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Highly sensitive fluorescence detection technology currently available for HTS

Christian Eggeling, Leif Brand, Dirk Ullmann and Stefan Jäger

Homogenous fluorescence methods are providing an important tool for HTS technologies. A wide range of different techniques have been established on the market, with read-outs ranging from total fluorescence intensity to statistical analysis of fluorescence fluctuations for biochemical assays or fluorescence imaging techniques for cellular systems. Each method has its own advantages and limitations, which have to be accounted for when designing a specific assay. Here, recently developed fluorescence techniques and some of their applications, with a particular focus on sensitivity, are summarized and their principles are presented.

Christian Eggeling
Leif Brand
Dirk Ullmann
Stefan Jäger*
Evotec OAI / Evotec Technologies
Schnackenburgallee 114
D-22525 Hamburg
Germany
*e-mail: stefan.jaeger@evotecoai.com

Today, fluorescence has started to emerge as the method of choice for tagging purposes in life science research and development, where it is continuously replacing radiolabelling. However, not all fluorescence techniques are amenable to HTS, where miniaturisation is playing a central role, especially when reagent and test compound savings are to be considered. When using bulk fluorescence techniques, robust assay systems can be miniaturized down to volumes of 10 µl and are still compatible with the requirements for ultra-high throughput screening (uHTS) [1]. When applying confocal microscopy the measurement volume is as small as 1 fl and assay volumes of 1 µl are established, based on recent advances in liquid handling [2,3]. As well as minute assay volumes, confocal techniques offer the key advantage of combining several different read-outs, which are inherent to the fluorescence signal [4–9]. The use of multiple, simultaneously acquired parameters improves the resolution of distinct species significantly, and hence it allows complex assemblies of biomolecular interactions to be studied.

This review focuses on highly sensitive fluorescence detection technologies amenable to HTS with some of their major applications, such as fluorescence lifetime techniques and the recently developed fluorescence fluctuation [e.g. fluorescence intensity distribution analysis (FIDA)] and imaging methods [e.g. Nipkow device and the fluorescence lifetime imaging method (FLIM)]. Finally, a list of commercially available HTS fluorescence readers is provided (Table 1).

Bulk fluorescence techniques

Prominent bulk fluorescence techniques use total fluorescence [10], time-gated fluorescence (such as LANCE™ [11] or HTRF® [12]), fluorescence polarisation (FP) [10], fluorescence lifetime [7] or a combination of the latter two, namely time-resolved anisotropy (TRA) [13]. The major advantages of these techniques are speed and ease of detection and analysis. Total fluorescence is often applied in enzymatic turnover assays when a substrate becomes fluorescent or quenched upon enzymatic modification. Time-gated fluorescence applies fluorophores with long fluorescence lifetimes (µs to ms), such as rare earth elements (lanthanides, especially europium chelates or cryptates). Here, the possibility of cutting out short-lived fluorescence is used to reduce significantly any auto-fluorescence or background signal. One frequent use is the combination with Förster resonance energy transfer (FRET), whereby donor-acceptor distances of up to 10 nm can be measured [10–12,14]. Finally, FP has its strength in detecting changes of mass upon a binding reaction [3,8,10,15]. The three techniques mentioned above are reviewed well within the literature [10,14], whereas lifetime and TRA are discussed below.

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Table 1. Detection systems for HTS applications (including cellular screening).

