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Towards Sorting of Biolibraries Using Single-Molecule Fluorescence Detection Techniques

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Abstract: The selection of specific binding molecules like peptides and proteins from biolibraries using, for instance, phage display methods can be quite time-consuming. It is therefore desirable to develop a strategy that is much faster in selection and sorting of potential binders out of a biolibrary. In this contribution we separately discuss the current achievements in generation of biolibraries, single-molecule detection techniques and microfluidic devices. A high-throughput microfluidic platform is then proposed that combines the propulsion of liquid containing fluorescent components of the biolibrary through microchannels, single-molecule fluorescence photon burst detection and real-time sorting of positive hits.

Key Words: biolibrary, phage display, selection, sorting, microfluidics, flow, single-molecule fluorescence, fluorescence correlation, photon burst.

INTRODUCTION

The general motto of this Special Edition of Current Pharmaceutical Biotechnology is "the way down from single genes, and proteins to single molecules". The editors have questioned where to start: either from the top down beginning with medicine or from the bottom up beginning with single molecules and their behavior? The answer of the editors is a typical compromise: working in both directions from the middle. We can extrapolate this way of thinking a little further and more generally to the field of imaging of cellular systems, even when we have constrained our topic by focusing on biolibraries as a biological system and single-molecule fluorescence detection as a helpful tool for sorting and selection.

There is a transition occurring in biology from the molecular level to the system level that promises to revolutionize our understanding of complex biological systems [1]. Understanding of genes and proteins remains centrally important and forms the basis of understanding the organization and dynamics of a biological system. Techniques and methods developed within the field of genomics and proteomics ranging from sequencing to yeast two/three hybrid methods, mass spectrometry and DNA/protein arrays, continue to be important and even need further development. However, breakthroughs in experimental devices, advanced software and analytical methods allowing *in vivo* imaging are required before the achievements of 'systems biology' can live up to their much heralded potential [1]. Only then will we be able to examine the structure and dynamics of cellular and organismal function. This will, in turn, lead to an understanding of the mechanisms that systematically control the state of the cell and multicellular structures. System-level insight will then allow the modification and (re)construction of biological systems having desired properties using genetic methods or by using mechanism-based drugs [1]. Therefore, understanding the properties of biomolecular networks is of central importance in basic biological science and in biomedicine of importance to human health. Modern microspectroscopic techniques (that can be considered as a combination of microscopic and spectroscopic techniques) are the method of choice for the above purpose as these techniques provide direct information on molecular interactions and dynamic events involving biomolecules with minimal perturbation of cellular integrity and function. Thus, the wealth of information and resources generated by efforts in genomics and proteomics can be directly translated into understanding the functioning of cells, tissues and organisms *in vivo*.

The further development of modern microspectroscopic techniques is also relevant for the subject that we want to emphasize here: biolibraries, how can we screen and sort them, and with which techniques? The same microspectroscopic techniques (single-molecule fluorescence detection in particular) can be used for screening and sorting of biolibraries as for optimizing cellular imaging systems. In both cases it is important to develop optimum contrast schemes between the reporting fluorescence signals and the background signals (actually eliminating background signals arising from Raman and Rayleigh scattering and spurious fluorescence) resulting in the highest level of sensitivity, precision and speed of measurements, temporal and spatial resolution. We will also need microfluidic systems for the downscaling of sorting and selection of biolibraries. These microfluidic devices also require techniques to control passage of the fluid as well as to determine the flow speed in minute volumina. Considering all possible microspectrosco-

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pic parameters fluorescence analysis of biolibrary components is particularly attractive, as its sensitivity is high enough to detect single molecules in aqueous solutions and its detection time short enough to enable on-the-fly measurements.

This minireview has been divided into the following topics: (i) biolibraries, (ii) single-molecule fluorescence techniques, (iii) microfluidic devices, (iv) possible ways of integrating topics i-iii into a working prototype.

