Micro- and nanofluidic systems for high-throughput biological screening

Jongin Hong[1,2], Joshua B. Edel[1,2] and Andrew J. deMello[1]

[1] Department of Chemistry, Imperial College London, South Kensington Campus, London SW7 2AZ, United Kingdom
[2] Institute of Biomedical Engineering, Imperial College London, South Kensington Campus, London SW7 2AZ, United Kingdom

High-throughput screening (HTS) is a method of scientific experimentation widely used in drug discovery and relevant to the fields of biology. The development of micro- and nanofluidic systems for use in the biological sciences has been driven by a range of fundamental attributes that accompany miniaturization and massively parallel experimentation. We review recent advances in both arraying strategies based on nano/microfluidics and novel nano/microfluidic devices with high analytical throughput rates.

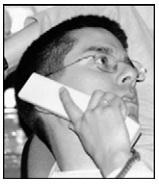
Introduction

Recent advances in genomics, proteomics, cellomics and metabolomics have provided an opportunity for the pharmaceutical industry to accelerate the pace of drug discovery using high-throughput techniques [1–11]. The sequencing of the human genome has generated several new molecular targets with unknown functions as well as those with known functions. Rapid progress in proteomic research has begun to enable an understanding of the physiological and metabolic pathways of cells, the cultivation of disease therapies and the discovery of new small-molecule drug candidates. Cellomics and metabolomics provide more comprehensive pictures of cells under complex physiological parameters. The increase in experimental complexity demands the organization, interpretation and utilization of experimental data in a methodical and rational manner and thus high-throughput screening (HTS) technologies have emerged to meet this demand. Current microplate-based HTS methods typically involve robotics, liquid handling devices, sensitive detectors and software for data processing and control. The combination of these components in theory allows many thousands of biochemical, genetic, proteomic or pharmacological tests to be performed in a parallel. Furthermore, HTS methods facilitate the understanding of human biological pathways that require large sets of experimental data to study genes, proteins and metabolites. The primary route to saving time and minimizing usage of biological samples involves miniaturization of existing technologies such as high-density wells on assay plates and nanoliter dispensing systems [8,10]. However, current robotic systems are burdened by several issues such as high costs, poor reliability of data, standardization of data types, rapid and accurate dispensing of very small liquid volumes and uncontrolled evaporation of dispensed liquids [10]. Moreover, current microplate-based HTS methods are generally

Corresponding author: deMello, Hong, J. (j.hong@imperial.ac.uk), A.J. (a.demello@imperial.ac.uk)

Jongin Hong
Jongin Hong is a postdoctoral research associate in the department of chemistry at Imperial College London. He received his PhD from Korea Advanced Institute of Science and Technology (KAIST) in 2005. Subsequently, he held postdoctoral training at Korea Institute of Science and Technology (KIST) and then a postdoctoral fellowship at the University of Pennsylvania in 2006. Dr Hong has published over 20 research papers in refereed journals and 3 book chapters. He has been engaged in the development of new instrumental platforms based on nano/microfluidics, nanophotonics and nanoelectronics to resolve chemical and biological problems. The focus of his current research involves developing droplet-based microfluidic systems for high-throughput biology and creating novel solid-state nanopore systems for single-molecule spectroscopy.

Joshua B. Edel
Joshua B. Edel has been appointed to a joint lectureship between the Institute of Biomedical Engineering and Department of Chemistry at Imperial College London since 2006. He received his PhD in Physical Chemistry at Imperial College London in 2004. Subsequently, he held postdoctoral training in Nanotechnology at the Cornell University with Prof. Harold Craighead and then a postdoctoral fellowship in the Rowland Institute at the Harvard University in 2005. Dr Edel has received numerous research awards and published over 30 research articles, 15 conference proceedings, 1 book chapter and 4 patents to his name. His core research focus is in the development of nanofluidic devices to study and further understand biophysical systems at the single-molecule level.



Andrew J. deMello
Andrew J. deMello has been on the faculty of the Chemistry Department at Imperial College London since 1997 and is currently a professor of Chemical Nanosciences. He obtained his PhD in Molecular Photophysics at Imperial College London in 1995 and subsequently held a post-doctoral fellowship in the Department of Chemistry at the University of California, Berkeley before returning to the UK. His current research programs are centered on the areas of miniaturized chemical analysis systems and ultra-high sensitivity detection. Prof. deMello has given over 120 invited lectures in the USA, Europe and Asia, has published over 100 research papers in refereed journals, and coauthored a book. He is also a cofounder and director of the Imperial College Spin-out company Molecular Vision Ltd. He has received multiple awards including the SAC Silver Medal by the Royal Society of Chemistry (2002) and the Clifford Paterson Lectureship and Medal by the Royal Society (2008).

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inaccessible to academic investigators owing to significant instrument and maintenance costs. Most industrial applications of HTS target a relatively small number of disease-relevant pathways and proteins, whereas many academics wish to use HTS methods to study a wide variety of biological pathways in diverse organisms.

As noted, the development of micro- and nanofluidic systems for use in the chemical and biological sciences has been driven by a range of fundamental attributes that accompany miniaturization. These include the ability to handle small volumes of samples and reagents, low fabrication costs, reduced analysis times, operational flexibility and easy automation and facile integration of functional components [11–36]. Such systems can be used to probe physical phenomena and mechanisms that are not observable on a macroscopic scale. Microfluidic systems manipulate or process tiny volumes of fluids in channel whose dimensions are most conveniently measured in tens or hundreds of micrometers. One key feature enabled by micromachining methods is the integration of different functional components such as sampling, sample pretreatment, sample transport, biochemical reactions, analyte separation, product isolation and detection. Accordingly, in theory, microfluidic systems enable serial processing and analysis and, furthermore, can accomplish massive parallelization through efficient miniaturization and multiplexing [17–36]. Extensive details on the physics governing microfluidic system can be founded in several excellent review articles elsewhere [12–15].

More contemporary research efforts have started to address the scaling down of microfluidic systems to create features with cross-sectional dimensions most conveniently measured in nanometers. These nanofluidic devices herald new opportunities for fundamental studies of biological phenomena [37–43]. One of the most important aspects of nanofluidics is an ability to confine and probe molecules at the single-molecule level. This contrasts with conventional approaches that study large molecular populations. Consequently, micro- and nanofluidic technologies seem to be an emerging opportunity to develop new platforms that may resolve many of issues faced in current HTS and offer the possibility of significant technological breakthroughs in the drug discovery process. Herein, we review some recent developments and applications of surface patterning using micro- and nanofluidics and novel micro- and nanofluidic devices exhibiting high analytical throughput.

Drug discovery and modern day HTS systems

Drug discovery entails a series of serial and/or parallel operations that include target selection, hit identification, lead optimization, compound synthesis, characterization, screening, assays for therapeutic efficacy and preclinical studies in animals [1–11]. Both natural product and chemical libraries are screened to determine which compounds interact with a given target. Successful compounds (called hits) exceeding a certain threshold value in a given assay are progressed into leads, which are confirmed by a more detailed assessment of chemical integrity, accessibility, functional behavior, structure–activity relationships (SARs) as well as biophysicochemical and absorption, distribution, metabolism and excretion (ADME) properties. The lead components that demonstrate activity and selectivity in the secondary screens are tested in progressively more complex systems that include cells and whole animals before reaching clinical trials. At this point, only a few drug candidates continue into the drug development stage for clinical trials, manufacturing and product management. Therefore, robust, rapid and efficient methods are required to vet an extremely large number of initial candidates and thus minimize the attrition of chemical entities in the costly clinical phase of drug development. These demands have encouraged the advancement of microplate-based HTS technologies including small molecule library design and assembly, robotics, assay development and data handling. Today, libraries containing millions of compounds are routinely screened with single compounds of high purity in 384-, 1536- and 3456-well formats.