System	Features/applications	Manufacturer
Insight	Multi-detection confocal reader, free formats (1536/2080 NanoCarrier)	Evotec Technologies
MF20/10S	Multi-detection confocal reader, SNP typing analysis	
Opera	High-resolution confocal cell imaging device	
ACUMEN	Laser-scanning microscope, point scanner, homogeneous + cell-based assays	The Automation Partnership
AlphaQuest™ microplate analyser	Optimised for highly sensitive detection of AlphaScreen reagents	PerkinElmer Life Sciences
EnVision™	Multilabel plate reader	
Fusion™	Universal microplate analyzer, also for AlphaScreen assay system	
ImageTrak™ epi-fluorescence system	Cellular imaging platform for kinetic and endpoint assays	
Victor^{3}™-V	Multilabel counter FLINT, TRET (LANCE), polarization, luminescence	
ViewLux™	Microplate imager, cooled CCD camera, TRET (DELFIA, LANCE)	
CLIPR™	Luminometer, CCD camera Cell-based assays, SPA	Molecular Devices
Molecular devices acquest™	Multimode detection system; FLINT, polarization, TR-FRET, luminescence	
Molecular devices FLIPR^{3}®	Fluorometric imaging plate reader, kinetic cellular assay systems, luminescence/aequorin option	
CyBi™-lumax 1536 S	Luminometer	CyBio AG
Hamamatsu FDSS	Kinetic fluorescent cell imager	Hamamatsu Photonic Systems
FLUO-/NOVO-/POLARstar OPTIMA	Multi-detection reader (five modes), FLINT and TRET for 1536-well plates	BMG Labtechnologies GmbH
RUBYstar	Time-resolved fluorescence, correction algorithms	
IN cell analyser 1000/3000	High-throughput cell imaging system	Amersham Biosciences
LEADSeeker™	Multimodality imaging system, cooled CCD camera, radiometric + fluorimetric toolboxes, well- + cell-analyser	
Plate::screen®	Automated multimode reader minilens array with 96 micro-objectives; 1,536 wells in 16 steps	Carl Zeiss Jena
Safire	Monochromator-based microplate reader, top and bottom reading	TECAN
ULTRA evolution	Multimode detection system, fluorescence, TRET, time-gated fluorescence, polarisation, luminescence, cell-based assay functionality	

Abbreviations: FLINT, Fluorescence intensity; SNP, single nucleotide polymorphism; TRET, Time resolved energy transfer.
Evotec Technologies (http://www.evotec-technologies.com); The Automation Partnership (http://www.automationpartnership.com); PerkinElmer Life Sciences (http://lifesciences.perkinelmer.com); Molecular Devices (http://www.moleculardevices.com); CyBio AG (http://www.cybio.de); Hamamatsu Photonic Systems (http://usa.hamamatsu.com); BMG Labtechnologies GmbH (http://www.bmg-labtechnologies.com); Amersham Biosciences (http://www1.amershambiosciences.com); Carl Zeiss Jena (http://www.zeiss.de); Tecan (http://www.tecan.com).

Fluorescence lifetime

Various scientific applications use the fluorescence lifetime because it represents an intrinsic molecular property of the fluorophore and it is able to detect minute changes in the fluorophore's direct environment [15]. The calculation of the fluorescence lifetime [fluorescence lifetime analysis (FLA)] is either achieved from the phase shift and demodulation of the fluorescence emission resulting from sinusoidal modulated excitation light (frequency-domain FLA) [16,17] or from the time-dependent decay of the fluorescence emission after using repetitive, brief excitation pulses, for example applying time-correlated single photon counting (TCSPC) [18,19] (time-domain FLA). FLA determines the lifetimes of the different fluorescent species present in the sample and takes into account the background and autofluorescence signals. Owing to its sensitivity and robustness (the fluorescence lifetime representing an intrinsic molecular property is independent of the setup or adjustment of the instrument), as well as the possibility to resolve and quantify different components of a sample (e.g. free versus bound ligand), the lifetime read-out displays a high statistical accuracy and is increasingly applied in HTS with measurement times below 1 s [7,13,20,21]. Therefore, FLA has also been introduced into various HTS platforms, such as the EVOscreen™ HTS platform (Evotec Technologies; http://www.evotec-technologies.com), the TECAN ULTRA FLT or Evolution reader (Tecan Group; http://www.tecan.com) or the FLARe system (Molecular Devices; http://www.moleculardevices.com). Although the latter is based on frequency-domain FLA, the former two apply TCSPC, which has been shown to be superior for HTS applications [7]. However, the applicability of FLA towards biological reactions is limited because changes in the fluorescence lifetime (owing to environmental quenching or polarity effects) are not always predictable. Therefore, new approaches in assay development use additional quencher labels to induce a lifetime change upon binding or enzymatic reactions [7].

TRA analysis

TRA combines the fluorescence lifetime with the polarisation read-out. Fluorescence lifetime data are collected simultaneously on two detectors that monitor the fluorescence emission parallel and perpendicular to the incident polarisation direction. Because the rotational correlation time, τ_r (the average time of a molecule's rotation cycle under the Brownian motion regime), is typically within the several hundred picoseconds to several tens of nanoseconds range, a mass-dependent change, for example upon a biochemical reaction, could be depicted in addition to a possible lifetime change [13,15,21,22]. This is achieved by a global analysis of the lifetime decays detected on both detectors. Because of the simultaneous determination of the intrinsic molecular fluorescence lifetime and rotational properties, the statistical accuracy in resolving and distinguishing different fluorescent species is further enhanced compared with FLA, which opens up future perspectives for its application in HTS [13,21].

