SCIENCE CHINA
Chemistry

• REVIEWS •

August 2011 Vol.54 No.8: 1185–1201
doi: 10.1007/s11426-011-4343-6

Advances of multiplex and high throughput biomolecular detection technologies based on encoding microparticles

ZHAO XiangWei, ZHAO YuanJin & GU ZhongZe*

State Key Laboratory of Bioelectronics; Southeast University, Nanjing 210096, China

Received April 13, 2011; accepted June 2, 2011

The rapid developments of genomics and proteomics have driven the demand for multiplex and high throughput analysis of large numbers of biomolecules in the fields of medical diagnostics, drug discovery, and environmental monitoring. Encoding the biomolecular binding events is the key technique to fulfill this demand, in which microparticles play the most important roles. This review outlines the development of multiplex and high throughput biodetections, and highlights the most recent advances in the field of encoding microparticles, together with problems that need to be resolved.

multiplex bioassays, multiplex labels, encoded microcarrier, binding assays, high throughput screening

1 Introduction

Since the completion of the Human Genome Project in 2001, the human being has entered an era of "-omics", the objective of which is to elucidate the whole intricate network of biomolecular interactions and unravel the mysteries of life. One of the distinctive characteristics of this era is data-richness, and the Human Genome Project has pushed individual biologists more in the direction of understanding life systematically, rather than getting stuck in a single gene, protein or other biological factors [1]. It means that not only a huge number of data are generated and mined but also more and more needs are acquired by biomolecular detection especially when it comes to their applications in biomedical diagnostics, drug discovery/screening, and environmental monitoring. At the same time, it drives the development of high throughput technology for multiplex assays, which can simultaneously detect multiple analytes in the same sample in a cost-effective way.

The binding of biomolecules or ligands like antibodies and oligonucleotides lies the basis of most of the biomolecular detection techniques since the introduction of biomolecular labels into immunoassays in 1950s [2]. Up to now, immunoassays are still the most commonly used type of diagnostic assay and undergoing fast growing for the analysis of biomolecules. For example, Figure 1 illustrates the principle of a solid-phase immunoassay in a double antibody sandwich format adapted from antigen-anti-body reaction for the quantification of an analyte, which is commonly known as Enzyme-Linked ImmunoSorbant As-say (ELISA). In this scheme, analyte-specific antibodies (capture

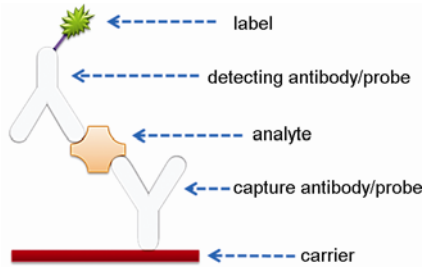

Figure 1 Scheme of a typical solid-phase immunoassay. Capture antibody immobilized on a solid carrier, analyte, and labeled detecting antibody form sandwich immunocomplex. The signal of the analyte is detected by the detecting label, which is an enzyme, fluorescence dye or radioactive isotope.

*Corresponding author (email: gu@seu.edu.cn)

antibody) are attached to a solid supporting medium (carrier), such as a polystyrene well in a microtiter plate. Antigens to be detected will bind to these antibodies after the sample is added. Then another kind of antibody (detecting antibody), which is specific for a different antigenic site on the antigen analyte and conjugated with radioactive, enzymatic or fluorescent labels, is added. After a second binding reaction, the concentration of the analyte is indicated indirectly by the label signal, and then quantified by referring to that of analyte standards. The sensitivity of these forward phase immunoassays is in the pg/mL range under optimal conditions. And which different assays performed in different wells, high throughput assays are carried out under the cooperation of automatic liquid operation workstation and signal readers. However, in this case most of the sample volume is wasted since only one analyte is analyzed in each well. This issue is particularly serious for the diagnostic applications involving analysis of quantity-limited samples from patients.

By virtue of detecting multiple analyte in the same sample in parallel while attaining the high sensitivity, multiplex immunoassays was developed in 1980s [2]. Traditional methods have employed different labels, usually fluorescence dyes, to distinguish multiplex binding reactions, but the categories of the dyes were limited. In contrast, spatial isolation of many immunoassays in the form of an spot array on a planar carrier offers much greater flexibility. This approach also underlies modern miniaturized microarray technology, such as DNA or protein microarray.

As one of the representative technologies of genomics and proteomics, DNA or protein microarray is fabricated with lithography or robotic spotter by immobilizing oligonucleotides or proteins onto a planar carrier surface such as glass and silicon to form microscopic spots (Figure 2). The detection scheme is analogous to that illustrated in Figure 1. But the probes or capture antibodies are encoded by their positions or coordinations on the carrier. Hence multiplex analytes can be analysed simultaneously since their quantities and identities can be detected with the label signals and their positions repectively. Although microarrays are marked by their high-density screening (multiplicity up to hundreds of thousands), deficiencies in data and array reproducibility, reduced reaction kinetics, high cost and poor flexibility are always haunting them [3, 4]. The main reason lies in the fixed planar arrangement of probes, due to which the binding of probe and analyte is diffusion-limited and the sensitivity is restricted by the low surface to volume ratio of the planar substrate [5]. One solution is to accelerate the reaction on position-encoded microsphere carriers by active flow in microfluidic chips [6–9]. And another alternative, provided that multiplex binding events can be identified, is to release them into the bulk sample solution in order to realize approximately homogenous reaction, which is called suspension array (Figure 3) [10]. In this case, new encoding strategy should be employed so that different "moving" binding events in the same solution can be distinguished. For example, xMAP technology of Luminex Corp. dyes the microsphere carriers with different fluorescence color and intensity in order to track the reactions [11].

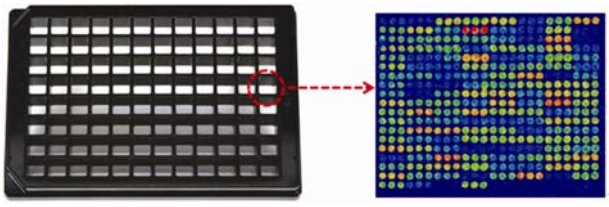

Figure 2 96 well glass bottom plates for multi-patient diagnostics. Each well contains high density arrayed features for multiplex assay, and the minimum spot and space size are 65 μm and 50 μm, respectively (Arrayit Corporation).

Meanwhile, during the last decades, huge advances are witnessed in the synthesis and applications of micro- and nano-materials, especially microparticles. And as a novel encoding material, a variety of microparticles were introduced into multiplex and high throughput biodetections, which resulted in great technology progresses. By contrast, microparticles hold the significant advantages over planar arrays in terms of the way they are produced and used. First, when they are used as carriers in multiplex assays, the kinetics of the binding will be improved by thoroughly mixing with analyte solution. Second, higher sensitivity and repeatability will result from the fact that microparticles with three dimensional morphology can be massively produced with uniform surface chemistry. Last, microparticles with different probes can be flexibly combined for customized applications, while expensive instruments are needed in case of microarray. Therefore, the key problem of suspension array based on microparticles is to encode the multiplex binding events without the position references. It is obvious that, referring to the scheme in Figure 1, either carrier or label can be encoded as long as the code and analyte signal can be interpreted in correlation.