Microchip technologies offer other platforms for HTS, such as microarrays and microfluidic devices. Microarrays allow the simultaneous analysis of thousands of chemical entities within a single experimental step and have become popular tools in drug discovery and life science research [44–49]. They are normally composed of biomolecules attached to a planar surface in defined locations. Biomolecules commonly immobilized on microarrays include oligonucleotide [45–47], polymerase chain reaction (PCR) products [45,46], proteins [44], lipids [47,48], peptides [44,47] and carbohydrates [49]. Currently, high-density microarrays can be purchased or custom fabricated. During the past few years, a variety of chemical compound microarrays with different surface chemistries and activation strategies have emerged.

By contrast, microfluidic devices can perform screening assays in a continuous fashion. These devices require a series of generic components for introducing reagents and samples, moving fluids within a microchannel network, combining and mixing reactants. To date, the microfluidic community has focused on demonstrating the efficacy of such components and integrating them into monolithic devices. More details on microfluidic devices and their development can be found in several excellent reviews [17–36]. It should also be noted that custom-made microfluidic chips and entire measuring systems are now commercially available. For instance, Tecan's LabCD-ADMET™ system is a miniaturized 'turn-key' system for the full automation of ADMET assays. The GyroLab™ workstation shares many of the benefits of the LabCD and is a flexible benchtop platform that automates almost every assay step from sample application to detection. Furthermore, SpinX Technologies' LabBrick aims to automate all steps involved in optimizing and running assays, using a 500-nl reagent volumes in a closed system.

The testing of analytes on living cells is also an important part of high-throughput drug discovery. Microfluidic devices enable manipulation of cells [22,26], maintenance of cellular environments close to physiological conditions typically found in biological systems [22,25], simultaneous characterization of cells under comparable conditions [30–32], and subsequent analysis. A fundamental paradigm shift in drug discovery, coined high-content screening by Cellomics, has motivated the development of cell-based high-throughput assays to analyze individual cells and subcellular processes under complex physiological conditions [1,3,7,8,10,11,25]. More recently, because of the long diffusion times and Taylor dispersion limitations associated with single phase microfluidic flow [21,27,36], the creation of microfluidic systems that exploit flow instabilities between immiscible fluids (to generate sub-nanoliter droplets in an immiscible carrier fluid) has defined a new experimental platform for performing a diverse

range of biological processes [27–29,33–36]. In particular, localization of reagents within discrete droplets is an effective way of suppressing dispersion of reacting volumes and allowing precise definition of reaction or incubation times in a bioreactor. This precision combined with rapid and reproducible droplet formation renders microdroplet technology an ideal platform for HTS. Nowadays, it is fair to say that nanotechnology is a dominant theme in the pharmaceutical drug discovery and development. The similarity between the typical dimensions of nanoscale components and the size of macromolecules, such as DNA, RNA or proteins, make nanofluidic devices powerful tools for genomics or proteomics. The ability to analyze biological systems at the single-molecule level opens new ways of investigation that are simply not accessible using techniques that measure averaged properties of molecular populations. Among single-molecule analysis methods, the use of nanometer-sized pores and holes (or wells) can provide a level of confinement that allows exploration of discrete molecular phenomena, while operating at extremely high analytical throughput and with perfect molecular detection efficiencies [37–43]. The unique feature of fluids within micro- and nanofluidic networks can provide insights on new ways to resolve the current challenges of HTS and to revolutionize all stages in drug discovery and development.

Novel surface patterning

Contact and noncontact printing technologies

The success of microarray technology in genomic and proteomic research has given new impetus to miniaturization methods that facilitate evaluation of a large number of chemical structures against hundreds of biological targets. Key parameters include the number of different probe sites (spots) per unit area and the number of probe molecules per unit area within an individual probe site. The probe sites and their spacing should be as small as possible, while allowing efficient molecular recognition. Spots can be formed using either pins or pen-like devices loaded with the appropriate solute and solvent (called contact printing) [50–56] or inkjet or other droplet formation technologies to dispense very tiny drops onto a surface (called noncontact printing) [56–60]. An exciting extension of the pen concept to micro- and nanoscale cantilevers is direct-write dip-pen nanolithography (DPN) introduced by Mirkin and coworkers (Figure 1a) [50–52]. In the DPN process, molecular inks coated on an AFM tip are transferred to a substrate, while the tip is held or laterally moved along a surface. Features sizes less than 15 nm can be routinely patterned by molecular self-assembly of the ink on the substrate. To increase the throughput of DPN, two different approaches have been used. The first involves individually actuated tips (by piezoelectric, thermoelectric and electrostatic mechanisms), and the second utilizes massive numbers of passive tips [51]. Espinosa and coworkers [53–55] have demonstrated a novel microfluidic AFM probe called the nanofountain probe (NFP) which possesses sub-100 nm patterning capability. It consists of a hollow tip, integrated microchannels and an on-chip reservoir (Figure 1b). When an ink solution is fed into the reservoir, it is driven by capillary action through the microchannel to the tip to form a liquid–air interface around the tip core. Molecules are transferred by diffusion from the interface to a substrate and a water meniscus is formed by capillary condensation. Recently, the same group demonstrated a multi-ink linear array of NFPs containing 12 cantilever probes [55]. In addition, a nanodispenser can be created by modifying a commercially available AFM probe. Focused ion beam (FIB) milling has been used to open a nanoscale aperture at the tip apex and to make a loading area for the liquid [56]. Meanwhile, Henderson and coworkers have commercialized multiple microcantilevers with open microfluidic channels (Nano eNabler^{TM} system, Bioforce Nanosciences Inc., Ames, IA, USA) constantly delivering a supply of biological and nonbiological liquids onto the surface for multiplexed biomolecular arrays. Upon contact with the surface, a small volume of liquid at the end of the cantilever (and held in a gap by surface tension) is directly transferred to the surface in an event typically requiring less than 1 ms. Capillary fluid flow instantly replenishes the volume at the gap, and the device is then ready to write the next feature. However, friction and resulting mechanical wear between the pen and the substrate during drawing limits spatial resolution and throughput. Massively parallel probe arrays and the integration of multiple reservoirs over large areas with multiple inks are still the key challenges for HTS applications.

By contrast, inkjet and other noncontact droplet formation technologies are able to dispense nanoliter-sized drops directly onto surfaces. Attractive features include additive operation, the ability to process a diverse classes of materials such as fragile organics or biological materials, flexible fluidic drawing through software-based printer-control systems, large-area printing and low-cost operation. However, they offer no control once the liquid has left the confinement of the ejection nozzle and, consequently suffer from issues related to drying and spreading of the ink on the surface and a coarse resolution (no smaller than 20–30 μm). Scanning ion conductance microscopy (SICM), developed to scan soft nonconducting materials using an electrolyte-filled nanopipette, has been used to write biological materials to surfaces potentially down to the single-molecule level (Figure 2a) [57]. The distance between a pipette and a sample is controlled by the ion current flowing between an electrode inside the nanopipette and an electrode in the bath. In more recent times, electrohydrodynamic jet (e-jet) printing has been shown to be a promising technique to create the fluid flows necessary for delivering inks to a substrate using a voltage applied between a nozzle and a conducting support substrate [58–60]. This approach has been explored for high-resolution patterning within the submicron range. Rogers and coworkers introduced a gold-coated nozzle functionalized with a hydrophobic self-assembled monolayer to minimize the probability for clogging and/or erratic printing behavior. Using such an approach with computer-coordinated control of the power supply and stages enables printing of complex patterns including digitized graphic images or circuit layouts (Figure 2b). To guarantee success using printing technologies, a perfect manipulation of chemical and biological material must be realized. This requires the use of surfaces or materials, which can adsorb, desorb, bind or prevent adsorption of such substances in localized regions, combined with the ability to switch between processes on demand or upon activation by a defined stimulus. A detailed discussion of these issues is beyond the scope of this review.