Bulk versus microscopic fluorescence techniques

Major efforts are being made to minimize the amount of material consumed in HTS. The easiest approach is to decrease the assay volume. However, because in macroscopic techniques the signal is averaged over most of the assay volume, the amplitude as well as the quality of the fluorescence signal will decline inevitably as the volume is reduced [3]. The two main effects in this respect are a decreasing signal-to-background ratio and a decreasing statistical accuracy. To overcome this problem, read-out methods based on the detection of single fluorophores have been introduced for HTS. Here the measurement volume is microscopically small (about 1 fl), thus miniaturisation does not alter the measurement statistics.

By using fluorescence techniques the most detailed information on biomolecular processes can be obtained by detecting fluorescence from single molecules. Therefore, fluorescence-based assays using single-molecule detection (SMD) techniques are evolving as an important tool in science [9,10,20,21,23]. These techniques use samples of highly diluted fluorophores and include not only the direct detection and analysis of single-molecule events (e.g. SMD) [20,22,24–28] but also spectroscopic analysis by means of applying fluctuation methods, such as fluorescence correlation spectroscopy (FCS) (see below) [29–33]. The major advantages of these techniques towards HTS are their high statistical accuracy, even at measurement times of about 1 s, and their low consumption of precious biological material.

Experimental setup: confocal microscope and sub-microlitre sample volumes

The sensitivity needed to observe fluorescence from single molecules calls for certain experimental requirements:

(1) Highly focused laser excitation combined with fluorophores expressing high quantum yields and a low tendency of photodestruction, such as rhodamines, allows for the highest fluorescence emission achievable in practice [34–36].

(2) A tiny detection volume of about 1 fl minimises the background signal originating from solvent scattering or impurities. Together with high precision optics and filters, which separate fluorescence efficiently from the

background signal, a high fluorescence- (or signal-) to-background ratio is realised.

(3) Finally, detectors with high detection efficiencies open up the possibility to monitor low signal intensities. Single-photon counting modules, such as avalanche photodiodes, efficiently convert the impact of single fluorescence photons into electronic counts.

All three prerequisites not only enable the observation of few fluorescence photons, as emitted from a single fluorophore, but also allow for the clear distinction of the fluorescence signal from the background level. The above conditions are realized within an optical setup, such as a confocal epi-illuminated fluorescence microscope, and have been described in detail before [37–39]. Commercially available equipment include the INSIGHT or MF instrument (Evotec Technologies), the ConfoCor system (Carl Zeiss Jena GmbH; http://www.zeiss.de), the IX81 inverted microscope (Olympus; http://www.olympus.com), the Leica DM series (Leica Microsystems; http://www.leica-microsystems.com), the Nikon C1 (Nikon; http://www.nikon.com) and the MicroTime 200 microscope (PicoQuant; http://www.picoquant.com). The excitation and detection instrumentation is straightforward. Although laser diodes (e.g. 635 nm PDL 800B, PicoQuant) match the demands for robust and easy-to-use excitation sources, avalanche photodiodes (e.g. SPCM-AQ, EG and G, Vaudreuil, Canada) offer single-photon counting with the highest possible sensitivity.

The tiny detection volume opens up new advantages for HTS because sub-microlitre sample volumes can be exploited. This drives the consumption of precious biological material to the lowest possible amount and allows high density sample carriers to be used, such as 1536- or even 2080-well plates, which now are routinely used within the EVOscreen™ uHTS system (Evotec Technologies). Further HTS systems include Asset™ (The Automation Partnership; http://www.automationpartnership.com), ZEISS UHTS System plate::explorer™ (Carl Zeiss Jena GmbH), Allegro™ (Zymark; http://www.zymark.com), SAGIAN™ (Beckman-Coulter; http://www.beckman.com) and Thermo CRS Dimension4™ (Thermo CRS; http://www.thermo.com). However, none of them exploit sub-microlitre HTS in routine use. By contrast, the sub-microlitre sample wells, with their large surface-to-volume ratio, require a special adaptation phase for the biological assay.