In this review, kinds of multiplex and high throughput detection technologies will be introduced together with the underlying encoding microparticles. And we would like to divide them into two main groups, encoding the labels and encoding the carriers, depending on their principle to carry out multiplex assays. Our focus is mainly on their codes generation related to manufacturing; encoding capacity

Figure 3 Scheme of suspension array. In each well, there are different microparticles and different immunoassay occurs on different microparticles instead of on different positions of a planar carrier.

which determines their application scopes; decoding mechanism which determines their feasibility and compatibility in applications; and their functionality to facilitate the assay operations as well as their applications.

2 Encoding the labels

Fluorescence dyes were widely used as labels in binding assays of protein or DNA since they have specific emission wavelengths and their intensity can indicate the analyte abundance. And multiple fluorescence dyes were usually employed for multiplexed target molecule detection in immunohistochemistry or fluorescence *in situ* hybridization (FISH). When they are adapted to encode multiplex detection of free analytes in solutions, measurements should be taken in order to separate them from excess ones that have not bound with the analytes. For example, in reverse phase multiplex immunoassays, multiple analytes need to be immobilized randomly on the carrier first and then detected by signals of multiple fluorescences after a thoroughly washing process. Here, the fluorescence dyes act as both encoding elements and binding reporters [12, 13]. However, the number of codes is limited since emission spectral width of conventional fluorophores is 50–200 nm and only four to six different fluorescence dyes can be used in the VIS-NIR region [14]. Hence, the kinetic problems of 2D planar microarray still exist.

2.1 Förster resonance energy transfer (FRET) encoding

In contrast to binding events happen on the 2D solid carrier and liquid interface, homogeneous reaction in solution phase simulates the state of nature interaction like antibody-antigen binding, DNA hybridization *in vivo*. Hence it possesses several inherent advantages, such as rapid reaction kinetics, convenient operation, and potential compatibility with *in vivo* or real-time applications. Distance- dependent fluorescence quenching by Förster resonance energy transfer (FRET) is usually designed to probe homogenous binding events. And by the virtue of fast-devel- oping nanomaterials, nanogold, quantum dots (QDs), nanowire and single wall carbon nanotubes are studied and applied in FRET to increase the transfer efficiency [15–19]. Fan and Song *et al*. designed a FRET system based on gold nanoparticles as superquencher and multiple fluorescence dyes as encoding labels of aptamers, which are oligonucleotides that can bind specifically with analytes like ligands [20]. In a multiplex assay as illustrated in Figure 4(a), at first, three aptamers, anti-adenosine aptamer, potassium-specific G-quartet, and anti-cocaine aptamer, are encoded by different fluorescence dyes at the 5' end respectively. And gold nanoparticles are modified with multiple 3'-thio-lated oligonucleotides whose sequences are complementary to those aptamers. After they form complex nanostructures by

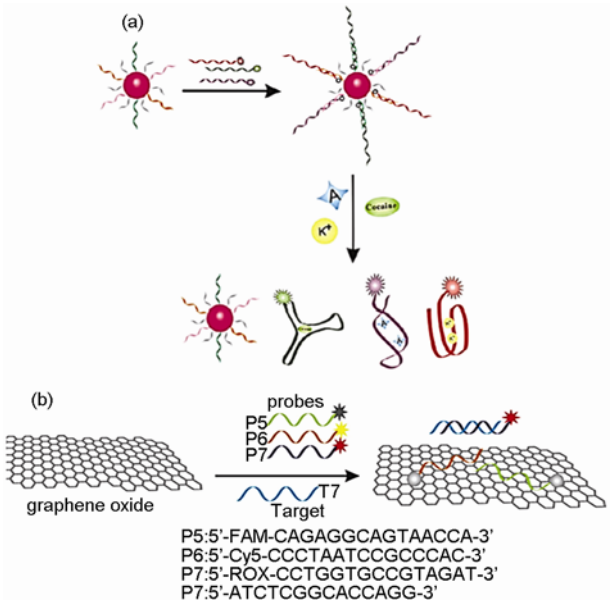

Figure 4 (a) Encoded aptamers for the multiplex signal-on detection of adenosine (A), potassium, and cocaine; (b) scheme for multicolor DNA analysis based on grapheme oxide as a selective quencher for charged single strand DNA.

hybridization, FRET occurs since the dyes are in close proximity to the gold nanoparticles. In the presence of adenosine (A), potassium, and cocaine, the aptamers will dissipate from the complex and bind with the analytes due to the higher affinity between them, and hence fluorescence will be detected in a "signal-on" mode. Meanwhile, multiplex DNA detection with sensitivity in nM and pM scale also can be realized by this means or using grapheme oxide as a selective quencher for charged single strand DNA [21, 22]. Perhaps, except for the fast binding kinetics, the largest advantage of this kind of encoding and multiplex detection maybe is the manner of mix-and-detection without washing steps owing to the "signal-on" mode, which saves a lot of time and holds a high throughput potential. Although, this encoding strategy can be transferred to multiplex protein detection, multiplicity is poor because of the limited number of optional aptamers and non-overlapping fluorescence dyes. In addition, multiple light wavelengths are needed to excite and detect the encoding fluorescence dyes.

2.2 DNA nanobarcodes

Recently, dendrimer-like DNA (DL-DNA) nanostructures (DNA nanobarcodes) have been synthesized by Luo's group with diameter less than 30 nm [23, 24]. The nanobarcode is assembly through hybridization of DNA strands from Y-shaped DNA (Y-DNA) as building unit, which has two ends labeled with different fluorescence dyes and one sticky end used for conjugation with probe molecule (Figure 5(a)). In this label encoding strategy, both dye type and dye number can be precisely controlled to generate multicolor fluo-

rescence intensity-encoded nanobarcodes. As shown in Figure 5(b), biotinylated probe oligonucleotides complementary to the target DNA sequences were bound to avidin-coated polystyrene beads. The target strands were then reacted with a mixture of beads coated with different probe sequences. Fluorescent nanobarcodes attached to oligonucleotides complementary to part of each target sequence are then hybridized to the target sequences attached to the bead. Each bead to which a target is bound now has a particular combination of fluorescent dyes and their intensities, the fluorescence spectrum of which indicates the DNA sequence on its surface. The label encoding strategy was applied in a 4-plex assay for the detection of DNA sequences from the pathogenic organisms *B. anthracis*, *Francisella tularensis*, Ebola virus and SARS coronavirus with detection limit low to 620 attomol. And the beads were successfully decoded using two-colour flow-cytometry or fluorescence microscope. The authors also showed their usage in blotting-based detection (Southern, northern and western) through gel imaging system. In comparison with conventional multicolor label encoding methods, the reaction kinetics of this method is promoted with beads as carriers. Also, the coding capacity (C) of nanobarcode is enlarged and determined by the color number (L) and the labeled branch number (P), and calculated by the following formula: $C = (P+L-1)!/[P!(L-1)!]$, where P is determined by the generation number (n) of DL-DNA ($P = 3x2^n$) [24]. In theory, 325 different nanobarcodes can be fabricated with three fluorescent colors and with a third generation (G3) DL-DNA. However, in practice, the number of codes that can be distinguished may be much fewer due to factors such as FRET, choices of dyes, equipment sensitivity and signal to noise ratios, and detection methods. Although increasing the generation number (n) will enlarge the capacity exponentially, the decoding readout becomes more complex and cost-ineffective.