Microchannel- and microwell-based patterning

Passive forces such as capillary forces in microfluidic systems provide a means to create a pressure difference in small conduits.

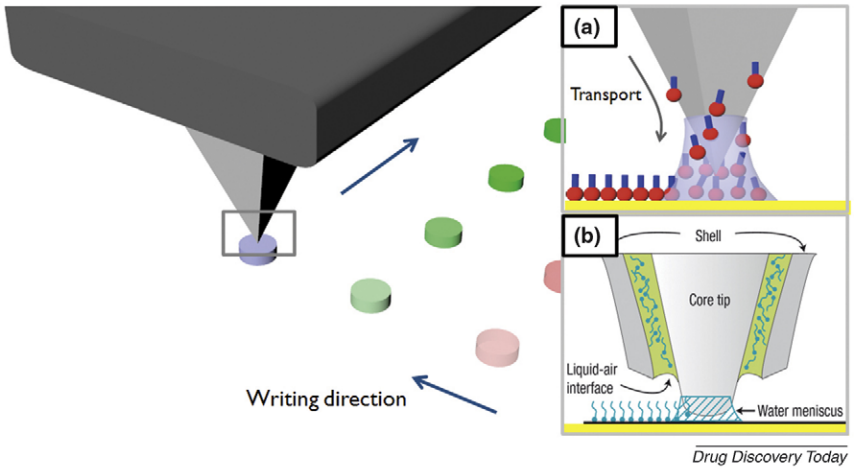

FIGURE 1

Novel surface patterning tools: (a) dip-pen nanolithography (DPN) and (b) nanofoundation probe (NFP) (reproduced with permission from [51], Copyright 2007 Nature Publishing Group).

Using this idea Delamarche *et al.* [61] proposed a new format for surface patterning and biological assays. In this, fluids fill a microfluidic network (μFNs) owing to the capillary pressure generated by the small dimensions of the channels and the hydrophilicity of their walls. One application of μFNs is shown in Figure 3a. A μFN patterns a series of antigens as narrow stripes onto a planar substrate. After a blocking step with bovine serum albumin (BSA), the antigens in each line may be recognized by specific analytes from a sample solution which is guided over the substrate using a second μFN. Figure 3a (right) shows the feasibility of such systems for HTS. The usage of $n \times m$ microchannels yields $n \times m$ binding sites, but only requires $n + m$ pipetting steps. The authors claim that the format necessitates a 10-fold reduction in analysis time, 100 times less sample and reagent, and a 1000-fold reduction in the area per site compared to the conventional 1536-well plate. Recently, similar approaches have been developed to screen for biological analytes in a combinatorial fashion and using various detection schemes [61]. Meanwhile, Khademhosseini *et al.* have developed structures to generate regions of low shear stress within microchannels that enable docking of various bioentities [62]. This approach has been used to seed multiple cell types (e.g. hepatocytes, fibroblasts and embryonic stem cells) inside different microwells. Reversible sealing of PDMS moulds enables sequential delivery of chemicals to each well (Figure 3b). In addition, conventional high-density microwell plates can be replaced with nanoliter through-hole arrays (OpenArray™, BioTrove Inc., Woburn, MA, USA). A series of vapor and liquid deposition steps covalently attaches a polyethylene glycol (PEG) hydrophilic layer (amine-coupled to the interior surface of each through-hole), and a hydrophobic fluoroalkyl layer to the exterior surface of the plate. Capillary pressure draws fluids into microchannels and surface tension holds the liquids in place and keeps them isolated from neighboring channels. The differential hydrophilic–hydrophobic coating facilitates precise loading and isolated retention of fluid in

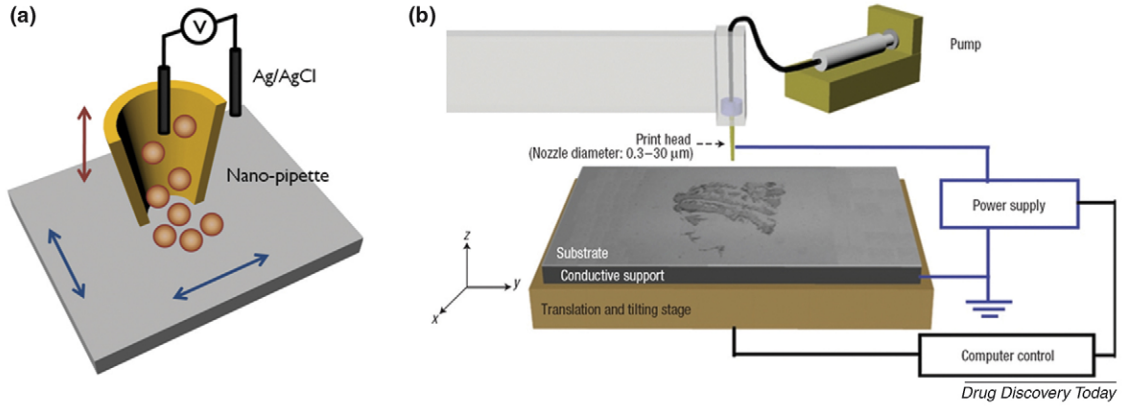

FIGURE 2

Schematic of (a) a nanopipette-based dispensing technique and (b) a high-resolution e-jet printer (reproduced with permission from [60], Copyright 2007 Nature Publishing Group).