Fluorescence fluctuation techniques

The signal detected by a confocal microscope setup fluctuates due to the diffusion of single fluorophore molecules into and out of the laser focus and detection volume, each causing a burst in fluorescence emission and, thus, in detected fluorescence photons. The direct observation of these fluorescence bursts (e.g. SMD) necessitates samples with fluorophore concentrations below 10^{-10} M, in which the mean number of fluorophores in the detection volume is well below one fluorophore at a time, that is a detected fluorescence bursts indicates the diffusion of only one single molecule through the focus. SMD has generated tremendous new results and insights into biological systems and will play an important role in future developments of detection techniques [20,22,24–28,40,41]. However, it needs acquisition times of at least several seconds because a reasonable amount of single-molecule events have to be gathered to reach a sufficiently high accuracy. Therefore, applications for HTS purposes apply other analysis methods based on the statistical analysis of the fluorescence fluctuations, which combine slightly higher fluorophore concentrations (10^{-10}–10^{-8} M) with much lower data acquisition times. Here, more than one fluorophore can be present in the detection volume, in contrast to SMD, where at most one fluorophore at a time is allowed.

Compared to FLA, the analysis of fluorescence signal fluctuations opens up the possibility of resolving and quantifying various components of a sample expressing different fluorescence and hence molecular characteristics. These characteristics are directly associated with the signal fluctuations; for example brightly fluorescing particles give rise to high fluorescence emission and detection rates and, therefore, to fluctuations with high amplitudes. Slowly diffusing fluorescing molecules remain in the detection volume and emit fluorescence over a long period of time, thus generating broader fluctuations compared with fast diffusing fluorophores. Additionally, the fluorescence fluctuations from highly concentrated molecules show much smaller amplitudes than from molecules of low concentration. Because the fluctuating signal is influenced by a large number of molecular properties, the statistical accuracy of the characterisation of a biological target will be increased by the simultaneous measurement of a variety of fluorescence parameters. Several different analysis methods have evolved over the past few years that are taking advantage of this molecular resolution [29–33]. In contrast to SMD, these methods use the whole signal data stream to extract the information and, therefore, the necessary data acquisition times can be lowered down to below 1 s, which finally led to their widespread application in HTS [3–6,8–10,13,20,21,42–44] and their integration into the FCS++ read-out portfolio of the EVOscreen™ HTS platform (Evotec Technologies). However, as outlined above, the analysis requires fluctuations of a certain amplitude, that is, the concentration of fluorescing molecules has to be close to the single-molecule level and special setups, such

as a confocal microscope, have to be used. The different fluctuation methods are outlined below:

(1) FCS analyses the temporal characteristics of fluorescence fluctuations by calculating the correlation function [29–33]. Hence, FCS is able to resolve components of a sample with different diffusion coefficients owing to different molecular mass. For example, the binding of a dye-labelled peptide to a larger protein is followed by an increase in the mean diffusion time. In this way, biological assays can be developed easily for HTS applications [10,21,42]. However, for measurement times as short as 1 s and for an increasing background signal, the correlation curves become severely noise limited and FCS loses its accuracy. Owing to this problem, further fluctuation methods have been developed for HTS.

(2) FIDA relies on a frequency histogram of the signal amplitudes of the fluctuating fluorescence intensity throughout a measurement of, for example, 1 s [30,45,46]. By analyzing the resulting distribution of signal intensities, FIDA distinguishes the species of a sample according to their different values of specific molecular brightness, q (number of detected fluorescence photons per molecule per second, measured in kHz), and quantifies each species by the determination of its absolute concentration, c (mean number of molecules in the detection volume). Furthermore, FIDA accounts for possible background signals, owing to scattering or auto-fluorescent impurities. Reactions resulting in a quenching of the fluorescence emission of a dye label or in the aggregation of dye-labelled molecules can easily be monitored by applying FIDA. Possible HTS applications have been introduced to measure enzyme cleavage as well as inhibition of ligand binding to acceptor proteins residing in vesicle membranes [5,6,9,43,46]. Because FIDA is based on a simple statistical process (building up a histogram by adding photon counts) rather than on a more complex numerical operation (calculating the correlation function as in the case of FCS), it is able to maintain good statistical accuracy down to acquisition times of 1 s.

(3) 2D-FIDA is based on the extension of the FIDA technique resulting in an improved performance. It is applied to a two-detector setup monitoring either different polarisation or emission bands of the fluorescence signal. In addition to the FIDA performance, 2D-FIDA achieves additional molecular resolution by the concurrent determination of two specific brightness values originating from both detection channels, q_1 (channel1) and q_2 (channel2) [47]. By observing the molecularly resolved fluorescence anisotropy, simple, as well as more complex, binding events and enzymatic reactions may be followed. Alternatively, such events may be resolved by labelling with two dyes that express different fluorescence emission bands. As a read-out either two-colour excitation by different lasers or one-colour excitation using a FRET dye pair can be chosen. Thus, the use of a second detector improves the power of FIDA to distinguish between molecular components and is, therefore, increasingly applied in HTS [9,44].

