Confocal optics microscopy for biochemical and cellular high-throughput screening

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In recent years, both academia and pharmaceutical industry have produced significant advances in confocal detection and spectroscopy by laser-induced fluorescence. Confocal fluorescence studies provide information on identity, size, diffusion coefficient and concentration of the fluorescently labeled entity. This enables the establishment of sophisticated biochemical drug screening assays using the multitude of fluorescence parameters that can be observed (e.g. molecular brightness, fluorescence lifetime, anisotropy, resonance energy transfer). In cellular screening assays, confocality introduces spatial resolution in the vertical direction and reduces background fluorescence from outside the focal plane. Confocal HTS systems focusing on femtoliter-sized observation volumes allow for assay volumes far beyond current limits.

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▼ Within pharmaceutical companies, there is increased pressure to use chemical compounds economically and to employ lower amounts of expensive bioreagents. To meet these new demands, HTS groups worldwide have made attempts to dramatically reduce the sample volume in drug screening assays. This miniaturization faces two major challenges: handling of minute volumes and the sensitivity of the typically optical detection. Optimization of liquid handling tools has preceded developments in detection: several sub-microliter pipetting and dispensing devices have been established in HTS routine use. However, all macroscopic fluorescence methods face the problem of increasing background with decreasing assay volumes. By contrast, the femtoliter-sized confocal observation volume enables miniaturization without loss of data quality [1].

Confocal optics

For the optical detection of fluorescence emission from individual molecules, unwanted background must be rigorously minimized. Most importantly, the volume from which light is collected has to be made as small as possible because background from solvent Rayleigh and Raman scattering as well as from fluorescent impurities cannot be completely suppressed. Confocal optics [2] typically employ a high numerical aperture objective lens to focus the excitation laser light. The restriction to fluorescence emission from the confocal volume is achieved by guiding the emitted light through a pinhole (Figure 1a). To obtain high fluorescence sensitivity, low noise detectors, such as avalanche photodiodes, are employed. Using the confocal detection principle, femtoliter-sized sample volumes that enable single-molecule studies can be analyzed.

Fluorescence studies at the single-molecule level can be performed on localized individual biomolecules, for example, while attached to surfaces. In an alternative approach, however, the emission from an open confocal volume, through which the biomolecules diffuse, can be monitored. Sample preparation is much simpler in this case, as samples are simply dilute, typically nanomolar, solutions of the molecules being studied. This technique is, therefore, preferred in biochemical drug screening assays, as described later. Alternatively, the confocal sample volume can be scanned through living cells for the study of fluorescent biomolecules in their native environments.

Fluorescence fluctuation spectroscopy techniques used in biochemical drug discovery

The fluorescence signal from a few molecules inside a small volume shows temporal intensity

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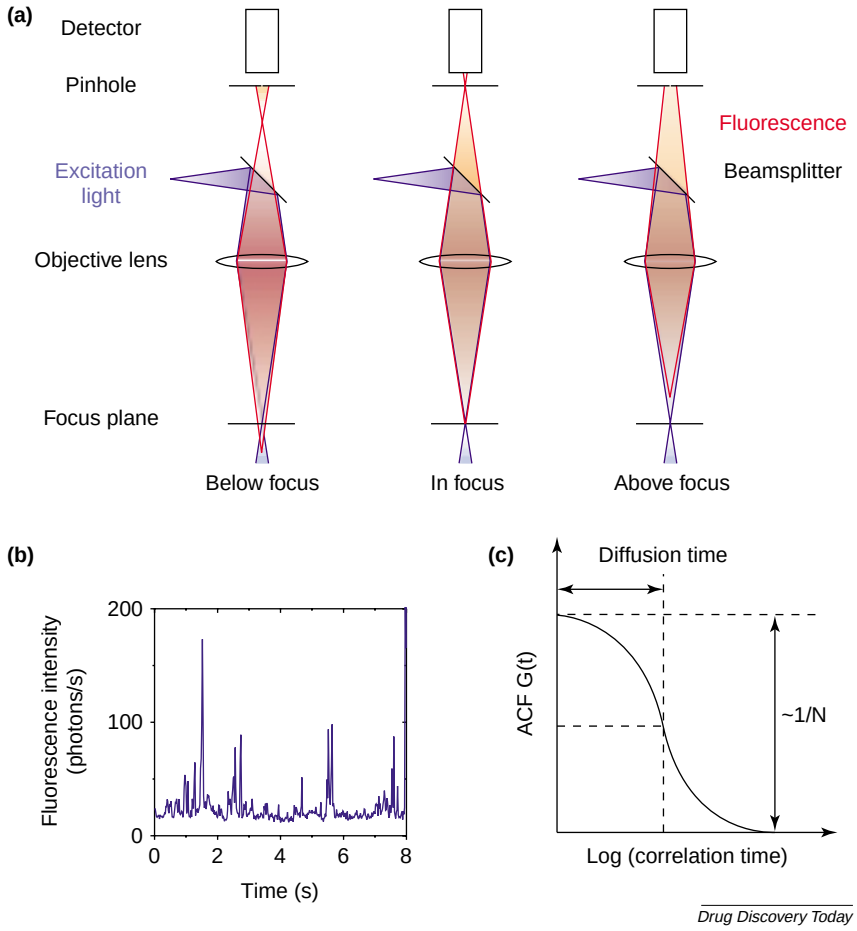