BIOLIBRARIES

The organization and dynamics of biological systems rely on an intimate interplay of (macro-)molecules. Proteins, nucleic acids and other compounds interact with each other to transduce signals or to build up macromolecular structures that shape cells and organelles. Cell functioning largely depends on protein interaction networks or as outlined in [2] 'the entire cell can be viewed as a factory that contains an elaborate network of interlocking assembly lines, each of which is composed of a set of large protein machines'. Unraveling protein interaction patterns is therefore of key importance to increase our understanding of cell function. Efforts in the fields of genomics and proteomics have increased our knowledge in this field considerably. It remains, however, required to use techniques to identify interacting partners using the appropriate techniques. Peptide and cDNA libraries have shown to be of great significance for this purpose when used in combination with techniques to find interactors such as yeast two/three hybrid systems, phage display and protein and DNA arrays.

Recent developments in microspectroscopy as well as new expression and labeling strategies enable a more specific and sensitive selection of peptides and proteins from peptide and cDNA libraries expressed on phage or bound to a solid surface as, for instance, used with arrays. Regarding the latter it has now been shown that overexpression of cDNAs in expression vectors using cell microarrays can be used to identify genes in diverse cellular processes [3]. A glass slide is printed with cDNA in an aqueous gelatin solution. The slides are then incubated with mammalian cells and transfected using a lipid transfection agent. Clusters of ~30-80 cells actively express the (defined) gene products that can be visualized using a variety of microspectroscopic techniques.

Regarding the expression of cDNA libraries on bacteriophage one can use filamentous and lytic phages. Filamentous phages such as M13, fd and IKE infect *E. coli* bacteria through their gene III protein (gIIIp) that binds to pili. Upon replication of the phage genome new phages are assembled and excreted from the bacteria without cell lysis. All five proteins present in the phage coat have been used for display. However, for cDNA phage display the gIIIp and gVIp proteins are most important, using gIIIp for expressing N-terminal and gVIp for C-terminal fusion proteins. Instead of filamentous phage an increasing use is being made of lytic phages such as phage λ and T7. These phages infect the bacterial cell and replication and assembly is followed by a real phage burst resulting in cell lysis. The protein forming the 'head' of the phage is used for display. The choice of the display system for cDNAs depends on the goals to be achieved as well as personal preferences and experiences. Jespers *et al*. [4] were the first to use cDNA phage display. They displayed a cDNA library from the hookworm *Ancylostoma caninum* on the gVIp of M13 and found two genes encoding novel members of two different families of serine protease inhibitors. Many applications of cDNA phage display have followed since, including the isolation of lectins [5], lysosomal proteins [6], SH2 proteins [7], allergens [8-10], antigens [11], proteins involved in various signalling pathways [12, 13] and many more.

Not only cDNA libraries but also peptide libraries are important in elucidating protein interaction networks and signalling pathways (for a review see [14]). Peptide library approaches can be broadly grouped into methods employing either synthetic or encoded libraries. Synthetic libraries are bound to a solid support such as beads or microarrays. In many screening strategies fluorescent receptor proteins are being used. Encoded libraries are usually displayed in bacteriophages.

In most of the studies applying phage display, panning procedures or variations thereof have been used for selecting genes encoding interacting proteins. Several rounds of selection are usually required to end up with a number of phage clones one can handle for characterization. This may result in a loss of rarely expressed genes and of genes encoding proteins having a lower affinity for the bait protein. The latter may be a consequence of truncated or partial cDNAs in the library. Interesting genes may thus be lost. Microspectroscopical methods facilitate the development of new selection strategies based on single-molecule detection and whole-cell imaging resulting in an integrated approach as outlined in the final paragraph.

SINGLE-MOLECULE FLUORESCENCE DETECTION TECHNIQUES

Fluorescence parameters of biomolecules are the quantum yield (*Q*) (or the fluorescence intensity), the lifetime of the excited state (τ), the radiative lifetime (τ_r), the emission and excitation (absorption) spectra, and the anisotropy [15]. All these parameters can be determined in bulk measurements using conventional fluorescence instruments that are mostly commercially available. In contrast, there is also a wealth of information available from single-molecule fluorescence measurements that resulted in novel applications notably in analytical chemistry, pharmaceutical sciences and biotechnology. Here we will highlight Fluorescence Correlation Spectroscopy (FCS) and, in general, single-molecule fluorescence detection techniques, as these techniques can be used to detect sparse, fluorescent molecules in a flow and to measure flow velocities of these molecules.