(4) Further FIDA-based methods, such as fluorescence intensity multiple distribution analysis (FIMDA) and fluorescence intensity and lifetime distribution analysis (FILDA), address the combination of the FIDA read-out (specific molecular brightness, q) with further fluorescence parameters, such as the mean diffusion time in the case of FIMDA and the fluorescence lifetime in the case of FILDA. Owing to the use of a second molecular parameter, both approaches gain further accuracy to distinguish between molecular components and have already been applied to various biological applications [13,20,30,48,49].

Figures 1 and 2 reveal the powerful possibilities of the FIDA-based methods in the field of HTS. An example of an assay ideally suited for FIDA analysis is presented in Fig. 1. The binding of a labelled ligand to an antibody expressed on the surface of a bacterium is schematically shown in Fig. 1(a). Accumulation of the ligand on the bacterial surface does not induce a significant change in the overall fluorescence intensity of the sample. However, there are, on average, 100–1000 receptors per bacterium that can bind the labelled ligand. As a consequence, the count rate per particle is enormously increased upon binding, which enables monitoring of the reaction using FIDA [Fig. 1(b)] [43]. In Fig. 2, the inhibition of a peptide-protein interaction is monitored using 2D-FIDA. The anisotropy values of the binding event are determined with and without the presence of fluorescence impurities. Background fluorescence is a major issue in HTS, owing to the possibility of intrinsic auto-fluorescence being inherent to the investigated drug candidates. When the anisotropy is determined from the overall signal, as is usually the case in fluorescence experiments, its values are erroneous owing to the presence of a background signal [Fig. 2(a)]. Figure 2(b) shows that the addition of an auto-fluorescent component can be corrected by calculating the molecular resolved anisotropy of the sample using 2D-FIDA. It is worth mentioning that the auto-fluorescence is much more pronounced for microscopic methods, such as fluctuation methods, than for macroscopic techniques, which apply different fluorophore concentrations (10^{-8} M for the former and up to the μM range for the latter technique). Because compounds are added at a rather high concentration (several μM), the

ratio of assay signal to auto-fluorescence signal will be much better for the macroscopic techniques. By contrast, these techniques do not offer potential auto-fluorescence correction steps.

One-photon versus two-photon excitation

Whereas one-photon excitation uses a single photon to excite the fluorophore, in two-photon excitation (TPE) or multi-photon excitation (MPE) this excitation is accomplished by the simultaneous absorption of two or more long-wavelength photons. During the past decade, MPE has increasingly been applied in biophysical chemistry, and especially in cellular imaging [39,50–60]. The simultaneous absorption of more than one photon requires a high density of excitation photons, which is usually obtained by tightly focusing short, pulsed (femto- to pico-seconds pulse width) laser light in a confocal microscope. By contrast, excitation usually occurs in the range of 800 nm and the dependency of the MPE-fluorescence is non-linear, for example squared in the case of TPE, which offers the following unique features:

(1) Because, in general, TPE and MPE fluorescence is emitted and detected far below its excitation wavelength (in TPE experiments with coumarin-120 excitation is at 700 nm and detection is around 400 nm [55]), one does not have to bother about any background signal owing to solvent scattering, and/or auto-fluorescence issues may be reduced [58].

(2) The squared intensity dependence of the excitation probability confines the MPE-fluorescence to the central part of the excitation focus only. This minimises possible out-of-focus signals and out-of-focus photodestruction. Most confocal setups even abandon the use of the pinhole when applying MPE.

(3) MPE results in a deeper penetration of thick samples, especially when measuring in a cellular environment. This makes MPE interesting for every biological application and opens up new approaches for high-resolution cellular imaging, SMD and FCS, for example by applying it to UV dyes and cellular or tissue environments [39,50–60]. Even HTS approaches using MPE have been proposed [57].

