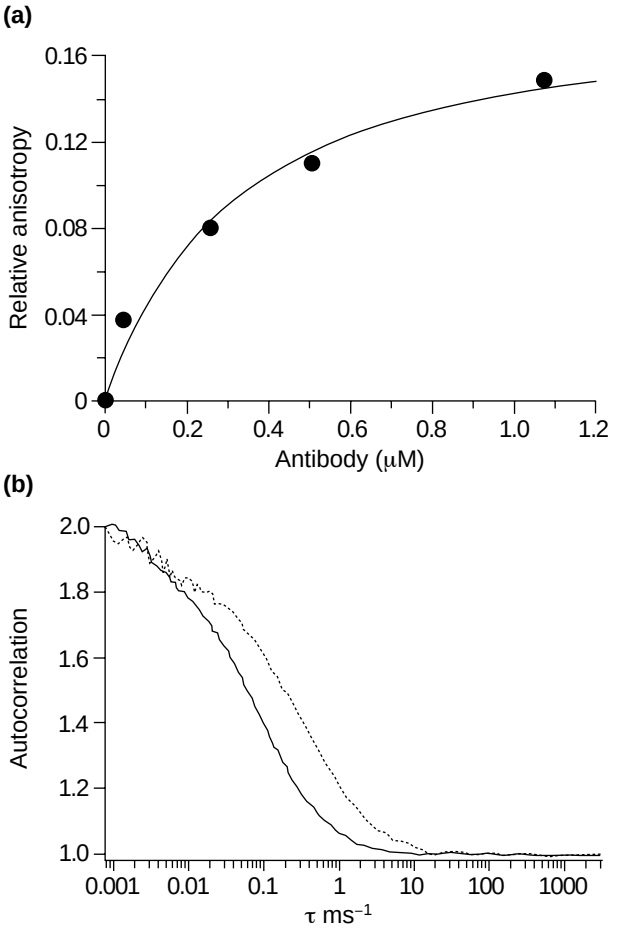

Figure 6. *(a) Fluorescence anisotropy equilibrium titration of EDANS-(PO_3^{2-})–Y-peptide with antiphosphotyrosine antibody (Ab). (b) Normalized autocorrelation curves for free TMR-(PO_3^{2-})–Y-peptide (solid line, τ = 128 µs) and the TMR-(PO_3^{2-})–Y-peptide–Ab complex (dashed line, τ = 488 µs).*

equilibrium, the TMR-peptide was added to a final concentration of 26 nM. After 20 min reaction time the detection antibody (2.5 μM) – stop reagent – was added as a premixed solution. Translational diffusion measurements followed with 1.6 s scanning time per well.

Practical assessment

This non-automated demonstration screening run resulted in 10,332 assays performed in 46 h. (More than 12 million individual droplets of ~1 nl were dispensed, corresponding to an average drop frequency of 70 drops s^{-1}). The test run was performed in a double-blind study with two times five positive compounds spiked into the 8000 samples that were run in parallel in the ELISA assay format. Inhibitors were identified in the initial screening run from the percentage of inhibition of the CD45 enzymatic activity. One of the major advantages of FCS-based HTS is the possibility for parallel biophysical characterization of positive hits in the primary screening run. No other screening technique described so far allows the immediate mechanistic characterization of inhibitors by determination of highly accurate kinetic and thermodynamic data at the same quality level as FCS. After confirmation of a positive hit by measurement of dose–response curves (see Fig. 7 for a typical example of a dose–response curve from a positive hit in the CD45 assay), compounds which are still found to be active can be very efficiently characterized by determination of their kinetic and thermodynamic interaction and/or inhibition parameters.

One important parameter in enzymatic assays is the association rate between inhibitor and enzyme. If the ligand/competitor association is much slower than the kinetics of the enzymatic reaction, the test compounds have to be preincubated with the enzyme to separate association and substrate cleavage. A plot of the inverse time constants of the association reaction versus the total inhibitor concentration under pseudo first-order conditions provides the association rate in accordance with $1/\tau = k_{+i}\,[I]_0 + k_{-i}$ (Fig. 8).

Single-turnover evaluation

In classical steady-state enzyme kinetics the time-consuming approach to derive physical constants (k_{cat}, K_m) from enzymatic reactions is using the Michaelis–Menten model. The prerequisite for this formalism is that the substrate concentration must be much higher than the enzyme concentration such that the substrate concentration effectively remains

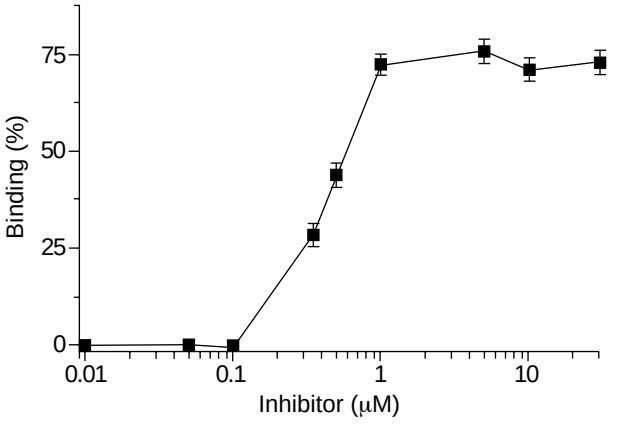

Figure 7. *Determination of a typical dose–response curve of a CD45 phosphatase positive hit found in the initial HTS.*