Figure 1. (a) The observation volume in confocal microscopy. The laser excitation light (shown in blue) is focused through the objective to a diffraction-limited point. The confocal pinhole in the detection path ensures that only fluorescence light (shown in red) emitted from the focal plane is detected (shown in the middle). Light coming from below or above focus can not be registered (see left and right). The combination of focused excitation and a pinhole in the detection path establish the confocal sample volume of approximately one femtoliter (10^{-15} l), from which the fluorescence is detected. **(b)** Fluorescence intensity time trace. For each time point, the fluorescence intensity signal is determined by the fluorescent particles in the observation volume. The fluctuations are caused by either a change of the number of observed molecules (e.g. through diffusion) and/or a change of their fluorescent properties (e.g. due to chemical reaction). In this example, bright lipid vesicles (labelled with several fluorophores per vesicle) cause the large spikes in the time trace, and dimmer particles (free fluorophores) cause the smaller fluorescence fluctuations. **(c)** An autocorrelation function (ACF). The photon bursts in (b) can be analyzed for their duration by the ACF, which yields information about the diffusion time through a confocal observation volume. Small molecules diffuse faster and show a shorter diffusion time, larger molecules diffusing slowly show a longer diffusion time. Moreover, the amplitude of the ACF provides information about number of fluorescent particles diffusing through the confocal observation volume. A large amplitude (~1/N) indicates a low concentration of particles, a lower amplitude indicates a higher concentration of particles.

fluctuations. In contrast to most other techniques, where these intensity fluctuations are considered as noise in the measured signal, fluorescence fluctuation spectroscopy uses the fluctuations themselves to obtain information about the processes that generate them. The fluctuations are caused by changes in the number of observed molecules and/or by changes in the fluorescent properties of the observed molecules. The processes causing the fluctuations can be diffusion or drift of the molecules, flow of the sample, and/or chemical reactions. Thus, fluorescence fluctuation experiments give access to information, such as the number of molecules, molecular concentration, molecular size, diffusion coefficient, rate coefficients of inter- and intra-molecular reactions, and molecular brightness.

Because fluctuations of the intensity signal are analyzed, it is desirable to maximize them. For a large number of molecules in the observation volume, fluctuations are averaged out. Consequently, the total number of fluorescent molecules in the observation volume has to be kept low to maximize fluctuations. This can be achieved by a low fluorophore concentration and/or small observation volume. Typical fluorophore concentrations used in fluorescence fluctuation spectroscopy are in the nanomolar range, and the femtoliter-sized observation volume can be realized by confocal optics, as described previously.

The primary data generated by a fluorescence fluctuation spectroscopy experiment are a record of fluorescence intensity as a function of time (Figure 1b). These data contain information about the number of molecules in the observation volume at a given time and about their fluorescent properties. The information can, in principle, be extracted with two different (and partly complementary) statistical approaches: (i) analysis of the autocorrelation function and (ii) analysis of the photon counting histogram. Both methods, as well as additional techniques, are described in more detail in the following sections.

Fluorescence correlation spectroscopy (FCS)

Fluorescence correlation spectroscopy (FCS), introduced by Magde, Elson and Webb in 1972 [3–7] and applied for the

first time by the same group in 1974, uses the autocorrelation function (ACF) to analyze fluctuation data. The ACF is defined in Equation 1.

$$G(\tau) := \frac{\langle \delta F(t) \cdot \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2} \quad \text{[Eqn 1]}$$

where F denotes fluorescence intensity, δF fluorescence intensity fluctuations, t time, τ correlation time and $\langle\ \rangle$ denotes the time average. It calculates the probability to detect a photon at some time ($t+\tau$) if there was a photon at time t. In other words, the ACF correlates the fluorescence at the time-point t with the time-point ($t+\tau$). For freely diffusing molecules moving in and out of the observation volume, a high correlation of the fluorescence signal is expected while a molecule stays in the volume. Independent molecules should be uncorrelated and the ACF will drop to zero, at times larger than the typical residence time in the observation volume, as shown in the schematic ACF in Figure 1c. This is called the diffusion time of the molecule; it is dependent on the size of the molecule and the observation volume. Small molecules diffuse fast, the diffusion time is short, and their ACF decays at a short correlation time. As larger molecules diffuse more slowly, their diffusion times are longer and, therefore, the decay of the ACF is shifted towards longer correlation times.