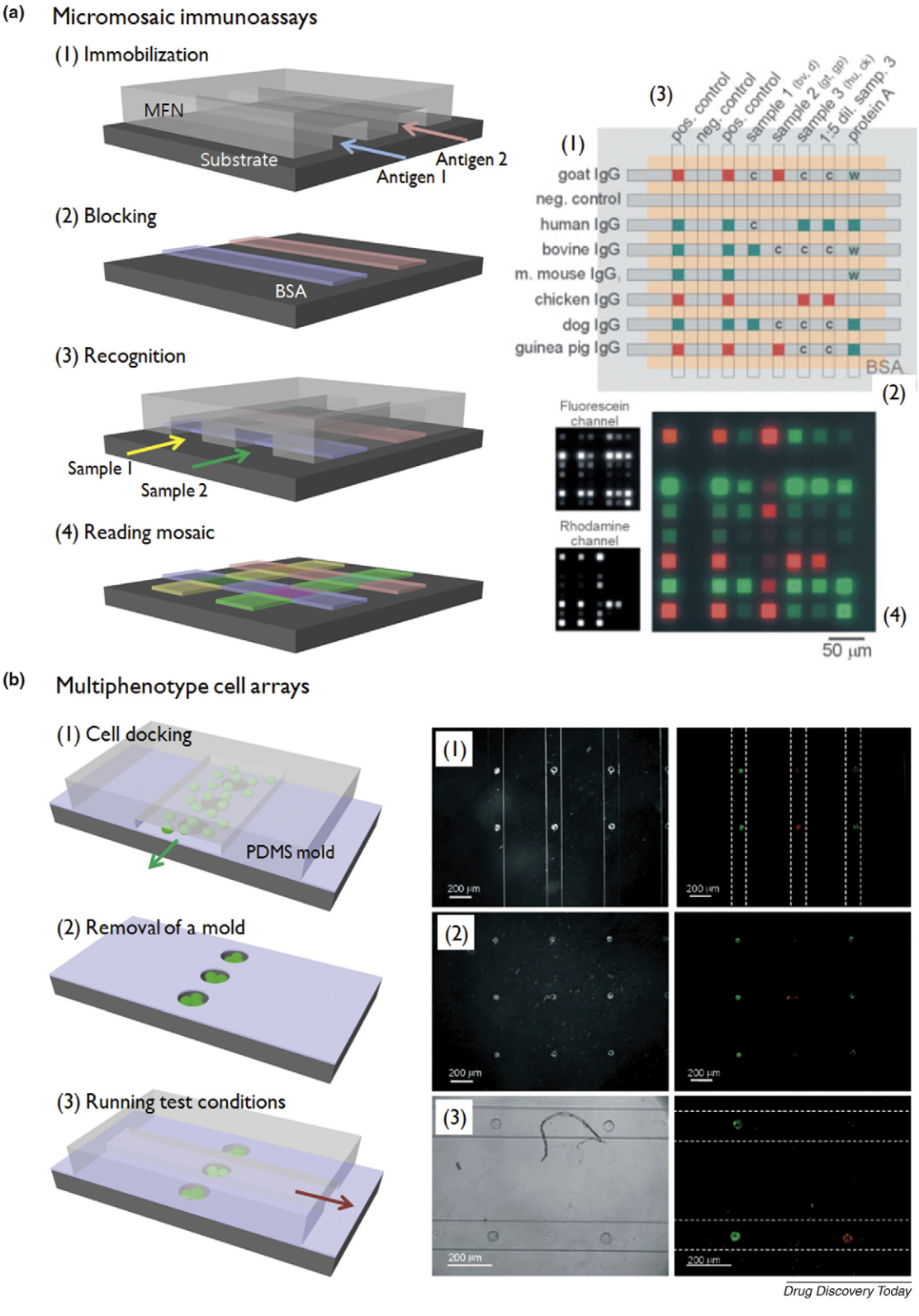

FIGURE 3

(a) Surface patterning based on microfluidic channel and combinatorial micromosaic immunoassays. The device is used to interrogate the binding between surface-immobilized receptors with fluorescently labeled antibodies (reproduced with permission from [61], Copyright 2005 Wiley–VCH Verlag GmbH and Co. KGaA) and (b) cell-docking within microchannels containing microwells. Multi-phenotype cell arrays are fabricated by reversible sealing of PDMS molds onto microwell patterned substrates (reproduced with permission from [62], Copyright 2005 The Royal Society of Chemistry).

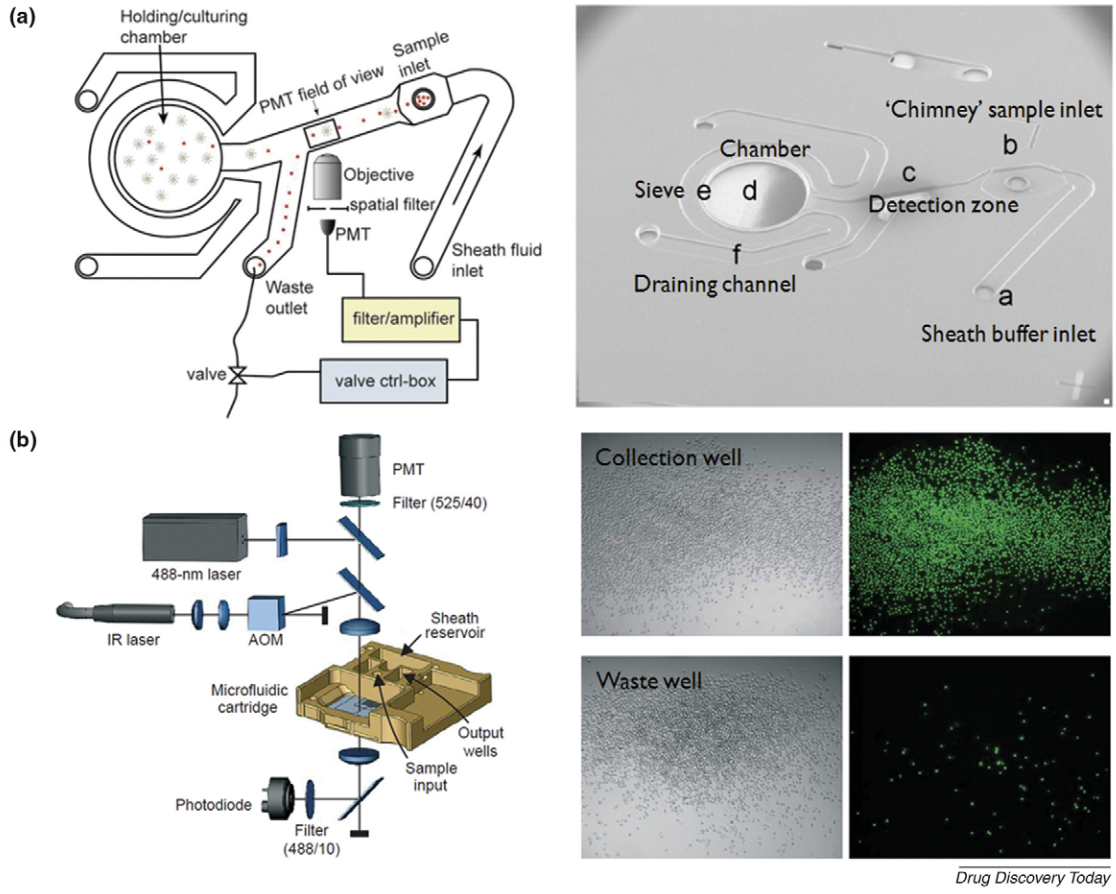

FIGURE 4

High-throughput microfluidic cell sorting: (a) a microcell sorter chip with integrated hold/culturing chamber (reproduced with permission from [77], Copyright 2007 The Royal Society Chemistry) and (b) an optically switched microfluidic fluorescence-activated cell sorter. GFP-expressing HeLa cells are sorted to the collection cell (reproduced with permission from [80], Copyright 2007 Nature Publishing Group).

each channel. This platform can reduce the capital cost of thermocycling and plate reading, labor costs, reagent consumption and analysis times when compared to microwell-based HTS systems.

Novel microfluidic devices

One key feature of microfluidic systems is the integration of different functional units for sampling, sample pretreatment, sample transport, biochemical reactions, analyte separation, product isolation and analysis in a continuous flow manner. Two particular developments in microfabrication techniques have accelerated the creation of integrated microfluidic systems. One is soft lithography using elastomeric polymers, such as polydimethylsiloxane (PDMS). PDMS is an inexpensive, flexible, optically transparent (down to 230 nm) material, which is compatible with biological studies [63–67]. This fabrication approach allows quicker, less expensive creation of prototype devices. The second development relates to simple methods for fabricating pneumatically actuated valves, mixers and pumps by multilayer soft lithography (MSL) [68–72]. Monolithic elastomer actuators restrict fluid flow in a channel by the pressurization of an adjacent channel. When pressure is introduced into 'control' channels, elastic membranes expand into the fluidic channel. A regular valve having a round-profiled fluidic channel allows complete sealing of a channel, while a sieve valve having a square-profiled fluidic channel offers partial closure. A peristaltic pump with three elastomer valves works by applying air pressure in a sequential fashion [73]. Such elastomeric actuators have enabled the design and realization of large-scale integrated microfluidic systems for high-throughput processing of smaller volumes with higher degrees of parallelization [68–70].