FCS was introduced in the 1970s as a method for measuring molecular diffusion, reaction kinetics and flow of fluorescent particles [16-19]. The underlying principles of FCS laid the foundation for a whole series of methods that are collectively referred to as fluorescence fluctuation spectroscopy. In the early 1990s we could observe a renewed interest in FCS owing to considerable progress in instrumentation (stable lasers, confocal excitation and detection, avalanche photodiodes, high-speed correlators, faster computers,

etc.) [20], offering novel applications in biotechnology [21, 22].

FCS measurements can be carried out in an optical, confocal microscope. In FCS small spontaneous deviations from thermal equilibrium in an open system are reflected by fluctuations in the fluorescence intensity induced, for instance, by fluorescent molecules diffusing into and out of a well-defined observation volume generated by a focused laser beam. The laser beam continuously illuminates a fixed region within the sample. Although fluorescent particles throughout the excitation volume are excited, only the fluorescence from particles is detected through a pinhole positioned at the image plane of the excitation volume. The observation volume is smaller than the excitation volume and, depending on the size of the pinhole and the magnification of the objective, amounts to less than one femtoliter. The observation volume has an ellipsoidal shape with the long axis being ca. 3-10 times longer than the short axis (this ratio of length and radius of the observation volume is called the structural parameter). The detector is either an avalanche photodiode or a sensitive photomultiplier operating in single-photon counting mode. Generally a pinhole is not required when 2-photon near-infrared excitation is used. The simultaneous absorption of two low-energy photons leads to an excited state and fluorescence only in the very focus of the laser beam. The excitation and observation volumes are then the same and more spherical in shape.

Each time a fluorescent molecule enters the observation volume a burst of fluorescence photons is detected. When diffusion is the only dynamic process causing intensity fluctuations, the duration of this photon burst reflects the time a particle needs to diffuse across the observation volume. Autocorrelation of the intensity trace results in an autocorrelation curve which can be analyzed to yield the average number of particles in the observation volume and the average diffusion time. The diffusion time τ_{dif} describes the dwell time of a particle in the observation volume, which is related to the diffusion coefficient D_{tran} via $\tau_{dif} = \omega_{xy}^2/(4D_{tran})$, ω_{xy} is the distance from the center of the observation volume in the x, y plane at which the detected fluorescence intensity has decreased by a factor e^2. The amplitude of the correlation function, $G(0)$, represents the average number of molecules N found in the observation volume: $G(0) - 1 = 1/N$.

Alternatively, the amplitude of the emission bursts contains information about the molecular brightness of the particle, since bright particles will on average give rise to larger fluorescence bursts than dimmer ones. Let us consider a living cell expressing a receptor-protein construct with GFP in the plasma membrane. The receptor can form dimers in equilibrium with receptor monomers. The dimer containing two GFP molecules will emit twice the intensity as a monomeric receptor. The frequency of fluorescence intensities can be plotted against the time-binned fluorescence intensities. This is known as photon-counting histogram (PCH) analysis [23] or fluorescence-intensity distribution analysis (FIDA) [24]. These concepts have essentially the same meaning and were developed simultaneously and independently. Analysis of this PCH yields the molecular brightness and the number of the particles. The molecular brightness can be defined as the number of detected fluorescence photons per molecule per second. In case of the presence of dimeric receptor species in equilibrium with monomeric ones one should observe a PCH of two species and their relative concentrations.

Fig. (**1**) gives a pictorial view of the time- and amplitude-dependence of photon burst detection of single molecules in a focused laser beam.

Besides measurements of local concentrations, diffusion times and brightness values, fluorescence fluctuation spectroscopy is capable of observing a whole range of other dynamic processes that give rise to fluctuations in the fluorescence signal. Comprehensive reviews [25-27] and a book [28] dedicated to this technique clearly demonstrate its versatility.