However, there is a trade-off to MPE, which limits its applicability, especially with respect to HTS. First, although much progress has been made over the past few years, integration of the more complex laser systems (indispensable for TPE) into a fully automated HTS platform is rather difficult. Second, in-focus photodestruction of the fluorophores limits the maximum possible fluorescence emission. Therefore, SMD and fluorescence fluctuation analysis

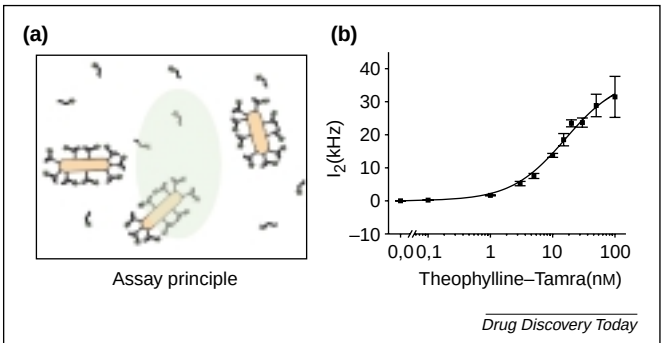

Figure 1. An example assay for FIDA: binding of Tamra (tetramethyl-rhodamine)-labelled theophylline to a polyclonal antibody linked to the surface of a bacterium by an exposed protein A derivative. The principle of the assay is shown in panel **(a)**. Upon binding, the bacteria accumulates labelled theophylline. Analysis using FIDA resolves the brightness values q_x of free (x = 1) and bacteria-accumulated (x = 2) theophylline: q_1 = 2.5 ± 0.1 kHz and q_2 = 1560 ± 27 kHz, resulting in about 600 theophylline molecules binding to one bacteria. In the subsequent titration series of labelled theophylline these brightness values were both fixed and the read-out was calculated as light flux from the complex: $I_2 = q_2 \times c_2$, which is shown in panel **(b)**. The binding is characterised by an apparent binding constant of K_D = 16 ± 2 nM, determined by a hyperbolic fit to the data in **(b)**. For further details see [43].

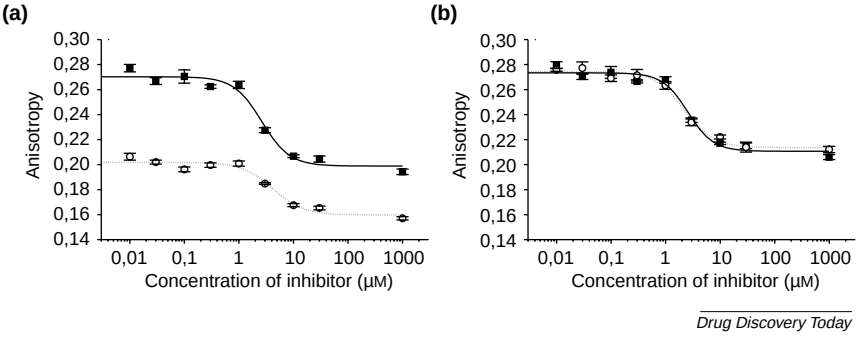

Figure 2. Binding of a small cyanin5-labelled peptide to the SH_2-domain of the Grb2-protein is monitored by a change of the fluorescence anisotropy. Unlabelled peptide (inhibitor) was titrated, with (open dots) and without (black dots) addition of fluorescent impurities (1 μM rhodamine 800). The anisotropy was determined from the overall signal, panel **(a)**, and from 2D-FIDA, panel **(b)**. The latter analysis is able to separate the fluorescence signal of the labelled peptide from the impurities. A dose-response fit of the data resulted in IC_{50} values of 2.6 ± 0.4 and 4.2 ± 1.1 μM for the pure [solid line, panel **(a)**] and the impure [dotted line, panel **(a)**] sample and 2.2 ± 0.3 μM for the impure corrected [dotted line, panel **(b)**] sample. Oligino *et al.* [67] report an IC_{50} of 2 μM for the same peptide motive. For more details on the assay system see [48].

techniques, such as FCS and FIDA, are limited and less optimal for TPE/MPE compared with one-photon excitation [35,55,61].

Fluorescence microscopy and imaging techniques

Although fluorescence-based soluble assays are well established for miniaturized HTS, in recent years there has been an ever increasing demand for the development of automated screening systems for cellular applications. However, cell-based applications in HTS form a considerable challenge to both instrumental design and fully automated data handling and evaluation.

To follow physiological pathways in detail, fluorescence staining techniques, such as the internalisation of synthetic dyes into cells or the fusion of the green fluorescent protein or its variants to the protein(s) of interest, are required. Fluorescence staining in cellular biology opens up ways to highlight specific areas selectively within the cells, for example membrane or cytosol.