Miniaturized fluorescence-activated cell-sorting (μFACS)

Much effort has focused on developing miniaturized fluorescence-activated cell-sorting (μFACS) devices with high-throughput rates [74–84]. For example, Wolff *et al.* designed and developed a novel pressure-driven μFACS device with advanced functional components including a chamber for holding and culturing sorted cells and monolithically integrated waveguides for cell analysis (Figure 4a) [77]. They reported a 100-fold enrichment of fluorescent beads at a throughput of 12,000 cells per second while sorting a mixture of fluorescent latex beads and chicken red blood cells. In addition, Wang *et al.* demonstrated a high-throughput cell sorter with an 'all-optical' control switch for living cells (Figure 4b) [80]. Optical forces, which depend on the optical power and relative

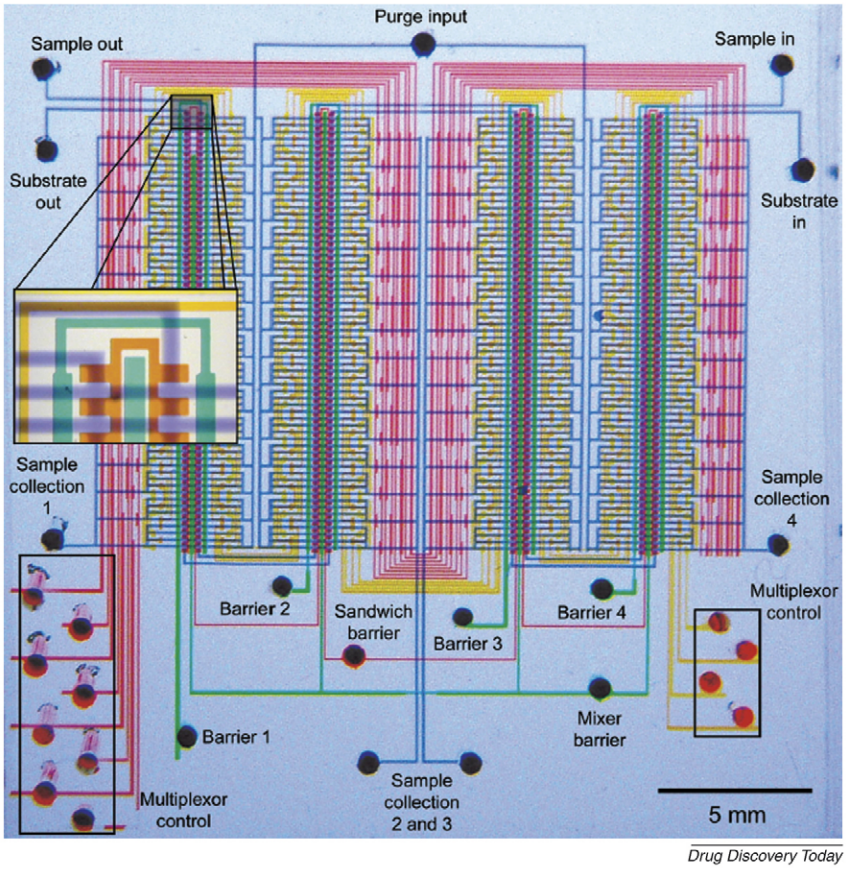

FIGURE 5

Microfluidic large-scale integration: optical micrograph of a microfluidic chip for parallelized high-throughput screening of fluorescence-based single-cell assays (reproduced with permission from [70], Copyright 2007 Nature Publishing Group).

optical properties of the particle and its surrounding medium, were used to deviate the cell from the flow stream towards the target output channel. The authors showed that sorting runs of cell populations ranging from as few as 1000 cells up to 280,000 cells can be completed in less than an hour. Cheung *et al.* have also developed a microfabricated impedance flow cytometer that incorporates deflective dielectrophoresis barriers and controlled pressure-driven liquid flows, and characterizes cells by the ratio of impedance at a high frequency to that at a low frequency [81]. More detailed information on novel chip-based flow cytometers can be found in Ref. [16].

Microfluidic large-scale integration for multiplexed systems

Multiplexed analysis is accompanied by several challenges including molecular encoding/decoding and the need to assay sensitivity, specificity and reproducibility in complex mixtures. Thorsen *et al.* have made enormous strides in multiplexing technology with MSL and developed elastomeric PDMS devices with thousands of valves and hundreds of individually addressable chambers [69]. They demonstrated a microfluidic system for parallel HTS which integrated 2056 valves with 256 compartments containing bacterial cells expressing an enzyme of interest (Figure 5). In later studies, they developed mechanical valve-based systems for the automation of serial and parallel bioprocesses including cell isolation, cell lysis, DNA purification and DNA recovery without any pre- or post-sample treatment on a single microfluidic chip [70]. They also implemented a microfluidic bioreactor having two alternating states: (a) continuous circulation and (b) cleaning and dilution. This functionality allowed long-term monitoring of small populations of bacteria with single-cell resolution over hundreds of hours [71]. Recently, a high-throughput microfluidic device containing 2400 unit cells controlled by 7233 valves has been developed to detect low-affinity transient binding events on the basis of mechanically induced trapping of molecular interactions [72]. This allowed mapping of binding energy landscapes of four eukaryotic transcript factors to predict *in vivo* function and to test basic assumptions about transcription factor binding.

To address the simultaneous quantification of different targets within a single sample and self-assembly of microstructures on the micrometer scale, continuous-flow lithography with *in situ* photopolymerization of liquid-phase photocurable materials has gained much attention [85–87]. PEG prepolymer solutions flowing in a microchannel are crosslinked by UV light filtered through either a photomask or a computer-controlled programmable two-dimensional spatial light modulator. Pregibon *et al.* fabricated multifunctional particles bearing over a million unique codes of 0 and 1 by a single process and then successfully demonstrated multiplexed, fluorescence detection of 500 attomoles of DNA oligomers