Because of its small, confined detection volume and its large sensitivity FCS is especially suitable for measurements in microfluidic devices. It is therefore not surprising to see that FCS has been used to determine flow profiles and flow speeds of fluorescent molecules and particles in microcapillaries or microstructured channels [29-31]. The theoretical framework for FCS analysis of transport by flow, superimposed on transport by diffusion, has been given in [19]. When there is active transport in the form of laminar flow, the autocorrelation function $G(\tau)$ also contains a 'flow' component with a characteristic time τ_{flow}, that is the average flow time of the fluorescent particles through the detection volume [29, 32]. The flow velocity v is given by $v = \omega_{xy} / \tau_{flow}$.

The minimum flow velocity that can be measured is determined by Brownian diffusion. When the flow velocity is too small, it becomes difficult to distinguish between diffusion alone and diffusion superimposed on flow. Therefore, τ_{flow} must be distinctly shorter than the diffusion time, τ_{dif}, in order to recover the flow velocity from the analysis. In our facilities at Wageningen University the recorded autocorrelation curves are analyzed using in-house developed global analysis software enabling the determination of parameters τ_{dif} and τ_{flow} (see [30] for details). In Fig. (**2**) we present an example of autocorrelation traces of *E. coli* bacteria expressing Yellow Fluorescent Protein (YFP) and the recovered flow velocity from the analysis [30].

Although analysis of FCS curves allows accurate determination of flow velocities, the flow direction of particles being transported through the observation volume cannot be established exactly. A few groups have circumvented this problem by employing two focused laser beams that are spatially separated by a defined distance and by cross-correlating the respective emission signals [32, 33]. In case of directed flow, each single fluorophore successively passes the two focused beams aligned in the direction of the flow and resulting in a cross-correlation curve with a distinct maximum that corresponds to the transition time between the volumes.

Of course, one can determine the flow rate of fluorescent particles in a continuous liquid flow very accurately from FCS analysis (see Fig. (**2**)). However, if one wants to use flowing, fluorescent particles for sorting and deflection in a microfluidic device, there is simply no time for generating a

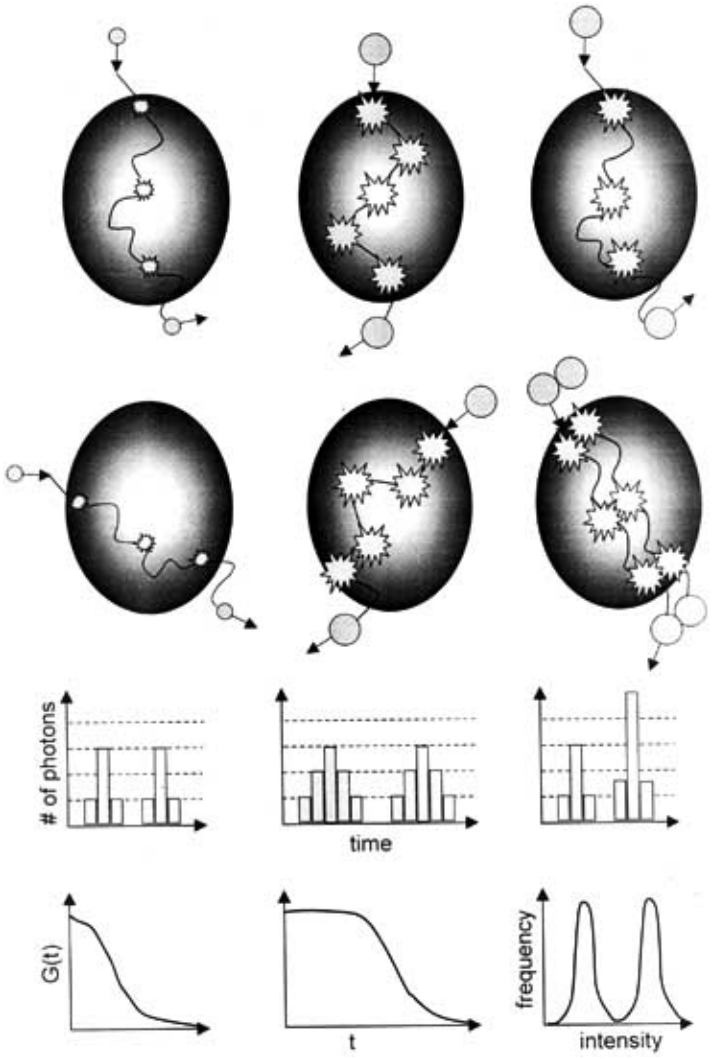

Fig. (1). Schematic view of photon burst detection and analysis of single fluorescent molecules in a focused laser beam.