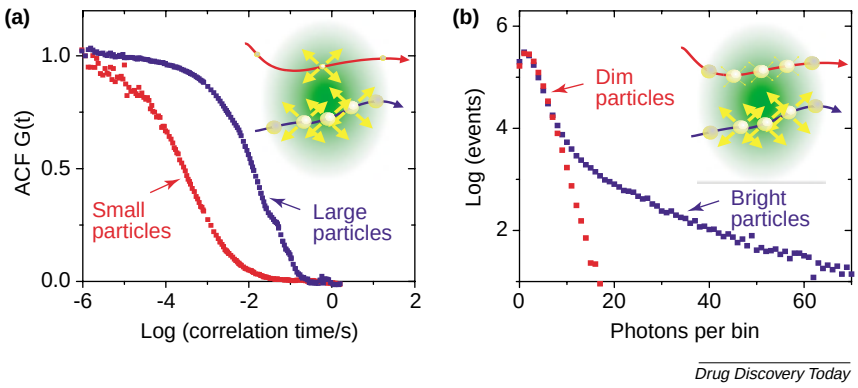

Figure 2. Application of FCS **(a)** and FIDA **(b)** to a receptor–ligand binding assay. **(a)** Autocorrelation functions (ACFs) are shown for two samples: the ACF of a fluorophore-labeled peptide (red) and of this peptide bound to its receptor in large lipid vesicles (blue). The unbound peptide traverses the confocal observation volume fast (red track in inset), and its ACF correspondingly shows a shorter diffusion time. If the fluorophore-labeled peptide is bound to its receptor in the large vesicle (blue track in inset), it will diffuse through the confocal volume more slowly, and the ACF yields a longer diffusion time. **(b)** In a photon counting histogram (PCH), the photon-bursts are analysed for their intensity, so that two components can be distinguished with regard to their individual molecular brightness. The emitted photons are counted for a short time interval (bin width here is 100 μs) and plotted as a histogram. The PCH for a fluorophore-labelled peptide is shown in red, and the PCH for peptide ligands bound to multiple receptors in a lipid vesicle is shown in blue. Each lipid vesicle contains 50-100 receptors, and the receptors were saturated with ligands in this example. The unbound fluorescent peptide emits less photons during its passage through the confocal volume (red track in inset) than the vesicle with multiple fluorescent peptides bound to its receptors (blue track in inset) for different wavelength detection.

As stated earlier, fluctuations are larger if the number of molecules is smaller. The amplitude of the ACF is proportional to the inverse number of the molecules in the volume (~1/N in Figure 1c). Lower concentrations, thus, give a larger signal in FCS measurements (i.e. a large amplitude is associated with a low concentration of particles while a smaller amplitude is associated with a higher concentration of particles); FCS enables us to distinguish between different molecules in the sample with regard to their size and to measure their concentrations.

A typical biochemical application of FCS in an HTS environment is a ligand binding assay, which measures the binding of a ligand to the target and its displacement by tested compounds. An example of the application of FCS for such a biochemical assay, the binding of a peptide to its receptor, is shown in Figure 2a. The peptide ligand is fluorescently labeled so that its diffusion through the obser vation volume can be measured. The free ligand is relatively small and diffuses quickly through the confocal observation volume (red data points). As the receptor is incorporated into large lipid vesicles, binding of the ligand to its receptor results in a longer diffusion time through the observation volume (blue data points). The situation of small, fast diffusing and large, slowly diffusing particles is schematically shown in the inset in Figure 2a. Further applications of FCS for HTS have been reviewed by Auer *et al.* [1].

In principle, FCS can be used for any binding assay associated with a change in the size (mass) of the detected molecule on binding; however, as translational diffusion is proportional to the hydrodynamic radius, and thus, only to the cube-root of the mass, the mass difference should ideally be rather large. Hence, an eightfold increase in the molecular mass changes the diffusion time only by a factor of two. Thus, FCS measurements are sensitive only to large changes in the molecular mass. Typically, ligands are small and target molecules are large, so the condition of a large mass ratio is fulfilled by many binding assays. From this point of view, large membrane fragments or lipid vesicles

are optimal as slowly diffusing components in FCS measurement. However, a problem that is associated with experiments on particles that diffuse extremely slowly is the long data acquisition time needed to generate enough statistical events for the calculation of the ACF. This disadvantage of FCS can be partially compensated for by a scanning approach – a technique that achieves a reduction of the measuring time without loss of data quality. It is based on an active search for membrane fragments in the sample by moving the focused laser beam or the entire sample. Using the scanning approach, many more membrane fragments or vesicles with bound ligand can be detected in the same measuring time. For scanning FCS, the diffusion ACF must be supplemented with an additional exponential factor containing the scanning velocity [7,8].

FCS is an excellent method for the estimation of absolute concentrations of fluorescently labeled species and allows us to quantify free and bound ligand, based on the mass difference between these two species. When several diffusing species are simultaneously present in the sample, the ACF is the sum of the ACFs of each species weighted by the square of its fractional intensity, which depends on its molecular brightness and concentration. For example, in a mixture containing identical concentrations of two species, but one having twice the molecular brightness of the other, the weights differ by a factor of four.