2.3 Nanostring encoding

Another similar label encoding technology, nanostring, based on combination of fluorescence dyes was proposed by NanoString Technologies Inc [25, 26]. In this technology, firstly, unique pairs of capture and reporter probes are designed and constructed to detect target mRNAs. As shown in Figure 6(a), after a homogenous hybridization reaction with total RNA in solution, tripartite structures are formed and comprised of a target mRNA bound to its specific reporter and capture probes. The single strand DNA backbone is annealed to a unique pool of seven dye-coupled RNA segments (nanostring) corresponding to a single code of the target mRNA. Unhybridized reporter and capture probes are removed by affinity purification, and the remaining complexes are washed across a streptavidin coated surface and captured by biotinylated oligonucleotides annealed to the 3' repeats. Then, an applied electric field extends and orients each complex in the solution in the same direction. The

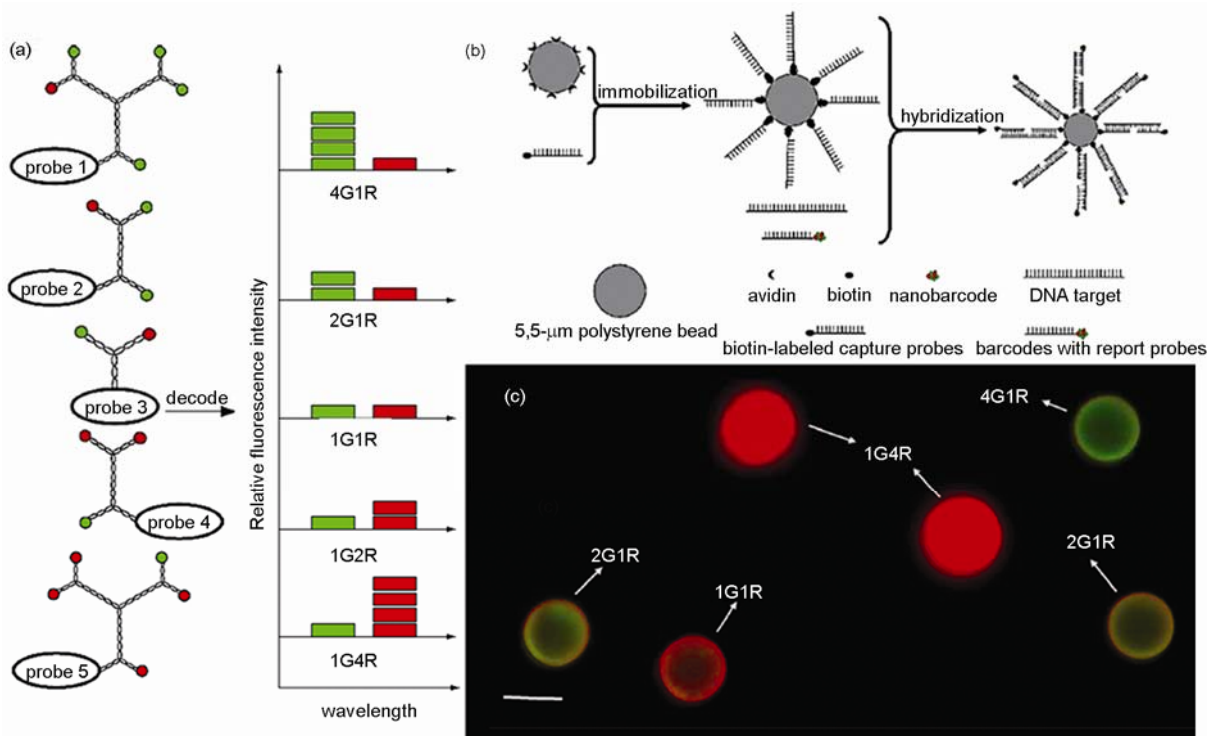

Figure 5 (a) Schematic illustration of barcoding of the DNA dendrimer fluorescent labels by fluorescence intensity ratio; (b) DNA nanobarcode, DNA target and polystyrene microbeads form sandwich structure in multiplex detection; (c) four targets were detected using DNA nanobarcodes and microbeads. Scale bars, 5 μm.

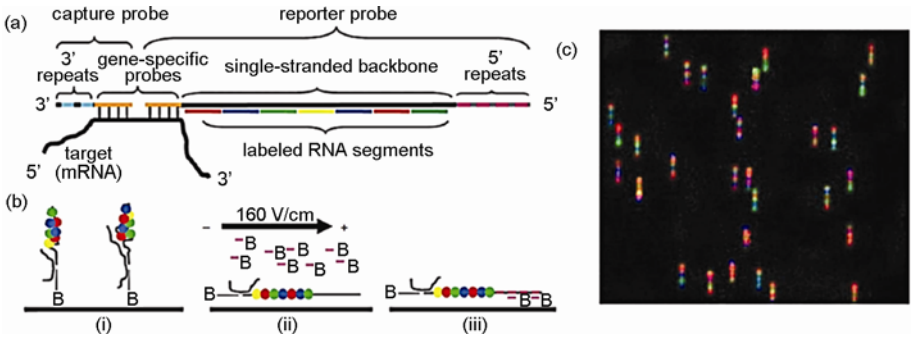

Figure 6 Illustration of nanostring encoded assays. (a) Complex structures formed after hybridization of a target mRNA, its specific reporter and capture probes; (b) biotinylated 3' and 5' repeats are used to fix the starched tripartite structure on a streptavidin coated slide; (c) the nanostring codes are imaged with a fluorescence microscope equipped with high resolution and magnification lens.

complexes are then immobilized in an elongated state (Figure 6(b)) by biotinylated oligonucleotides annealed to the 5' repeats and imaged (Figure 6(c)) with a fluorescence microscope. Each target molecule of interest is identified by the color code generated by the ordered fluorescent segments present on the reporter probe. The level of expression is measured by counting the number of codes for each mRNA.