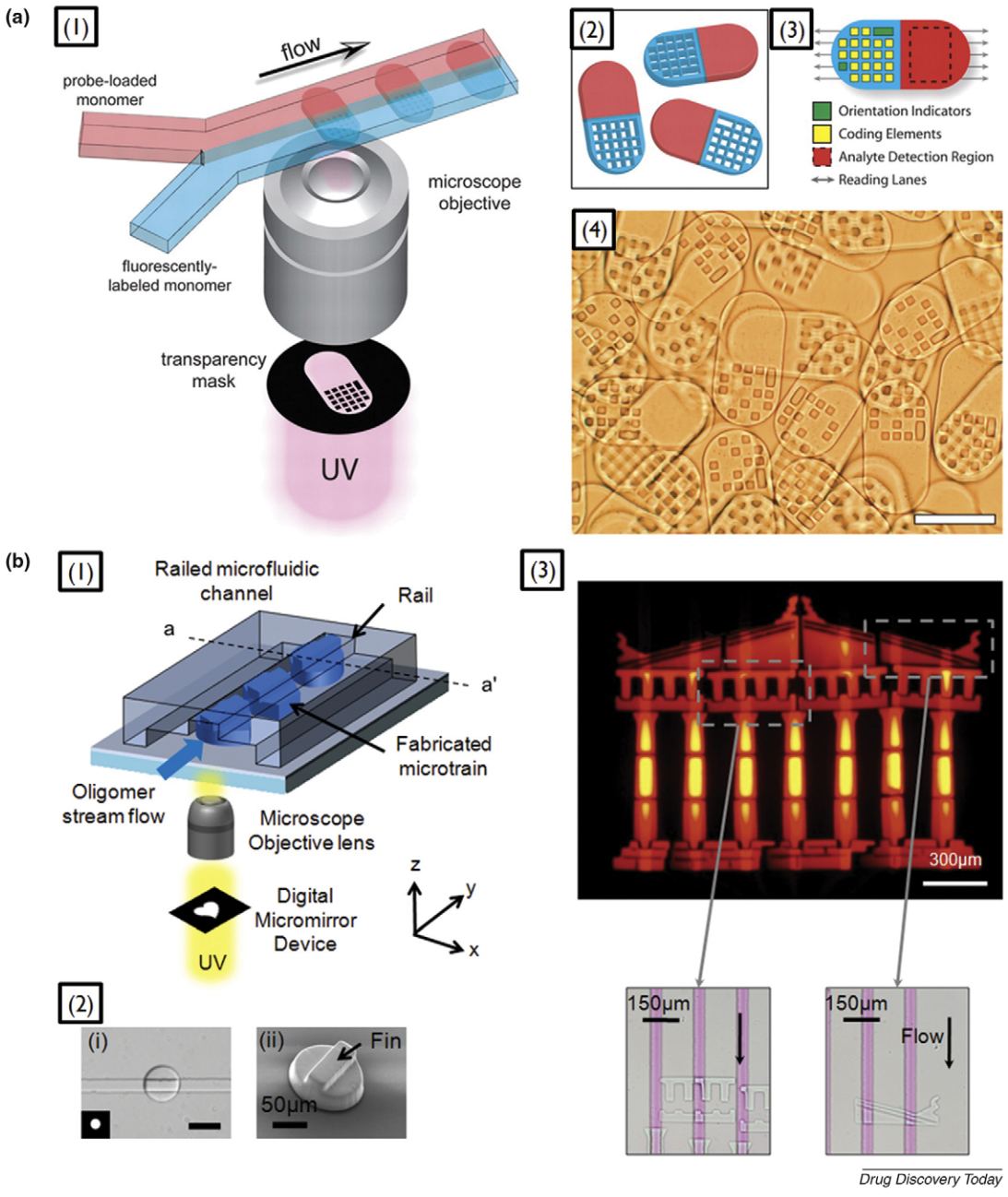

FIGURE 6

(a) Multifunctional encoded particles produced by continuous-flow lithography with *in situ* photopolymerization of liquid-phase photocurable materials: (1) schematic of dot-coded particle synthesis, (2) half-fluorescent particles, (3) particle features for encoding and analyte detection and (4) differential interference contrast images of particles. A scale bar in (4) indicates 50 μm (reproduced with permission from [85], Copyright 2007 American Association for the Advancement of Science). (b) Concept of railed microfluidics as a method to guide and assemble microstructures inside fluidic channels using a optofluidic maskless lithography system: (1) schematic of a railed microfluidic channel, (2) fabricated 'micro-train', (3) Greek temple assembly containing 37 microstructures. (Courtesy of S. Kwon, Seoul National University, South Korea).

(Figure 6a) [85]. Recently, Kwon and colleagues devised a new way to guide the self-assembly of microstructures within microfluidic networks, which works by using a guiding 'rail' mechanism. Complex structures could be assembled into larger structures (e.g. an ancient Greek temple, the Eiffel tower and a computer keyboard) as they were pushed by a flowing liquid to the end of the channel (Figure 6b) [87]. This technique could also be used to manipulate arrays of silicon microchips and collections of living cells.

Cells on chips

Laminar flow within microchannels can be exploited to selectively deliver compounds to defined regions of a cell, potentially allowing the evaluation of the stochastic nature of cellular responses

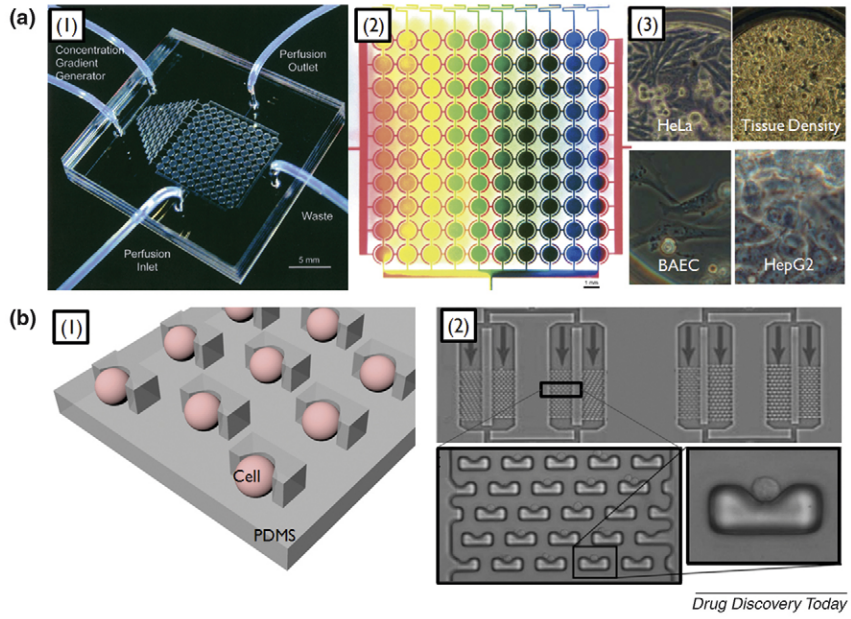

FIGURE 7

Cell-based microfluidic systems: (a) microfluidic cell-culture arrays with integrated concentration gradient generators; (1) photograph of the microfluidic cell culture array, (2) concentration gradient across 10 columns and (3) a variety of cell types growing in the device (reproduced with permission [25], Copyright 2006 The Royal Society Chemistry, and [88], Copyright 2004 Wiley Periodicals, Inc.). (b) dynamic single cell culture arrays; (1) a schematic of the device and (2) a photograph of the cell trapping device (reproduced with permission from [91], Copyright 2007 The Royal Society Chemistry).

and the effect of chemical gradients or drug interactions [25,32,88–93]. Recently, Hung *et al.* developed a microfluidic cell-culture array with an integrated concentration gradient generator for long-term cellular monitoring (Figure 7a) [88,89]. Gradient generation permits many growth conditions to be analyzed in a combinatorial fashion and is well suited for high-throughput experimentation where a large number of conditions need to be screened in parallel. The authors also demonstrated high-density regular arrays of single cells isolated in a purely hydrodynamic fashion within a microfluidic device (Figure 7b) [92]. Cells were held by obstacles or incorporated into the channel. The trapped cell on each obstacle can be used for biological studies on a single-cell level and combined with patch clamping. High-content screening has been considered a core method in the early stage of drug discovery for secondary compound screening and toxicology screening. Indeed, Ye *et al.* demonstrated an integrated microfluidic device for investigating cellular responses in human liver carcinoma (HepG2) cells under complex physiological conditions [93]. The device is composed of multiple drug gradient generators and parallel microscale cell chambers that function to dilute liquid, culture cells, stimulate cells and label cells. This enables rapid extraction of information related to cell response across several drug candidates.