In addition to the different diffusion times, differences in the molecular brightness of multiple species in the assay make the evaluation of the ACF complicated. Under well-controlled conditions, these effects can be entangled. In one special case for FCS enabling simplified analysis, all fluorescent species in the assay should have the same molecular brightness and differ by their molecular masses only. The second special case can be achieved if the molecular brightnesses of two species are vastly different; here, the dimmer species can be neglected, and the ACF represents only one species. The difference in molecular brightness is used for the second type of statistical evaluation of fluorescent intensity time traces, as described in the following section.

Photon counting histogram (PCH) or fluorescence intensity distribution analysis (FIDA)

Instead of analyzing the temporal fluctuations of the fluorescence signal (as done in FCS), the statistics of the intensities of the generated photon bursts can be analyzed. Photons that are emitted during passage of the fluorophore through the confocal observation volume are counted for a short time interval (time bin) and plotted as a histogram. A dimmer fluorophore emits less photons per time bin during its passage through the confocal volume than does a brighter fluorophore (see inset in Figure 2b). If the time bin is shorter than the diffusion time of the fluorophore, the fluorophore size does not influence the histogram. Thus, two components can be distinguished with regard to their individual molecular brightness. The distribution of the numbers of photons per bin-width can be analyzed by two slightly different statistical methods. One method, analysis of photon counting histograms (PCHs), has been developed by Chen, Müller and coworkers [9], and the other, fluorescence intensity distribution analysis (FIDA), by P. Kask, K. Palo and co-workers from Evotec OAI (http://www.evotecoai.com) [10].

FIDA/PCH analyses enable us to distinguish between different molecules in the sample by their brightness and to measure their concentrations. The application of FIDA to a HTS ligand–receptor binding assay necessitates changes in the molecular brightness on binding, which may arise from changes in the fluorescent properties of the fluorophore-labeled species, binding to a multivalent receptor or binding to vesicle/membrane fragment/particle with multiple receptors. In Figure 2b, a PCH diagram is shown as an example of a peptide binding to its receptor. The peptide ligand is fluorescently labeled; so its photon statistics on passage through the observation volume are shown as the red data points. As multiple receptors are incorporated into the lipid vesicles, the vesicle carries more fluorophore-labeled ligands on binding of the ligand to its receptor. The vesicle with multiple bound ligands emits many more photons during its passage through the observation volume (blue data points) than the free ligand. The scheme of dim and bright diffusing fluorescent particles in the confocal observation volume is shown as an inset in Figure 2b.

Applications of FIDA to membrane receptor assays were published previously [11–13] and in our work (report in preparation). Most known sources of membrane receptors bear multiple receptors and hence, FIDA appears to be an ideal method for such assays. Membrane receptors, like G-protein coupled receptors, can be obtained in cell membrane fragments from cell culture or partly purified, and reconstituted in lipid vesicles or enriched in virus-like particles (VLiPs™; Evotec OAI). As for the FCS experiments, the scanning approach reduces the measuring time without loss of data quality and has been used successfully in FIDA measurements [11–13].

Further fluorescence fluctuation spectroscopy techniques used in drug discovery

Futher techniques have been developed that combine the basic methods FCS and PCH, and might also include additional observable factors, which help to identify different species in complex biological samples and provide

additional information about the properties of the species or their binding.

Fluorescence cross correlation spectroscopy (FCCS); this technique extends FCS to enable cross correlation analysis of two colors [4,14]. For example, if ligand and receptor are labeled with two different fluorophores, the binding event is indicated by the simultaneous presence of both labels in the confocal volume.

Confocal fluorescence coincidence analysis (CFCA); this is a recently developed technique, which emphasizes short analysis times and simplified data evaluation [15]. It is, therefore, particularly useful for screening applications and/or measurement on living cells where small illumination doses need to be applied.

Fluorescence lifetime analysis; this could be also used with confocal optics. It gives insight into changes of the excited state by monitoring the fluorophore lifetime in the nanosecond time range.

2-dimensional fluorescence intensity distribution analysis (2D-FIDA; Evotec OAI); this extends FIDA to the combined analysis of two simultaneously recorded brightness distributions [16]. 2D-FIDA might be configured for two-color, anisotropy or FRET applications, and enables high quality and high content data.

Fluorescence intensity and lifetime distribution analysis (FILDA; Evotec OAI); this is an advanced analysis technique, yielding simultaneous information on the fluorescence lifetime and molecular brightness of multiple fluorescent species [17].

Biomolecular dynamics in the confocal volume

Proteins are enormously complex physical systems that are characterized by a huge number of conformational states, and transitions among these states are intimately linked to their function. Single molecule spectroscopy, the measurement of intensity fluctuations, emission spectra, fluorescence lifetime and polarization in the confocal observation volume can provide valuable information on these dynamic processes [18]. Molecular interactions, enzymatic activity, reaction kinetics, conformational dynamics, as well as alterations in the chemical environment of the fluorophore can be monitored. For G-protein coupled receptors (GPCRs), the most frequently addressed drug targets in pharmaceutical industry, this method has the potential to distinguish between partial, full, inverse agonists or antagonists [19].