The number of effective codes depends on a combination of factors that include the length of the DNA backbone, the minimum spot size that can be resolved under current imaging conditions. In order to minimize spectral overlap of fluorescence dyes during imaging, the authors used seven backbone positions (Figure 6(a)) and four colors to generate nanostring codes. The encoding capacity of the technology in this form is therefore $4^7 = 16{,}384$ codes, a relatively large encoding capacity suitable for big scale gene expression assays, such as expression profiles used to find disease indicators in clinical settings. The authors also demonstrated their high sensitivity (0.1–0.5 fM detection limit) comparable to real-time PCR. But the shortcomings of the technology is also obvious by the complex assay process and the long detection time (up to 6 h) due to slow and high resolution codes scanning, which may lower its analysis throughput greatly.

2.4 Surface enhanced raman spectroscopy encoding

Whereas, fluorescence dyes are widely used in molecule labeling or encoding, their drawbacks such as photobleaching, photoquenching, narrow excitation with broad emission profiles, spectral overlapping and multiple excitation requiring in multiplexed assays are obvious. Hence, alternative labels are in pursuit all along, among which are Raman dyes or tags. They have very stable unique spectral fingerprints containing high information content with narrow peak widths (20 cm^{-1}) and can be excited at any wavelength. Although the intensity of their Raman spectra is very low since the typical Raman cross section is in the order of 10^{-30} cm^2/molecule, it can be overcome by surface-enhanced Raman scattering (SERS) with million- to trillion-fold increase when Raman active molecules are in very close proximity to roughened noble metals like gold or silver nanoparticles [27, 28]. For example, commercialized NANOPLEX™ biotag of Nanoplex technology, latterly acquired by Oxonica limited, are composed of Raman active molecule tagged gold nanoparticles encapsulated with silica shells (Figure 7(a)) [29]. Irradiation of these tags with monochromatic light yields the SERS spectrum of the reporter. To encode different tag, one simply employs a different reporter molecule. Owing to narrow spectral features and large spectral window of SERS, it is possible to create many distinct and simultaneously quantifiable tags. In a typical multiplex assay, the biotags, analytes and magnetic beads immobilized with capture probes (Figure 7(b)) form two-particle sandwich complexes, which are then concentrated and detected at a specific location within the reaction vessel in the presence of the sample matrix and excess free detection particles. It is amazing that this simple assay permits fast homogenous reaction, no-washing, multiplexed detection and quantification (Figure 7(c–f)) of proteins and DNA sequences. Undoubtedly, the encoding technology has great potentials not only in robotic high throughput screening, but also in miniaturized point-of-care or field applications such as lateral flow immunoassays. According to the company's data, the protein detection sensitivity can reach pg/mL in the presence of biological matrices such as serum, plasma and whole blood. However, the encoding capacity of NANOPLEX™ biotag depends on the level of different spectral precisions required for specific applications and hence the cost of the decoding instrument. Usually, it is around 10 for low-cost handheld decoding readers.

In comparison with single noble metal nanoparticles, their aggregations can dramatically increase the SERS signal intensity by several orders of magnitude, according to theoretical calculation [30, 31]. Su *et al.* used aggregates of silver nanoparticles as encoded labels for multiplex analysis based on organic compound-induced metal colloid aggregation and coalescence in a simple and highly scalable process.

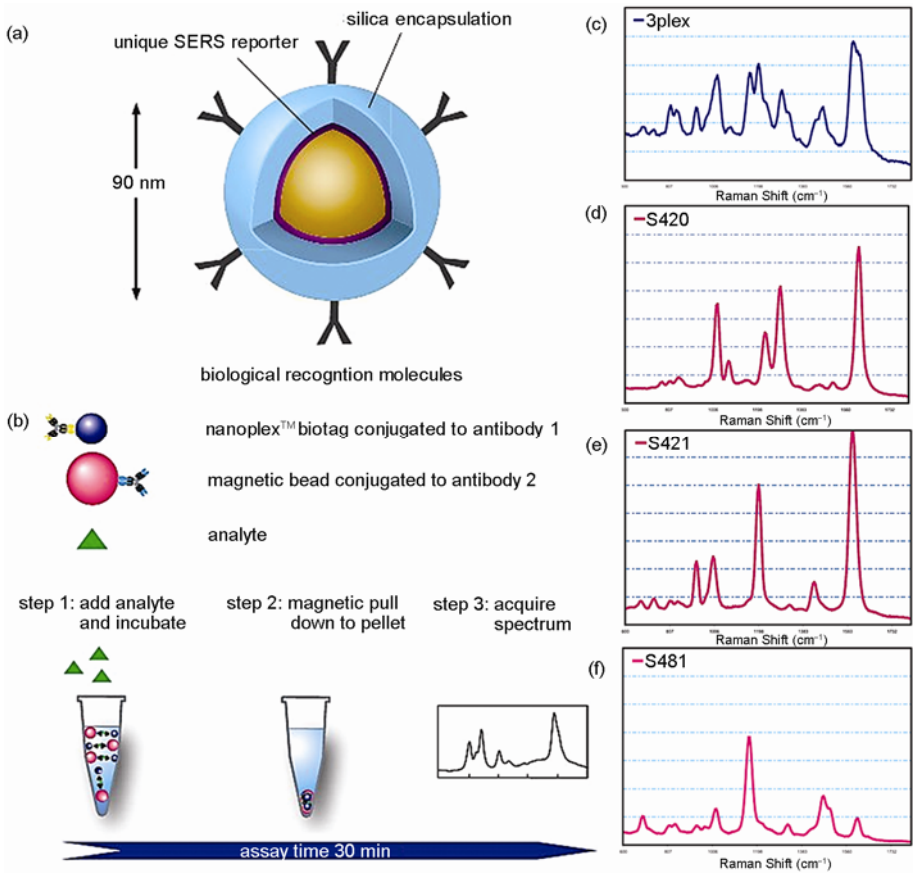

Figure 7 (a) Diagram of Nanoplex™ biotags with gold core; (b) multiplex assay procedure using Nanoplex™ biotags as encoded labels and magnetic beads as carriers; (c) composite spectra of three different tags as measured in a multiplexed assay; (d)–(f) spectra of each tag quantitatively deconvoluted by proprietary software. [www.oxonica.com]

While the SERS of 50 organic compounds could provide a large number of signatures as high as one million, it seems hard to realize since the spectra of the Raman compounds are dependent on their concentration and usually complicated by containing unwanted features. Mirkin group has applied SERS encoding in ultrasensitive and multiplex detection of DNA [32]. In the approach, gold nanoparticle probes are coated with Raman-dye-labeled oligonucleotides to generate spectroscopic codes for individual targets of interest. The presence of the target is confirmed by silver staining catalyzed by Au and then the amplified Raman signal of the dye. The unoptimized detection limit of this method is 20 femtomolar for DNA, and only single-wavelength laser radiation is needed to scan a highly multiplexed array with numerous target-specific Raman dyes, showing great values for gene express profiling.