Light microscopy

In standard fluorescence microscopes a sample of sedimented or adherent stained cells is excited by an appropriate lamp or LED. A set of selectable colour glass filters ensures a rough monochromaticity. An objective lens magnifies a certain area of interest and maps it onto an eyepiece or a camera to be viewed and studied in detail. To obtain high-contrast images, the focal plane of the optics has to be adjusted to the centre of the cellular layer. By means of phase contrast methods, standard fluorescence microscopes offer possibilities to distinguish whether individual objects or groups of objects show fluorescence and even allow for the detection of brightness differences. However, fluorescence microscope images always suffer from blurring owing to light from regions above and below the focal plane. To provide the highest image quality and contrast, as well as spatial resolution even in the axial direction, suppression of these out-of-focus contributions is required.

In an attempt to suppress out-of-focus fluorescence contributions, mathematical methods of image deconvolution have been described exemplarily [62]. However, as time-consuming image acquisition and expendable subsequent processing are required for this method, ways of experimental avoidance of out-of-focus blur are preferable in practice and can be achieved by introducing confocality.

Confocal scanning microscopy

The principle and advantages of confocality have already been described above for epi-illuminated microscopes. Transferring the advantages of confocality from a 'point-like' towards an 'area-like' detection, and hence enabling confocal image microscopy, led to the development of confocal laser scanning microscopes (cLSM), for example the Olympus FluoView series (Olympus), the Leica TCS series (Leica), the Nikon C1 (Nikon) or the LSM 5 series (Carl Zeiss Jena GmbH) [37]. The functional principle of a cLSM is to move the excitation laser beam rapidly within the xy-plane via a scanning mirror pair before being coupled into the objective lens. All points of the image plane are subsequently illuminated for a certain time interval. After the measurement, a confocal fluorescence image is recalculated out of the known position-count rate relation. cLSMs generate high quality and highly resolved confocal fluorescence images. However, as only a single focal volume element is scanned over the image area, data acquisition is rather time consuming and hardly performed in less than 10 s, which makes laser scanning microscopy not ideal for high throughput drug screening.

To overcome that limit, the principle of 'line scanning' was applied within the ArrayScan HTS reader (Cellomics; http://www.cellomics.com). In contrast to 'point scanning', a full line of the sample is illuminated and mapped onto an appropriate pixel line on the charge coupled device (CCD) camera. A CCD camera is a highly sensitive detection tool consisting of a large number of photosensitive microelements, enabling the detection and digitalisation of a whole image area simultaneously. With the ArrayScan, the illuminated line is dragged over the whole cell sample, thus requiring only one scan direction. However, full confocality may not be achieved by this technique.

Nipkow microscopy

High resolution confocal images can also be acquired by a different approach, using a so-called 'Nipkow-disk' unit (e.g. Yokogawa; http://www.yokogawa.co.jp) being mounted onto fluorescence microscopes [37,63]. Confocality is assured by rotating a Nipkow-disk pair. One of these disks is equipped with a series of approximately 20,000 microlenses, whereas the other corresponding one is placed with approximately 20,000 pinholes. A detailed description of the working principle is given elsewhere [37,63,64]. An expanded laser beam passes a microlens array and illuminates about 1000 microlenses. This series of fractional beams is focused onto the according pinholes and passes the objective lens giving rise to approximately 1000 independent confocal volume elements within the sample. The fluorescence light emitted from each of these volume elements is collected by the objective lens, passes the according pinhole and is separated from the excitation beam by a dichroic mirror. All fluorescence is directed to a CCD camera. By rotating the Nipkow-disk pair, a highly resolved, full confocal image acquisition is enabled within 50 ms acquisition time.

A one-channel instrument for performing cell microscopy based on the Nipkow principle was launched with the UltraVIEW™ system (Perkin-Elmer Life Sciences; http://lifesciences.perkinelmer.com).

Recently, an imaging microplate reader combining both confocal image acquisition on two independent optical channels and ultra high throughput capability has been developed (Opera system, Evotec Technologies). This reader is equipped with a fast autofocus system, keeping permanent control to the axial depth of the focal plane, thus ensuring that the cells are hit within their equatorial section. The emitted fluorescence may be separated chromatically and detected simultaneously by two CCD cameras. The principle of a rotating microlens disk was even used for performing cell and tissue imaging by MPE [64]. In contrast to the above described Nipkow-disk pair, a pinhole disk is no longer needed, as already described in the section on TPE.