good signal-to-noise FCS-curve (note that the autocorrelation traces in Fig. (**2**) were measured during one minute). Therefore, one should simply capture the burst of emission photons when fluorescent molecules transit a focused laser beam. Since almost two decades large progress has been made in the detection of single fluorescent molecules in liquids at ambient temperature (Keller's group in the USA has made pioneering efforts as reviewed in detail in [34]). Let us first estimate the number of photons that can be detected from a single fluorescent molecule that transits a laser beam tuned to an optical transition. During the transit time in the beam the molecule undergoes cycles of photon absorption and emission giving off a photon on most cycles. The maximum number of photons emitted in a burst is approximately equal to the transit time divided by the fluorescence lifetime. For a transit time of 1 ms and a lifetime of 1 ns this maximum number is 10^6. In practice, however, photodecomposition limits this number to ~10^5 photons even for very stable molecules. The detection efficiency of the optical microscopic systems used in single-molecule studies amounts to ~1%. Therefore we expect a burst of ~1000 photons when a single, strongly fluorescent molecule crosses the laser beam. An example of a photon burst emitted by *E. coli* bacteria (containing many YFP copies) flown through a micro-capillary is given in Fig. (**3**) [30]. In this example the minimum bin time in which the photons are counted and stored is 13.5 ms (this time is limited by the electronics of the commercial instrument). In this relatively long bin time bursts as high as 10^6 photons/s can be observed. When the bacteria were flown through the capillary with a velocity of $5x10^{-2}$ $m.s^{-1}$, photon bursts of $5x10^4$ photons/s were observed corresponding to ~675 photons counted per bin. This number is based on the average of many particles and is not representative for the photon burst of a single particle. We can estimate the number of passing particles through the laser beam from the lower panel of Fig. (**3**). The average photon intensity was 27000 photons/s. When we assume that one particle emits 100 photons, ~270 particles/s are passing through the laser beam, corresponding to 3.6 particles counted in one bin.

Several other groups have reported on detection of single molecules in microstructures (see, among others, [35-41] and even in submicrometer-sized fluidic channels [42].

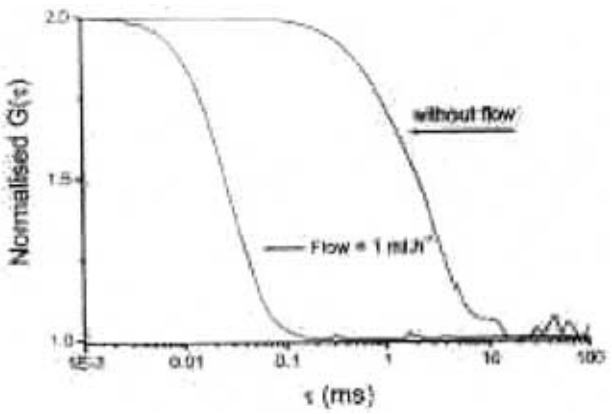

Fig. (2). Normalized autocorrelation traces of *E. coli* bacteria containing EYFP with and without flow measured during a one-minute measuring period (adapted from [30] with permission of the American Chemical Society).

There is considerable interest in using microfabricated fluidic devices as a tool for microflow cytometry (or micro-fluorescence activated cell sorter, μFACS). The particles are transported electrophoretically by applying potentials to the fluid reservoirs. For interrogation of the fluorescent particles use is made of on-the-fly detection of fluorescence photon bursts. After burst detection the particles are deflected in another channel by changes in the applied high-voltage settings. The groups of Quake and Ramsay have made important contributions in this field [43-45]. Fluorescent bacteria could be separated from a background of non-fluorescent bacteria. Further details are given in the next section.