Instrumentation for fluctuation fluorescence spectroscopy in a HTS environment

Several companies supply devices that enable fluctuation fluorescence spectroscopy measurements, but Evotec OAI is the most advanced in applying this technology to HTS. The Insight™ reader (Evotec Technologies GmbH; http://www.evotec-technologies.com) detects fluorescent molecules with single-molecule resolution. Submicroliter miniaturization without loss of signal quality becomes possible by the use of confocal optics. Parallel analysis of multiple fluorescent dyes is enabled by multiple laser sources and detectors. Detection of single molecules at different wavelengths or polarization states with nanosecond time resolution is made possible by the use of two highly sensitive single photon detectors. High-speed signal processing boards support a real-time calculation of all incorporated methods. Measurement times for large particles, such as membrane fragments, are reduced by 2D beam scanning. Evotec Technologies GmbH indicates a typical readout time per well of ~1 s, which fulfills the requirements of ultra-HTS.

The basic version of the Insight™ reader is equipped with three lasers: an Ar+ laser for excitation at 488 and 514 nm, a HeNe laser (543 nm) and another HeNe laser (633 nm). It supports the FCS, FIDA, FCCS and 2D-FIDA modes.

For fluorescence lifetime measurements, another version of the Insight™ reader is available, which additionally contains a mode-locked frequency doubled Nd:YAG green laser (532 nm) and a pulsed red laser diode (635 nm). This last reader also contains a software extension that supports the fluorescence lifetime analysis and the FILDA technique.

Confocal cellular screening

Advantages of confocal cellular imaging

Fluorescence microscopy has been widely used in cell biology as a non-destructive and sensitive technique for the visualization of intracellular structures and biomolecular translocations. The imaging of intracellular structures has benefited substantially from the introduction of confocal microscopy. Fundamentally, confocal optics dramatically improve the spatial resolution in the vertical direction, greatly reducing interference from adjacent object features above or below the focal plane (Figure 4a). For example, confocal optics enable the observation of cells that are adherent to the bottom of a microtiter plate well without interference from dead cells, free fluorophores or autofluorescent particles above the cellular layer. In a standard microscopic image, light is also collected from a layer outside the focal plane. This increased optical resolution is particularly important to permit the visualization of the complex subcellular membrane, vesicle and organelle systems within eukaryotic cells. The detailed study of intracellular translocation of target biomolecules, for example, the translocation of a transcription factor from the cytosol to the nucleus in response to a stimulus, is facilitated by this approach.

'High (Information) content screening'

The emerging field of 'High (Information) Content Screening' (HCS; main topic of the meeting of the Society for Biomolecular Screening 2002; http://www.sbsonline.com) is based on high resolution imaging of fluorophore-stained cells. Typically, several fluorophores can be observed in parallel (multiplexing). Image analysis software automatically quantifies intracellular translocations of fluorophore-labeled biomolecules. Apart from protein trafficking, HCS can provide information on apoptosis, morphological changes (e.g. neurite outgrowth), cellular movements and other phenomena that result in an overall change of the fluorescent cellular image.

Like other non-confocal imaging systems, confocal readers enable single-cell imaging. Single-cell imaging provides the additional advantage of being able to analyze the response of a heterogeneous cell population to a drug stimulus, for example. The individual cells of a population might differ with respect to their developmental stage, their stage in the cell cycle, their state of transfection or by natural variability. Single-cell analysis enables the study of cellular responses that only occur in a subset of a cell population. Furthermore, certain drug effects on single cells might be cross-correlated with other phenomena, such as apoptosis. Both the study of intracellular transport pathways and single-cell distinction benefit from the good spatial resolution of confocal readers.

Drug discovery applications of confocal cellular imaging

To localize specific biomolecules within a cell using fluorescence microscopy, the biomolecules must be labeled with an appropriate fluorophore. Labeling can be achieved through chemical, antibody-mediated or endogenous (fusions with fluorescent proteins) methods. If the biomolecular transport starts from the plasma membrane, the biomolecule of interest can be labeled (e.g. with a fluorophore-labeled antibody or ligand) from the extracellular side before it begins its journey into the cell. However, if the epitope for an antibody-based fluorescent labeling is not exposed to the extracellular medium, the cells must be fixed and lysed before the fluorescent antibody can be applied. This fixation procedure restricts the possibilities of the cellular imaging to end-point measurements. Alternatively, endogenously synthesized fluorophores, such as the green fluorescent protein [20] or its differently colored relatives [21–24] might be attached to a protein-encoding sequence on the DNA level. The resulting fluorescent fusion protein and its movement kinetics can be so visualized in a non-destructive way. The novel fast cellular confocal readers that are described below enable kinetic measurements with a high temporal resolution.