Automated image analysis/confocal microscopy in uHTS

To allow for automated screening operation, fast data handling and a variable and widely flexible image analysis have been developed for the Opera system. Based on appropriate object recognition algorithms, the resolution and identification of subcellular structures is enabled to quantify biological reactions (e.g. expression level, receptor activation, translocation events and binding reactions, among others). Figure 3(a) shows the binding of a fluorescently Tamra (tetramethyl-rhodamine)-labelled ligand to the β_2-adrenergic receptor constitutively expressed in Hek 293 cells. In channel 2, the number and location of the cells are determined using a cytosolic dye (unspecific signal at around 630 nm, red channel). In channel 1, the specific binding event of the labelled ligand to its receptor is detected simultaneously (at around 530 nm, green channel). Figure 3(b) denotes the ligand-binding titration and the according apparent K_D determination by a hyperbolic fit. A value of 4.0 ± 0.3 nM was calculated, compared with 0.7 ± 0.08 nM, as determined by 1D-FIDA from membrane fractions [5]. This demonstrates that, in this case, the whole cell approach leads to a slightly different result. Nevertheless, screening for agonists or antagonists will be possible for both assay types and which assay system is preferred will depend on the user.

FLIM

So far, confocal microscopy or image acquisition always means 'intensity imaging' because the fluorescence intensity per image pixel is the only parameter being investigated. By contrast, an additional useful parameter in microscopy is the fluorescence lifetime. To perform confocal fluorescence lifetime imaging, a lifetime value has to be assigned to each image pixel. Thereby, the realisation of FLIM appears different for either scanning or Nipkow microscopy [16,65]. At least a fast-pulsed excitation source is required for both types.

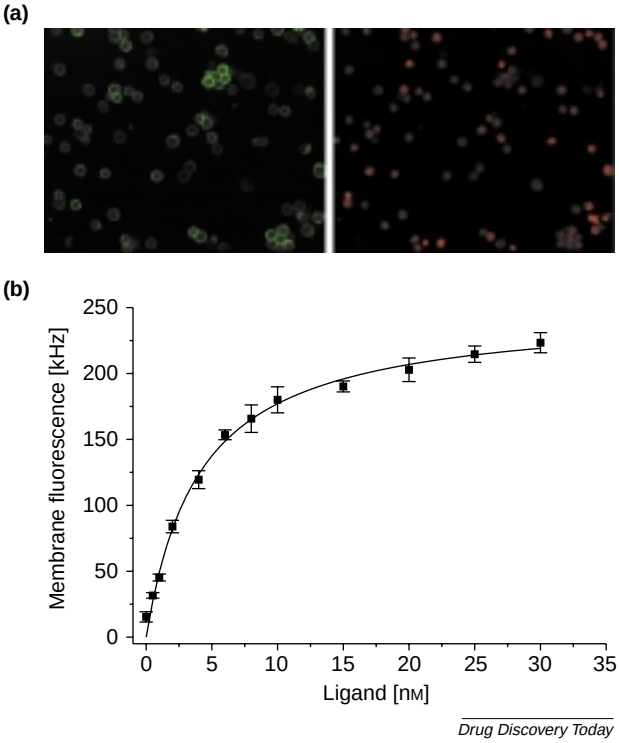

Figure 3. (a) Hek 293 β_2 cells imaged by the Opera system; **(b)** Apparent K_D determination of the ligand-binding event. For details see text.

For cLSMs, the 'point-like' acquisition of fluorescence intensity data has to be combined with a simultaneous excitation-to-detection delay-time acquisition, resulting in the possibility of assigning a measure of the fluorescence lifetime to each of the image pixels [65]. Scanning FLIM has been realized by, for example, Carl Zeiss and Nikon. A different approach for image acquisition is required when using a CCD camera. Thereby, a stack of images is acquired, each displaying fluorescence emitted within a certain time window of several hundred picoseconds width, but at different distances with respect to the excitation pulse, thus sampling the data collection over the decay profile. The subsequent data processing allows for a pixelwise determination of the fluorescence decay, which finally assigns the appropriate lifetime values to every single point of the acquired image [66]. An appropriate FLIM enhancement was recently developed for the Opera system (Evotec Technologies).

Summary

This review has outlined the capabilities of highly sensitive fluorescence detection technologies and its application to life sciences. The demand for deeper insight into complex biomolecular interactions for soluble as well as cellular systems has guided the development of highly resolved and precise fluorescent read-outs, culminating in fluctuation techniques for biochemical and confocal image analysis for cellular applications. Commercially available fluorescence readers using at least one of the above outlined techniques in HTS mode are presented in Table 1.

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