MICROFLUIDIC DEVICES

In the last couple of years microfabricated devices have shown a plethora of novel bioanalytical applications such as macromolecular separation, biomolecular sensing and biochemical assays. They all have in common the downscaling of various (bio)chemical processes, which comprise reaction kinetics, separation of reaction products, detection of biomolecules (sensing) and applications involving cells (cellomics). Microfluidic devices are composed of micrometer channels and microliter reservoirs that are capable of transferring and storing tiny amounts of liquids in volumes of nano- and picoliters. Integrated microfluidic systems combine channels of microscopic geometry with miniaturized pumps, mixers, valves, electric components and light detectors. Such integrated systems are known as Micro Total Analysis Systems (μTAS) or 'lab-on-a-chip' systems in which it is in principle possible to automate a complete analytical process sequentially from sample preparation, reaction, to separation and detection. Many aspects of μTAS are highlighted in recent literature. Fundamental technical issues still need to be solved before the devices can be industrialized [46]. The

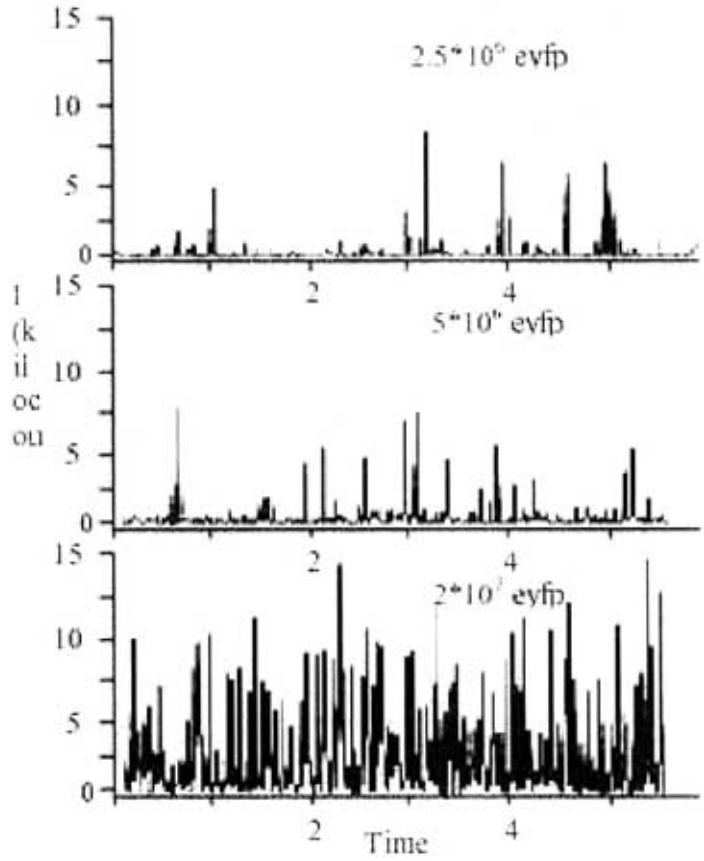

Fig. (3). Influence of the concentration of *E. coli* bacteria containing EYFP on the intensity at a flow rate of 1 $ml.h^{-1}$ (adapted from [30] with permission of the American Chemical Society).

fluid behavior in microchannels is completely determined by diffusion making equilibrium mixing (quadratically) faster with smaller sized channels [47]. Considerable details of μTAS have been reviewed recently [48, 49]. Chovan and Guttman [50] have summarized the application of microfabricated devices in biotechnology and bioprocess engineering. Very recently, Andersson and van den Berg [51] have published a comprehensive review on the use of microfluidic devices for manipulation of single cells (sampling, trapping, sorting, lysis, poration, fusion: in short cellomics).

Miniaturization offers various advantages over macroscale laboratory operation: (i) reduced sample volume and less reagents thereby reducing the costs of reagents, (ii) faster reactions and reagent mixing are possible, (iii) superior heat and mass transfer eliminating thermal side-effects, (iv) more accurate measurements, (v) low-cost compact system design in which more functionalities can be integrated or operated in parallel, (vi) as a result of (v) it is foreseen that microfluidics technology can be mass-produced, (vii) development of miniaturized high throughput screening systems [52, 53].