In a typical drug screening application for a cellular imaging system, the intracellular transport of fluorophore-labeled biomolecules is observed in response to an extracellular stimulus. As an example, the endothelin 1-induced internalization of a fusion protein between endothelin A receptor (ET_AR) and green fluorescent protein (GFP) has been studied using the Opera™ system (G. Gradl, pers. commun.; Figure 3). In the absence of endothelin, the ET_AR-GFP resides in the plasma membrane. Upon binding of endothelin, the ET_AR-GFP becomes internalized and translocated to a central endosomal compartment. Image analysis and object recognition software enable the distinction between the peripheral and the accumulated intracellular green fluorescence on the single-cell level.

Instrumentation for cellular confocal imaging in an HTS environment

Several systems are commercially available for confocal microscopic imaging of cells. However, the available confocal point scanning microscopes are generally too slow for drug screening applications. Three confocal high-throughput cellular imagers are marketed to fill this gap: the Opera™ from Evotec Technologies GmbH, the InCell Analyser™ from Amersham Biosciences (http://www5.amershambiosciences.com) and the Pathway HT™ from Atto Biosciences Inc. (http://www.atto.com). These systems achieve a readout time of ~1 s per well (varying for example with the required resolution of the microscopic image and the brightness of the fluorophores) and are described in detail later. For the sake of higher throughput, the Opera™ (Figure 4c) and the Pathway HT™ (Figure 4d) employ a Nipkow disk to project fluorescence from several confocal volumes in parallel to a CCD camera. In a similar approach to shorten the imaging time, the InCell Analyser™ employs line scanning through a confocal slit (Figure 4b). This new generation of HTS-compatible confocal imaging readers combines high temporal with high spatial resolution. All three systems support an autofocus mechanism that keeps the microscope objective focused to the cellular layer adherent to the bottom of the well. The Pathway HT™ and InCell Analyser™ provide an environmental chamber that maintains user-defined temperature and carbon dioxide levels, thus, enabling live cell experiments; for the Opera™ this feature will be available in the near future.

The Evotec Opera™ possesses three laser sources (488 nm, 532 nm and 633 nm) and two CCD cameras for the detection of two fluorescence emission wavelengths. Using parallel two-color excitation (488/633 nm or 532/633 nm) and two parallel detection channels, it enables the simultaneous observation of two cellular phenomena.