Droplet-based microfluidic systems

As discussed, droplet-based microfluidic systems have been introduced as a fundamental experimental platform for high-throughput experimentation. These systems enable the generation and manipulation of liquid droplets in an immiscible carrier fluid. Such encapsulated droplets can mimic artificial cells and probe the kinetics and biology behind life's fundamental reactions. Indeed, these systems have been applied to a range of biological problems including enzymatic assays [11,37], protein crystallization [27,29,37], real-time binding kinetics [27,36] and cell-based assays [11,27,36]. Compared to single-phase flow, the localization of reagents within discrete and isolated droplets is an extremely effective way of enhancing reaction yields for mixing and mass transfer limited reactions and narrowing residence time distributions [21]. Moreover, the ability to controllably form droplets with changeable reagent composition and generate droplets at rates in excess of 1 kHz means, theoretically, that millions of individual reactions or assays can be processed in very short times. In addition, microdroplets can be fused, subdivided, sorted, isolated or incubated to establish a multifunctional analytical device. To date, several research groups have investigated and solved many of the engineering tasks behind such systems, and more details on droplet operations and applications can be found in the following review articles [11,27,33–36]. Figure 8a illustrates the utility of droplet-based microfluidic systems for performing single-cell enzymatic assays [94]. Here, a single femtoliter volume droplet is generated using a T-junction injection geometry. Before the formation of the droplet, a single cell can be selected from aqueous solution and moved by optical trapping to the interface between the aqueous phase and the carrier fluid. These techniques make it possible to investigate each cell under controlled and physiologically relevant environments. Additionally, Huebner *et al.* have demonstrated the detection of yellow fluorescent protein (YFP) expression by encapsulating single *E. coli* cells in droplets coupled to efficient laser induced fluorescence detection. Here cell occupancy in each droplet is controlled by varying the two flow rates of

a cell suspension and growth medium at a Y-channel configuration [95]. Moreover, Tan and Takeuchi achieved dynamic microarrays using a *trap-and-release* mechanism owing to the combination of fluidic resistance and optically generated microbubbles (Figure 8b) [96]. In this case, when the trap is empty, the main stream flows along a straight channel. Once the trap is occupied by a droplet, the main stream is redirected to a loop channel. To release the droplet, an IR laser is focused onto an aluminum surface to generate a microbubble. It should be noted that online droplet detection with high resolution and speed is one of the most significant challenges for realizing droplet-based HTS systems. With this in mind, Srisa-Art *et al.* have recently demonstrated high-throughput biological assays based on confocal spectroscopy and fluorescence resonance energy transfer (FRET) [97,98] and high-resolution fluorescence lifetime imaging in droplet-based microfluidics [99].

Importantly, droplet-based microfluidic systems are likely to be promising tools for directed molecular evolution. Aqueous droplets can be used to form cell-sized compartments (called as artificial cells) containing genes, RNA and proteins that they encode, and the products of their activity. Indeed, *in vitro* expression of green fluorescent protein has been achieved in a droplet-based microfluidic system [11,100]. We expect that the continuing evolution of control architecture and online detection will provide the essential impetus to ensure that droplet-based microfluidic systems become an indispensable tool in the biological sciences.

Novel nanofluidic devices

As stated, nanotechnology solutions are now being used in the field of drug discovery. The development of nanofluidic devices with crucial dimensions on the order of characteristic physical scaling lengths (typically 1–100 nm) heralds new opportunities for probing single molecules. Several crucial characteristics discriminate fluid flow at the nanoscale from flow in larger environments. First, natural scaling lengths, such as the Debye screening length and hydrodynamic radius, are similar to the dimensions of the

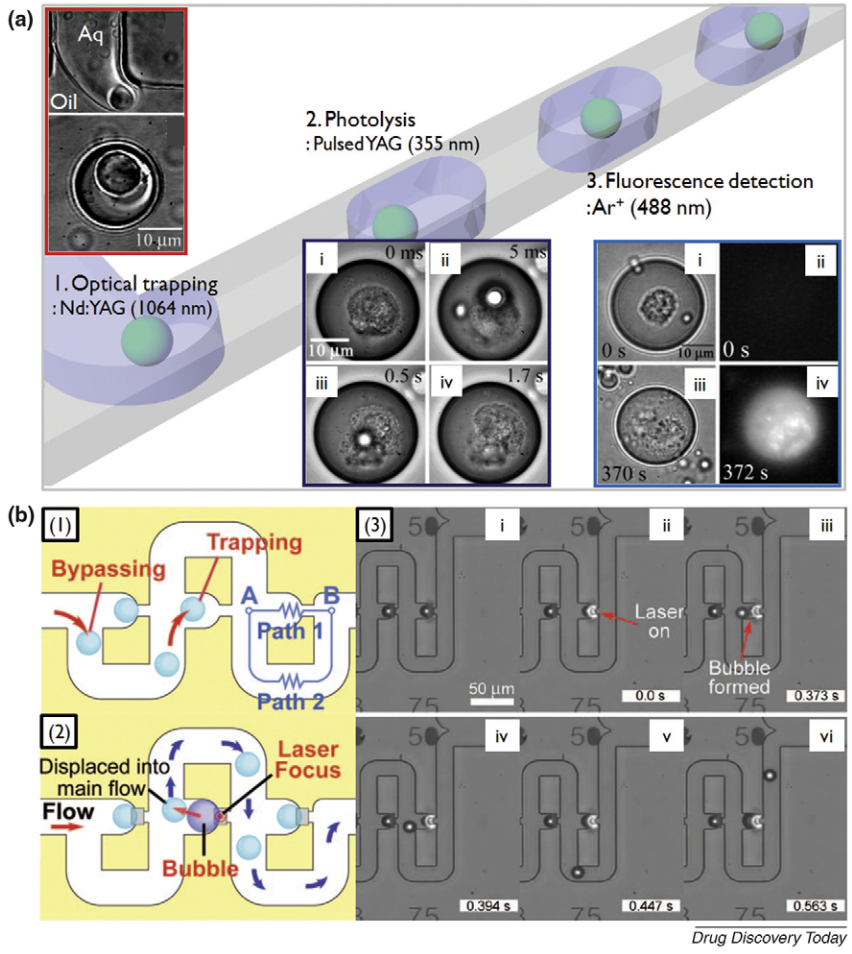

FIGURE 8

Droplet-based microfluidic systems: (a) an enzymatic assay for a single mast cell within an aqueous droplet; (1) an entrapped cell in a droplet. Optical trapping is used to transport and position the cell close to the water/oil interface. (2) rapid photolysis of a single cell. The energy pulse forms a plasma and cavitation bubbles which lyse the cell. and (3) after laserinduced cell lysis, β-galactosidase catalyzes the formation of a fluorescent product, which causes the droplet to become highly fluorescent (inset iv) (reproduced with permission from [94], Copyright 2005 American Chemical Society). and (b) a dynamic microarray based on a trap- and -release integrated microfluidic system; (1) trap, (2) release mechanism, and (3) sequence of high speed camera images showing the retrieval process using a bubble (reproduced with permission from [96], Copyright 2007, the National Academy of Sciences).