Although the aforementioned Raman labels are more stable than fluorescene dyes, they still suffer from degradation under long time laser irradiation and small Raman scattering cross-sections. Recently, by the virtue of nanomaterials, there is a trend that new Raman labels such as single wall carbon nanotubes are explored towards a direction of higher intensity and better robustness [33].

2.5 Bio-barcode

Mirkin *et al.* have also utilized bio-barcode assay (BCA) to overcome the drawbacks associated with microarray technology such as low reaction kinetics and low sensitivity [34–36]. It employs oligonucleotides as bar codes for multiple target DNA or protein analytes. In a typical assay as illustrated in Figure 8, gold nanoparticles are functionalized with bar-code DNA composed of universal sequence and target recognition sequence, and magnetic microparticles are functionalized with sequences that capture a different target region. In the presence of target DNA, the magnetic microparticles and the gold nanoparticles form sandwich structures that are magnetically separated from solution. Then, the bar-code DNA strands are released by ligand-exchange with dithiothreitol (DTT) and detected using the scanometric approach, approved by Food and Drug Administration (FDA) of USA. In the scanometric detection, the released strands, form sandwich structures again with target-specific capture probes on a microarray and universal sequence specific probes on another gold nanoparticles. With silver staining catalyzed by gold nanoparticles, the detection limits could be as low as 500 zM (10 strands in solution). This fast method obviates the need for PCR am

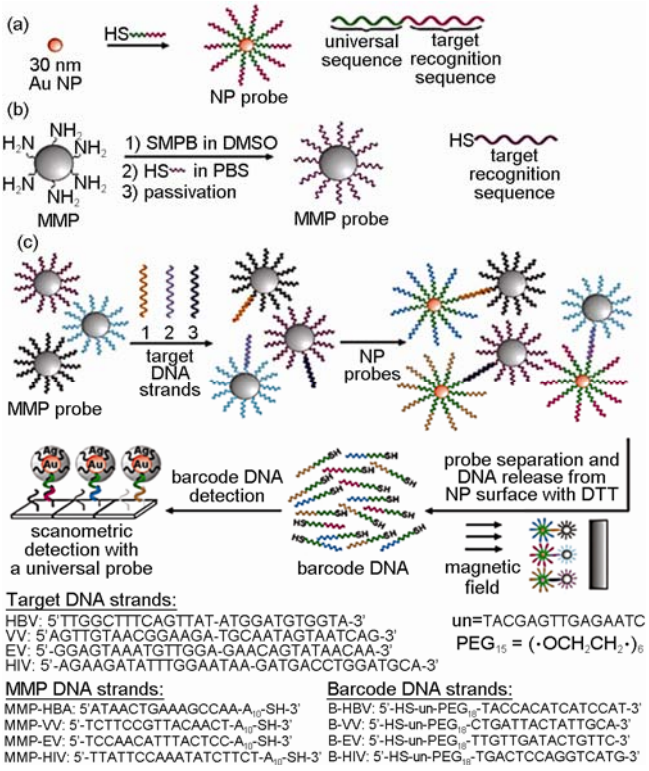

Figure 8 The procedure of multiplex protein detection by bio-barcode.

plification in usually DNA detection regardless of target concentration since the kinetics of the target binding process can be controlled by adjusting probe concentrations. The same scheme can also be adapted for multiplex protein detection with limits of detection that are 4–6 orders of magnitude lower than ELISAs, depending upon capture antibody and background signal. Although, it is claimed that unlimited bar codes can be synthesized for virtually any target of interest, for example, for a 10-mer oligonucleotide sequence, there can be 4^{10} codes, the merit of the BCA mainly lies in the ultra-sensitivity resulted from the unique signal amplification mechanism which is detected by scanometry. Significantly, the multiplexed biobarcode assay can be performed within a 96-well-plate in a high-throughput and facile manner. Also, it can be integrated with microfluidic chips to realize more miniaturized and labor-saving assays [37, 38].

2.6 Electrochemical label encoding

Since electrochemical detection is characterized by simplicity, low-cost, size-scalable, well-integratable features. Electroactive labels are commonly used in the detection of bio-binding events on solid electrodes in the same way as fluorescence labels in optical detection. For example, besides fluorescence, quantum dots also yield well resolved and highly sensitive stripping voltammetric signals. Wang *et al*. described an electrochemical coding technology for the simultaneous detection of multiple DNA targets based on QDs tags with diverse redox potentials [39]. Distinct DNA hybridizations thus can be detected by stripping voltammetric signatures of the encoding QDs, which can be systematically tuned by changing their compositions. The technology employs a sandwich assay in which target capture strands are attached to magnetic beads (Figure 9). Once the target DNA hybridizes to the capture strand, it is then labeled with oligonucleotide-functionalized QDs for the target. The sandwich system can be easily separated and transferred by magnetic field to an electrochemical cell where stripping voltammetric signals of encoding nanoparticles are detected. The magnitude of the stripping peak corresponds to the concentration of target DNA, thus making this method amenable to multiplexing and quantification. However, the encoding number is also limited as in the fluorescence encoding. The Wang group also put forward multiplex protein electrochemical detection by taking advantages of electroactivity and highly sensitive stripping response of the guanine (G) and adenine (A) nucleobases [40]. In a same manner as bio-barcode assays, the target proteins are sandwiched by magnetic bead probes and polystyrene microsphere probes coated with oligonucleotide barcodes, followed by alkaline release and acidic dipurinization of barcodes, and adsorptive chronopotentiometric stripping measurements of the free nucleobases. In this case, it is possible to create a larger number of identifiable oligonucleotide barcodes for electrochemical immunoassays by designing oligomers with different predetermined A/G ratios. And the coupling of carrier-loaded amplification of barcodes with the preconcentration feature of electrochemical stripping detection leads to extremely low detection limits down to pg/mL for protein molecules, which compares favorably with values obtained by ELISA, but still higher than bio-barcode assays mentioned above.