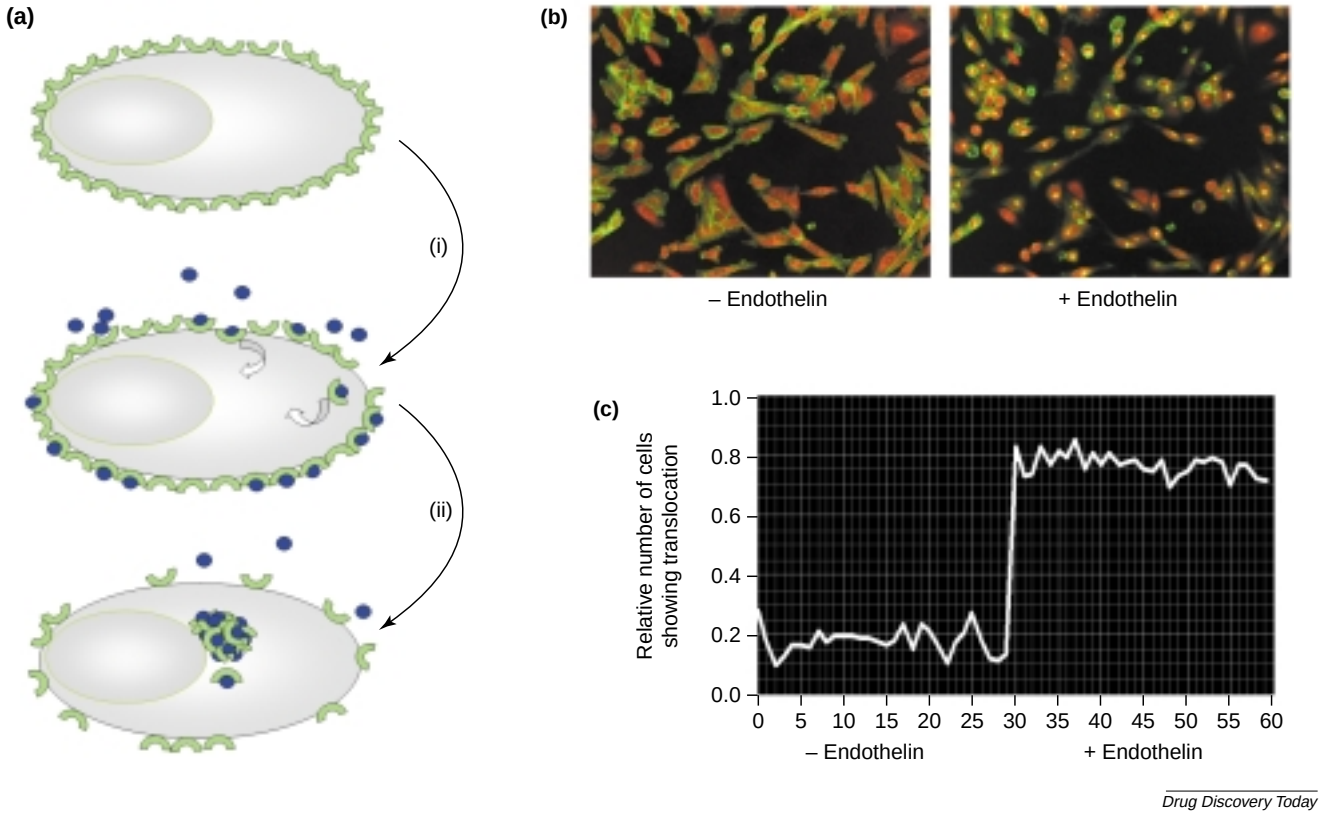

Figure 3. Agonist-induced internalisation of a fusion protein between endothelin A receptor (ET_AR) and green fluorescent protein (GFP) observed in the Opera system. **(a)** Scheme of the experiment: In the absence of endothelin (blue dots) the ET_AR-GFP (green semicircles) resides in the plasma membrane. Upon addition (i) and binding of endothelin, the ET_AR-GFP becomes internalized and translocated to a central endosomal compartment (ii). **(b)** In the absence of endothelin (left panel), the green fluorescence is mainly localized to the plasma membrane, 3 h after addition of endothelin (right panel), green fluorescence arises largely from a central spot in the cytosol. **(c)** Image analysis that distinguishes between the peripheral and the accumulated intracellular green fluorescence was carried out on the single-cell level. The results of this analysis for 30 microtiter plate wells that were not exposed to endothelin are shown for wells # 0-29, the results for 30 wells that had been exposed to endothelin for 3 hours are shown for wells # 30-59. Statistics of this analysis predict a Z' value [25] of 0.58 for an internalisation assay.

The Pathway HT™ is constructed around the Confocal Attofluor Ratio Vision (CARV) scanning technology of Atto Bioscience. In contrast to the Opera™ reader, the system employs two independent full-spectrum mercury arc lamps for illumination. Therefore, this setup provides the full spectrum of excitation wavelengths (340 nm to near-IR), using 16 excitation and 8 emission filters with independent dichroic mirror setting (Figure 4d). A single CCD camera enables the measurement of multiple fluorescent markers by taking fast sequential images at different wavelengths.

The InCell Analyser™ possesses three laser sources (365 nm, 488 nm and 647 nm) and three CCD cameras for simultaneous imaging (Figure 4b). Parallel use of the three excitation wavelengths and of the three detection channels enables observation of three cellular phenomena simultaneously.

Conclusions

Confocal fluorescence studies can be performed on fluorophore-labeled biomolecules in femtoliter-sized volumes in a biochemical assay solution or within living cells. Biochemical HTS assays based on fluorescence fluctuation spectroscopy are predominantly homogeneous assays. Due to the high sensitivity of the technique, only minute quantities – typically nanomolar concentrations – of the bioreagents are needed. Because of the confocal setup, these assays are well suited for extreme miniaturization, while maintaining the same background fluorescence. The reduced assay volume results in overall lower bioreagent costs and lower consumption of the analyzed compounds. The fluorophore-labeled entity in the sample might be analyzed for translational diffusion time, fluorescence lifetime, fluorescence brightness, fluorescence polarization or spectral characteristics. All of these parameters could change