Much progress has been made in the development of materials needed to make microfluidic devices and the various ways to direct liquid flow inside microchannels. Soft lithography using elastomeric materials has been shown to be an alternative to standard photolithographic and wet chemical etching including the fabrication of valves and pumps [54, 55]. The fluid is usually driven by pressure or vacuum or by electrical means (electro-osmotic flow or electrophoresis). The direction of fluid flow can be diverted by various methods such as mechanical valves or by changing voltage gradients. Flow control can be performed by using microfluidic valves of hydrogels in microfluidic channels in glass substrates that open or close depending on the pH of the solution [56]. The latter research group has been experimenting with surface-directed flow that allows aqueous liquids to be confined to hydrophilic pathways flanked by hydrophobic 'walls' inside microchannels [57]. With the advantage of having no moving parts such as pumps and valves, electrocapillary pressure as a technique to drive the flow along microchannels has been developed [58]. When working with cells glass microchannels have been coated with poly(dimethylacrylamide) to minimize cell adsorption [45]. Although electro-osmotic flow was inhibited in the latter case, the negative charge of the cells still allowed electrophoretic transport. Nanofluidic (instead of microfluidic) devices will be realized in the very near future owing to improvement in fabrication processes and advances in nanoscale sensing and actuation [55, 59].

In addition to microfabricated cell sorting devices described above [43-45], some other microtools for handling cells have been developed. Confinement of cells is based on dielectrophoresis that refers to the force on induced polarization or dipole charges in nonuniform electric fields [60]. Dielectrophoretic sorting of particles and cells has been performed in a system that operates in three stages: a beam-narrowing device for funneling and aligning the flow, a field cage to trap cells and a switch to direct particles in an output channel [61]. This 3-D microelectrode system for funneling, aligning, caging, switching has been further improved [62]. A microfabricated dynamic multi-trap array cytometer for use in parallel single-cell assays has been recently described [63]. When cells are introduced into the array of traps, it turned out possible to sort cells upon the basis of fluorescent dynamic (functional) responses to stimuli.

FUTURE DEVELOPMENTS: AN INTEGRATED APPROACH

The described developments in the fields of cell and molecular biology (biolibraries), (bio)physics (single-molecule fluorescence detection) and nanotechnology (microfluidic devices) can be integrated. This will lead to detection systems with an, as yet, unsurpassable sensitivity. Larocca and coworkers [64] have, for instance, developed an approach wherein bacteriophages are genetically modified to transfect mammalian cells. In this approach one uses genetic fusions between (characterized) cDNAs and fluorescent proteins that are expressed in the cell and can even be targeted to subcellular compartments. By combining this with appropriate imaging technologies and analysis software one can in a high-throughput fashion obtain direct information on gene expression patterns and use that information for cell sorting.

Another possibility comes from single-molecule fluorescence detection technologies. These offer new possibilities for the selection of phage displayed peptide and cDNA libraries. Upon binding of the phage to a fluorescent bait on a larger particle the diffusion time will considerably increase and the burst of photons will last longer when the fluorescent particle transits the focused laser beam. Thus, photon-burst detection methods can be developed for instance in combination with micro- or nanofluidics devices. For library versus library screening one can use phages that are labeled with two different fluorescent labels and detection of coincidence photon bursts [32, 65]. As demonstrated in [65] this method allows the detection of 50-100 fM of dual-labeled DNA in the presence of a 1000-fold excess of DNA labeled with a single fluorophor. We have recently developed a prototype microfluidic platform that allows sorting one- or two-colored fluorescent particles.

Screening cell arrays [3] can already be accomplished using various imaging approaches. The speed can possibly be improved by developing better steering electronics and light collection optics. However, developing appropriate control and on-line analysis software is of equal importance in this field to speed up the processing of data that come to thousands of genes expressed in this way.

Taken together, important developments are foreseen for a more rapid and reliable screening and sorting of biolibraries. This is necessary in this era of post-genomics where a rapid translation of gene sequences into gene and even cell function is of increasing importance. This not only allows us to rapidly process the wealth of information coming from the numerous genome-sequencing efforts, both those undertaken and under way, but also helps us to acquire insight in the functioning of protein machines and, as a consequence, cells.

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