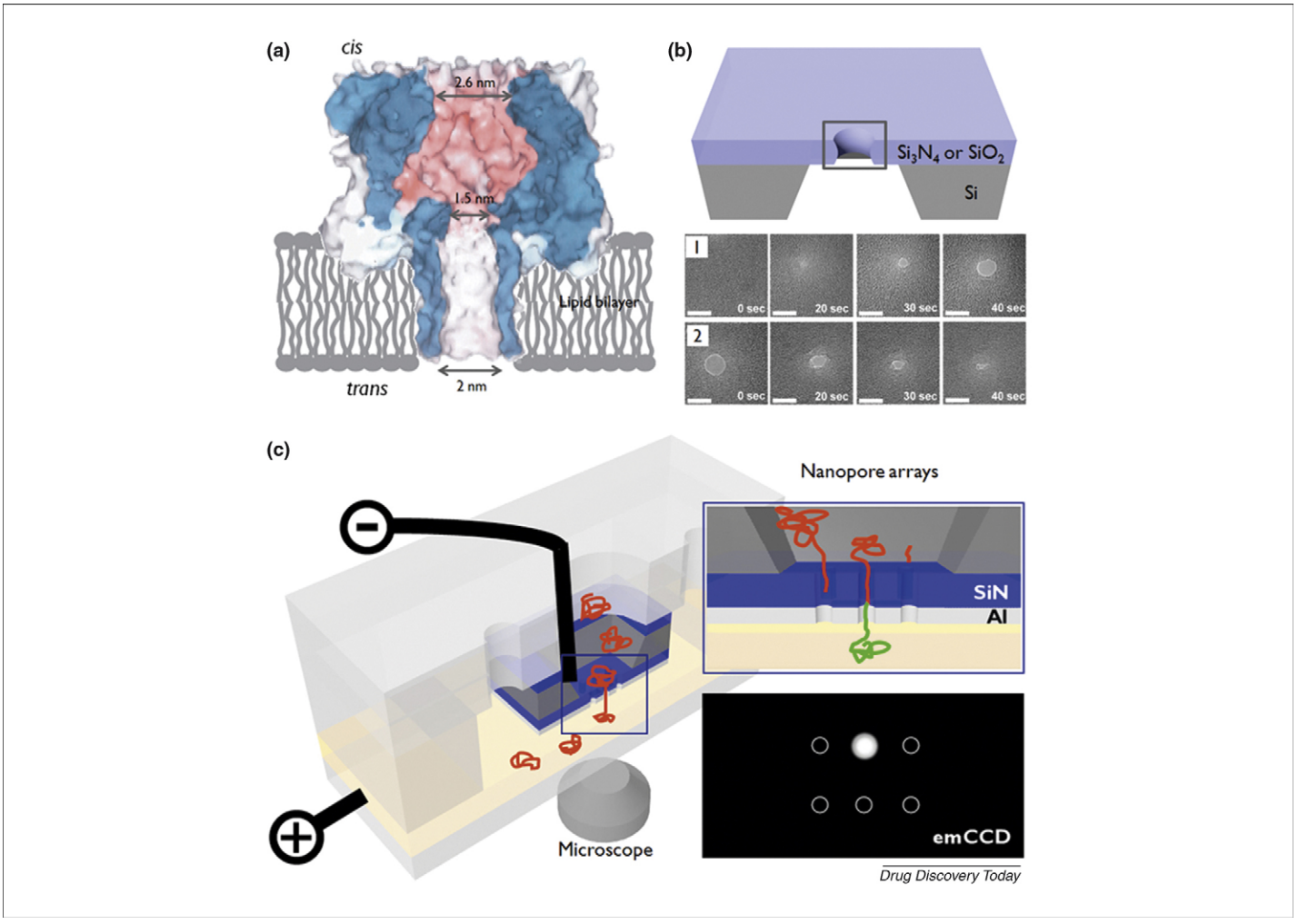

FIGURE 9

Nanopore-based single-molecule analysis: (a) the three-dimensional crystal structure of an α-hemolysin protein nanopore (reproduced with permission from [42], Copyright 2007 Nature Publishing Group), (b) a synthetic nanopore in a free standing membrane. A sequence of TEM images displaying the dynamics of (1) drilling and (2) contraction in the 50 nm-thick silicon nitride membrane. The scale bar is 5 nm (reproduced with permission from [102], Copyright 2007 IOP Pulishing Ltd.) and (c) a proof-of-concept device for highthroughput and parallel analysis at the single-molecule level.

nanostructure itself. Second, fluids can no longer be considered as continua but rather as ensembles of individual molecules. Third, typical surface-to-volume ratios are extremely high and so both local charge differences and surface roughness inside nanoscale conduits cannot be ignored. Finally, diffusion becomes an extremely efficient mass transport mechanism. Accordingly, these characteristics can be exploited to develop new analytical platforms for drug discovery and development. In this section, we focus on the use of nanometer-sized pores and holes (or wells) for HTS.

Recent studies have demonstrated that a nanopore can act as a single-molecule sensor to explore discrete molecular phenomena, while operating at extremely high analytical throughput. The majority of nanopore-based studies involve the use of a protein channel that spontaneously inserts itself into a lipid membrane. The toxin protein α-hemolysin is secreted by the bacterium *Staphylococcus* and each pore features a transmembrane channel with a width of 1.5 nm at its narrowest point (Figure 9a). The α-hemolysin nanopore is the archetype for rapid characterization and sequencing of nucleic acid molecules using high-resolution electrical read-out [37–39]. However, it is not possible to control the pore diameter or to use it over a wide range of pH, salt concentration, temperature and mechanical stress. An alternative to protein nanopores is the use of solid-state nanopores, which can be tuned in size with nanometer precision and display improved mechanical, chemical and electrical stability (Figure 9b) [41,42,101,102]. However, the fabrication of these nanometer-sized pores on solid-state materials represents a significant challenge, especially in the control and reproducibility of both the size and shape of the nanopores. To date, the analysis of translocation events is usually performed electrically by measuring changes in ionic conductivity. Molecules driven through a nanopore by an electric field physically block the pore and thus produce a temporal change in current. However, Chansin *et al.* have recently reported a novel approach for the optical detection of DNA translocation events through solid-state nanopores and this proof-of-concept study illustrates the potential for ultra-high-throughput and parallel analysis at the single-molecule level [103]. In essence, each individual sub-wavelength pore acts as a waveguide for fluorescence excitation with a metallic layer on

the free-standing membrane acting as an optical barrier between the illumination region and the analyte reservoir (Figure 9c). This configuration allows for high-contrast imaging of single-molecule translocation events through multiple pores and with minimal background or noise [104].

Nanoholes (or nanowells) that do not convey fluid flow have also been of significance in single-molecule analysis. These systems consist of sub-wavelength nanoholes in a metal film in the zero-mode propagation regime, where the short depth of penetration of the evanescent field is used to obtain a detection volume of the order of a few zeptoliters. These systems allow for probing single-molecule events at physiological concentrations [40,105] or on the surface of lipid membranes [106,107]. In addition, depending on the metal and the wavelength of interest, a periodic array of nanoholes exhibits extraordinary transmission of light resulting from the coupling of incident radiation and surface plasmons. A variation of the refractive index in the medium induces a shift in the transmission spectrum and so allows the detection of surface-binding events [108]. These nanohole arrays can also be used for surface plasmon resonance (SPR)-enhanced Raman spectroscopy because the strong electromagnetic field in the vicinity of the holes enhances inherently weak Raman signals [43,109]. Therefore, it is clear that nanofluidic devices have great potential for probing single-molecule events.

Concluding remarks

The development of micro- and nanofluidic systems for HTS continues to mature. Before micro- and nanofluidic devices replace existing assays and systems, several challenges still remain. These include facile interfacing between the micro- (or nano-) and macro-world, ease of device handling, system robustness, the ability to parallelize and ability to lower unit costs. If surmounted, future micro- and nanofluidic systems will be capable of performing complex processing and analysis operations in biological and drug discovery applications, on components ranging from DNA fragments to entire cells. Processing and analysis functions are likely to be performed on a single chip with high analytical throughput rates and in environments as close to *in vivo* conditions as possible. Furthermore, we expect that micro- and nanofluidic systems combined with computational tools and bioinformatics will form a core instrumental platform in many areas of biological analysis over the next few decades.

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