3 Encoding the carrier

As illustrated in the scheme of Figure 1, usually the carriers in a forward sandwich immunoassay act as solid phase, so that they can bear more functions such as separating the binding complex from the excess molecules and facilitating

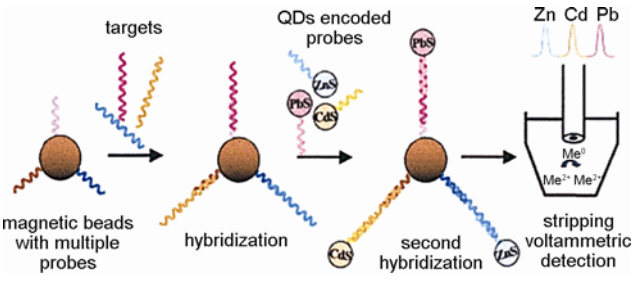

Figure 9 Multiplex electrochemical assay by QDs encoded probes.

facile interrogation of the molecular bindings. In comparison with the label encoding, there are a larger number of techniques have been proposed and extensively studied for encoding the carriers, such as fluorescence dye/QDs encoding, barcode encoding, electronic encoding, graphic encoding, about which there are already reviews elsewhere recommended by the authors [41, 42]. However, there are still not a perfect encoding technology that can fulfill the multiplex and high throughput requirements of biomedical applications until now. And the encoding technology still undergoes fast development with the advent of new strategies and materials. Therefore, in this part, we will put our emphasis on the newly advances in this field and address the problems that need to be solved.

3.1 Fluorescence color encoded nanoarrays

Although there are some drawbacks mentioned above of fluorescence dyes or QDs as labels, fluorescence color is the most well-established and widely employed encoding elements since their convenience both in encoding and decoding. In order to enlarge the encoding capacity, microspheres are dyed with multiple fluorescence dyes or QDs in different intensities in order to generate more color codes [10, 24, 25, 43]. But the dyeing progress is hard to control and the repeatability is very poor. Yan *et al*. used DNA tile, a programmable building block for self-assembly of micro- and nano-architectures based on the simple rules of Watson-Crick base paring, to construct 2D lattices as nanoarrays [44]. As illustrated in Figure 10(a), the nanoarray composed of three basic DNA tiles. A1 and A2 tiles are dyed with "red" Cy5 and "green" Rhodamine Red-X as encoding tiles, while probe tiles B (Figure 10(a)) carry single strand probes dyed with "blue" Alexa Fluor 488, which dangle out of the array plane through base-paring with the anchor strands that are single-stranded extensions of one of

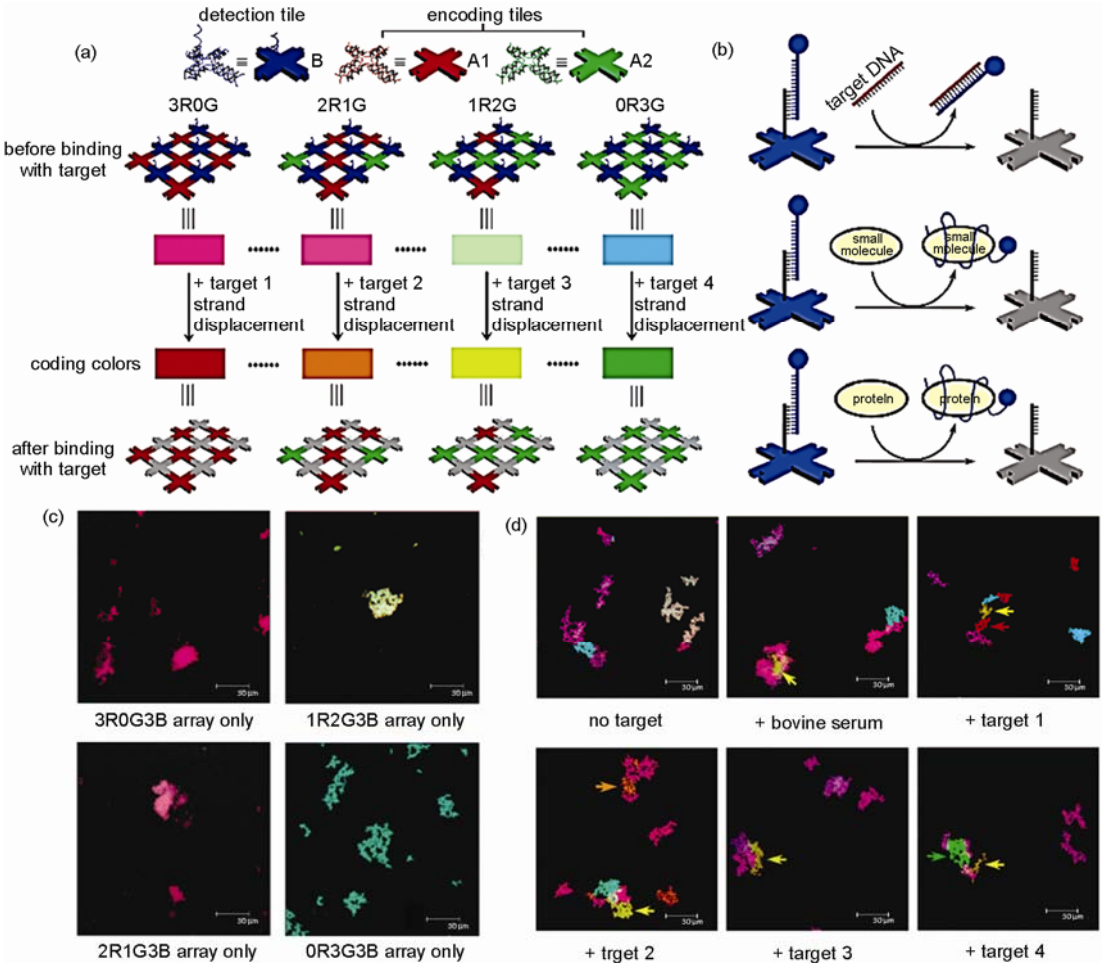

Figure 10 (a) Encoding strategy of nanoarray composed of different ratios of red and green fluorescence dyed DNA tiles, and blue fluorescence dyed detection tiles; (b) the "signal-off" mechanism of the detection tiles using oligos as probes for DNA and RNA, and aptamers for proteins and small molecules; (c) four fluorescence color encoded probes: Probes 1 (3R0G3B) and 2 (2R1G3B) are the complementary sequences of the two virus DNA sequences, severe acute respiratory syndrome virus (SARS) and the human immunodeficiency virus (HIV); probes 3 (1R2G3B) and 4 (0R3G3B) are the aptamer sequences that can specifically bind to human R-thrombin and adenosine triphosphate (ATP), respectively; (d) detection of oligos (4 μM) in bovine serum using probes in (c). In the present of only bovine serum, greenish yellow (1R2G) encoding color was revealed since a micromolar concentration of thrombin exists in serum. SARS DNA (target 1), HIV DNA (target 2), and the complementary strand to ATP aptamer (target 4) can all be detected without any ambiguity. Target 3 is the complementary strand to the thrombin binding aptamer. The arrows point to the appearance of the encoding colors. Scale bar 30μm.

the oligos within the DNA tile. The sticky ends of three kind tiles make them associate with each other alternatively to form 2D lattices. When target molecules appear, the blue dyed single strand DNA will bind with them and dissociate from the encoding nanoarrays, enabling a "signal-off" mode detection mechanism. Figure 10(c, d) shows an encoded detection in bovine serum using four nanoarray probes. In addition to the fast homogenous reaction speed (15–20 min) and washing-free due to "signal-off" mode, the advantage of this encoding method is that the codes coming from combination of different dye ratios can be precisely designed with a high reproducibility and yield due to the base-pairing rules. And the maximum encoding number is determined by the number of different intensity levels ("red" and "green") that can be distinguished by the fluorescence microscope detector in image-decoding. Therefore, the encoding capacity is governed by the similar mechanism of DNA nanobarcodes. And from Figure 10(c, d), we can conclude that the decoding is toilsome due to the overlap or stacking of nanoarrays if no image processing software is available.