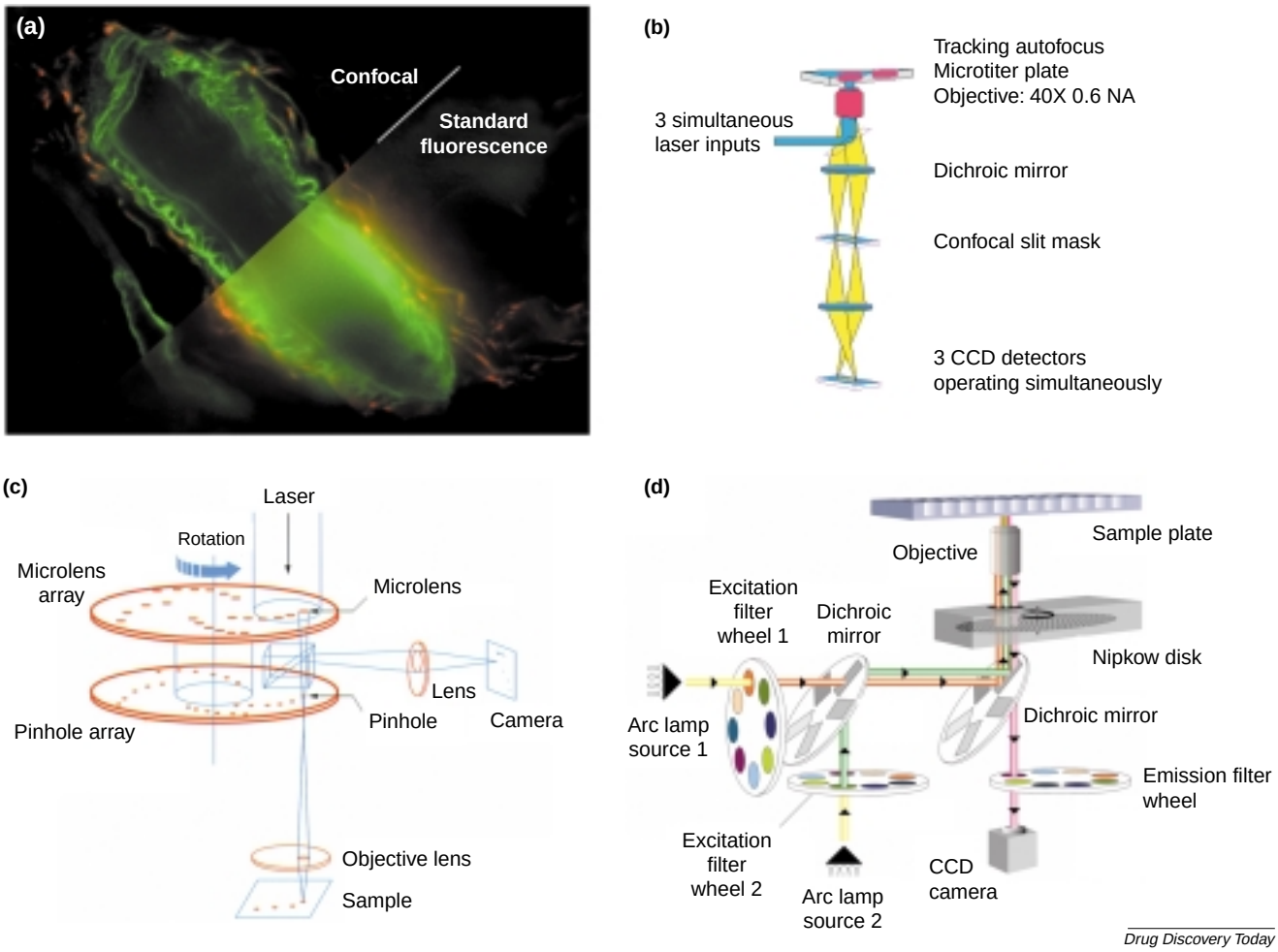

Figure 4. Light paths of three confocal imaging systems. **(a)** A large arteriole in a skin section was doubly stained for nerve with an antibody to protein gene product 9.5 conjugated to fluorescent marker Cy3 and to collagen type IV using marker Cy 2 (antibodies from Jackson ImmunoResearch Laboratories; http://www.jacksonimmuno.com). The image was captured sequentially in confocal and wide-field mode using the Atto Bioscience CARV scanning technology. The two images were then juxtaposed (courtesy of William R. Kennedy, University of Minnesota; http://www.umn.edu). **(b)** Light path of the InCell Analyser™. The excitation laser light is autofocused through an objective to the bottom of the microtiter plate. Fluorescence emission is collected by the same objective, then guided through a dichroic mirror and confocal slit mask to three simultaneously operating CCD detectors for different wavelength detection (scheme is courtesy of Gerd Erhard, Amersham Biosciences). **(c)** Light path of the Opera™. The excitation laser light is guided through a rotating microlens array that focuses the light through a dichroic mirror, a simultaneously rotating pinhole array and an objective lens to the sample. Fluorescence emission from the sample is guided back via an objective and pinhole array, then reflected towards a CCD camera (courtesy of Gabriele Gradl, Evotec Technologies GmbH). **(d)** Light path of the Pathway HT™. Light from two high intensity mercury arc lamps is sequentially passed through one of two 8-position excitation filters. The excitation light is reflected/passed through two selectable dichroic filter wheels, and projected through a broad-spectrum spinning Nipkow disk to the objective and onto the specimen. Fluorescence emission from the sample is collected by the objective, passed once again through the same spinning disk, through the dichroic mirror, and then through one of eight selectable emission filters to a CCD camera (courtesy of Phil Vanek, Atto Biosciences).

on binding of the investigated compound to the target biomolecule. From this multitude of fluorescence characteristics, the optimal parameter might be chosen as the basis for a drug screening assay format. Alternatively, multiparameter analysis can be performed to help distinguish between a technological artifact and a true pharmacological effect of the compound. Overall, multiparameter analysis is expected to substantially improve screening data quality.

In the pharmaceutical industry, fluorescence microscopy has been established as a widely applied tool to study the cellular effects of drug candidates. For cellular imaging, confocal optics provide a significant improvement in spatial resolution and data quality. The current generation of automated confocal imaging readers promises to deliver these advantages at a scale appropriate for HTS drug discovery applications. The available HTS-capable confocal

cell imagers achieve increased imaging velocity, either by line-scanning or by Nipkow disk-based multi-point scanning, both of which enable fast kinetic assays while maintaining high spatial resolution.

Confocal optics have the potential to benefit multiple stages of the drug discovery process. We predict a substantially increased use of this technology for highly miniaturized biochemical assays and for high resolution cellular imaging in the near future.

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