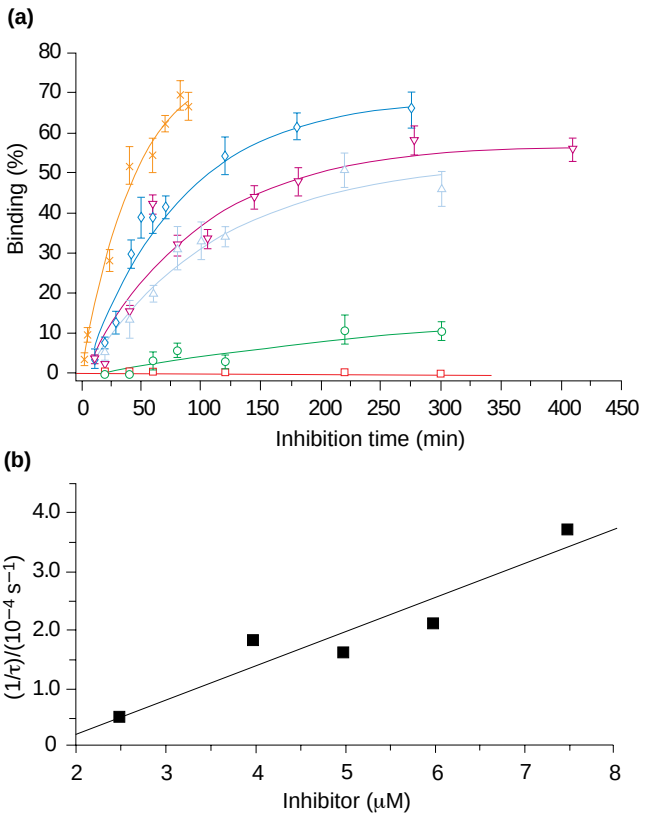

Figure 8. *(a) Association kinetics of 10 nM CD45–LCA at different concentrations of inhibitor: red, 1 μM; green, 2.5 μM; light blue, 4 μM ; pink, 5 μM ; dark blue, 6 μM and orange, 7.5 μM; error bars show SD. (b) Determination of apparent pseudo first-order association rate of an exemplaric inhibitor;* $k_{on} = 59\ M^{-1}\,s^{-1}$.

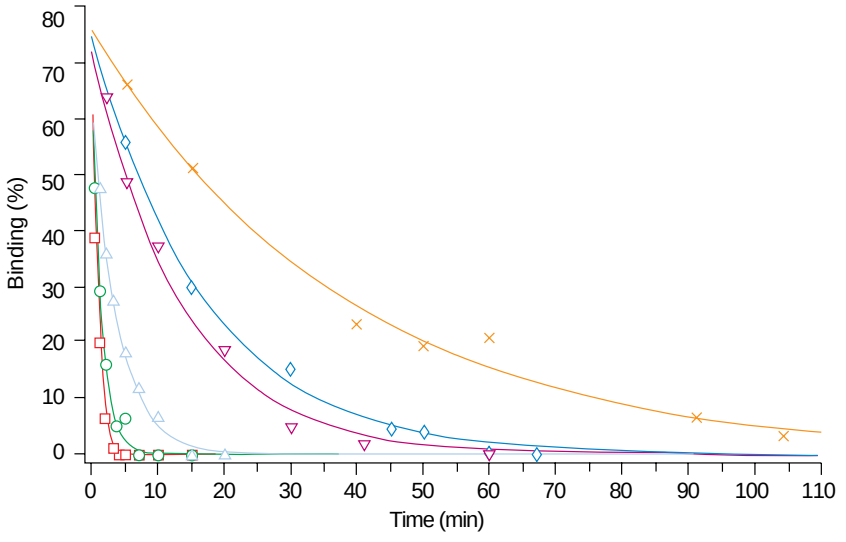

Figure 9. *Dephosphorylation kinetics of CD45-LCA in the presence of different concentrations of inhibitor: red, blank, t = 0.9 min; green, 1 μM, t = 1.5 min; light blue, 2 μM , t = 4 min; pink, 3 μM , t = 13.7 min; dark blue, 3.5 μM, t = 17.1 min and orange, 4 μM, t = 38.1 min.*

constant. Typically, FCS experiments are performed under single-turnover conditions. High substrate concentrations are disadvantageous because of aggregation phenomena and low solubility of substrate. The CD45-LCA assay described above clearly cannot be evaluated according to the general steady-state Michaelis–Menten approach. Therefore, a formalism was derived that allows for the determination of K_i values from the ratio of the time constants of the enzyme reaction with and without inhibitor (Fig. 9).

This formalism can be applied to all enzyme reactions where the turnover of small substrate concentrations is to be measured. It is particularly useful for FCS assays which, because of very high sensitivity and selectivity, allow enzymatic assays to be performed at low substrate concentrations.

Summary

The FCS assays we have developed thus far include examples of protein–DNA (Fig. 1), protein–peptide (Fig. 2), protein–protein, DNA–DNA, ligand–RNA, protein–RNA, protease, phosphatase (Figs 5–9) and 7-TM receptor targets (Figs 3,4). Experiments to investigate the utility of FCS to monitor cell surface and intracellular processes in living cells are ongoing. It is envisaged that hit profiling will be possible in the native cellular context using the same FCS assay used for the *in vitro* HTS screen. We expect that the power of FCS as a versatile *in vitro*, and ultimately intracellular, screening tool will become apparent as the industry-wide drive towards miniaturization approaches in assay volumes of 1 μl and below continues. The only limitation to homogeneous assays and confocal fluorescence detection is a maximal well concentration of fluorescent ligand of 1 μM, although, most FCS (and anisotropy) experiments are performed with nM concentrations of fluorescent ligand and the binding component (e.g. receptor) in molar excess, governed by the affinity of the interaction. Nevertheless, when the ligand is used in excess, 1 μM is well above biologically relevant levels. Finally, novel data processing algorithms developed at Evotec have removed the requirement for a mass change associated with the interaction of interest and the development of a parrallel FCS reader is expected to increase throughput by tenfold compared with the instrumentation used here.

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