3.2 Structure color encoded microcarriers

Different from fluorescence colors, structure colors come from the reflection of periodic nanostructures of dielectric material called photonic crystals, which can prohibit the propagation of electromagnetic waves in a range of frequencies [45]. The color or reflection wavelength is determined by the structural period and system refractive index of the dielectric system according to the Bragg's law, $m\lambda = 2nd\sin\theta$. Hence, they are tunable and resistant to photo destroy like photo bleaching or photo quenching, which make them a kind of ideal color encoding element. Sailor *et al*. first explored their applications as carriers in the encoded protein and DNA detection [46]. In their work, porous silicon photonic crystal flakes were fabricated by galvanostatic anodic etch of crystalline silicon wafers (Figure 11(a)). The thickness and porosity of the porous silicon were controlled by the current density, the duration of the etch cycle, and the composition of the etchant solution. By varying a computer-generated pseudo-sinusoidal current waveform, different colors were generated as reflection peaks in the VIS-NIR region with a full-width at half-maximum (FWHM) of 11 nm, which is much smaller than emission spectra of fluorescence dyes (more than 50 nm) and QDs (20 nm). And more encoding spectra were generated by etching multiple porous layers on a single flake. For example, like combination of fluorescence colors, a ten bit binary code is shown in Figure 11(c, d), composed of combinations of different reflection peaks and their intensities [47, 48]. Therefore, 4^{10}

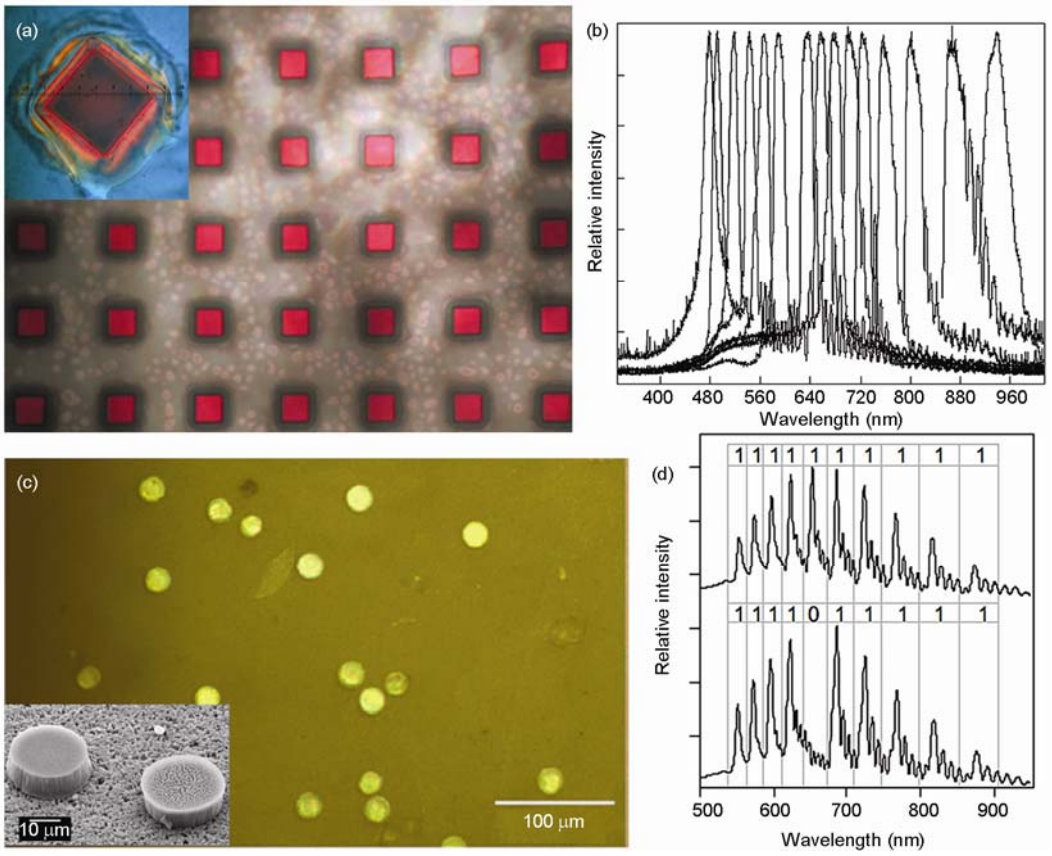

Figure 11 (a) Color image of porous silicon photonic crystal flakes; (b) reflection spectra of 15 encoding porous silicon samples; (c) color and SEM image (inset) of multilayered porous silicon flakes; (d) ten bit encoding spectra of two multilayer porous silicon samples prepared by multiple sinusoidal etch.

or 1,048,576 codes could be possible for ultrahigh level of multiplexing. However, the anisotropic 1D microparticles need to be properly oriented to avoid stacking or standing in the decoding process.

Our group have avoided this problem by doping pearl pigments, one kind of low cost 1D photonic crystal, into polystyrene microspheres and rendering them with uniform photo stable colors [49]. Another method is to use isotropic 3D photonic crystals such as photonic crystal beads. In comparison with "top-down" microfabrication technology, the "bottom-up" colloidal self-assembly maybe is the most cost-effective way to this purpose. We have also fabricated colloidal photonic crystal beads (PCBs) by droplet-templated self-assembly of monodispersed nanoparticles (Figure 12(b, c)). With a microfluidic device, uniform water-in-oil droplets of colloidal solution were generated, in which silica or polymer colloids formed ordered lattices by capillary force and Van der vas force during the dry process [50–52]. Then the mechanical strength of hence formed structure was enhanced by sintering or sticking of the nanoparticle intercourse so that they can bear any collision during the analysis applications [53]. The size and color of the opal photonic beads (Figure 12(d, e)) can be well controlled by the microfluidics and the structural period determined by the nanoparticle diameter. And inverse structure PCBs (Figure 12(f, g)) were also fabricated by inverting the nanoparticle assemblies with the interstices between them [54]. Owing to the isotropic morphology, no orientation is needed when they are used as microcarriers in encoded detection. In theory, one reflection spectrum or color in the UV-VIS-NIR range can be gained simply by self-assembly of one size of nanoparticles in the range of 100–500 nm. However, the FWHM of the beads (~25 nm) is relatively larger and the encoding number of photonic beads is limited less than 20 in the visible range.

In order to augment the encoding capacity, complex encoding strategy was introduced by combination of PCB and QDs with broad exciting and short emission spectra [55, 56]. For example, QDs are immobilized on the surface of PCBs by LbL (layer by layer) assembly (Figure 13(a)) so that the QD color and intensity are precisely controlled. In this case, the encoding number is $k(n^m-1)$, where K and m is the PCB and QDs color number respectively, and n is the intensities of the QDs that could be distinguished well. Hence, under conservative estimation, if 2 intensities and 10 PCB and QDs colors are used, the encoding number will be as large as $10(2^{10}-1)$ =10230. Actually, protein detection is usually of low level multiplexing in clinical applications. A more convenient way to enlarge the encoding number is to simply combine label-encoding and carrier encoding as illustrated in Figure 13(b), for which the code numbers will be their product (to be published), that is 100 codes for 10 PCB and QDs, which is equal to the maximum of Luminex 100/200.

Another advantage of PCB as encoded microcarrier is that the high surface to volume ratio (SVR) resulting from the ordered and porous nanostructure contributes a lot to enhanced assay sensitivity. Our research showed that the detection limit of assays based on PCB is about 100-fold lower than that on microarray for DNA detection. And the results of a multiplex detection of four tumor markers indicated that α-fetoprotein (AFP), carcinoembryonic antigen (CEA), carcinoma antigen 125 (CA 125) and carcinoma antigen 19-9 (CA 19-9), were assayed with detection limit of 0.68 ng/mL, 0.95 ng/mL, 0.99 U/mL, and 2.30 U/mL, respectively, which is comparable with those of high sensitive chemiluminescence ELISA assay Kits clinically used [57]. In addition, the photonic bandgap of PCB also provides another mechanism for signal enhancement of the detection [56, 58].

Whereas, the aforementioned encoded detections all have fluorescence dyes or QDs involved as labels. Typically, as shown in Figure 13(b), a multiplex detection using sandwich format immunoassays needs two steps of binding, each followed by several times of washings in order to remove unreacted entities. And then encoding signals and detection label signals are interrogated and acquired respectively, as shown in Figure 14(a). While in high throughput screening, this label-involved detection will be time-costing

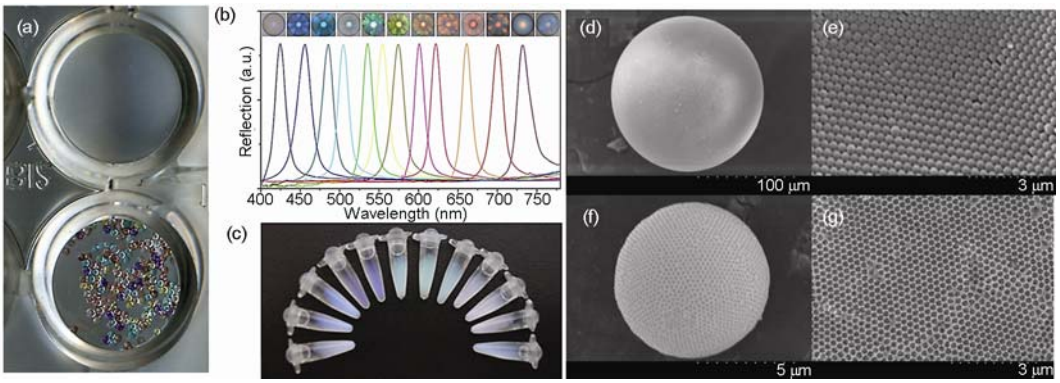

Figure 12 (a) 1D photonic crystal doped polystyrene microspheres in a 96-well plate; (b) and (c) reflection spectra of 12 kinds of colloidal photonic crystal beads in a centrifuge tube; (d) and (e) SEM image of opal colloidal photonic crystal bead and its surface structure; (f) and (g) SEM image of inverse opal colloidal photonic crystal bead and its surface structure.

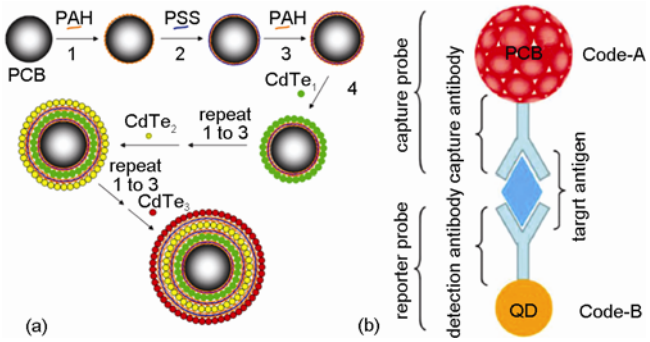

Figure 13 (a) Scheme of complex encoding by assembly of QDs on photonic crystal bead (PCB) ; (b) scheme of combination of label-encoding and carrier-encoding by QDs and PCB, respectively.

and impose high cost, complexities and difficulties on the atomization. What's more, labeling the probe brings an additional cost in spite of the activity of the probe being affected possibly. Hence, label-free detection, with which only one binding reaction is enough for the signal reading, is preferred in multiplex and high throughput applications.

With respect to photonic crystals, system refractive index or structure period changes caused by the biomolecular binding will lead to the shift of their refection peak, which makes photonic crystals a kind of promising biosensing material [59, 60]. In our research, we have used inverse opal photonic beads and films as encoded carriers for multiplex biosensing. The key lies in the fact that the peak shift caused by binding events will stop if the pore surface is saturated with bound complex. That's to say, the specific peak shift range determined by the original reflection peak of PCB carrier can be used to encode multiple label-free detection as long as the ranges will not overlap with each other, as illustrated in Figure 14(a). A multiplex label-free detection of three tumor markers, CA125, CA19-9 and CEA using three encoding inverse opal silica PCBs showed their priority over label-involved ones. Further improvements were gained in encoded detection of DNA or small molecule proteins by using hydrogel inverse opal PCBs, onto the skeleton of which recognizing moieties were incorporated to enlarge the peak shift by double response of period and refractive index to binding events. However, still limited to the FWHM of PCBs, the largest peak shift is less than 20 nm and the lowest detection limit for DNA is 10^{-9} M. Although larger peak shift preferred for high sensitivity will cut down the encoding number to less than 10 in the visible region for example, complex encoding with QDs can be adopted to offset it so that higher multiplex and label-free detection become available. Whether or not, multiplex and label-free detection based on encoded microparticles simplifies the assay procedure and lowers the cost for highly automated high throughput detections.

3.3 Bar-coded DNA tiles

It is a challenge to perform high sensitive and multiplex detection in a format of suspension array. Yan et al. proposed to use bar-coded for the detection of RNA without PCR amplifications [61]. The DNA tiles are prepared from the self-assembly of a circular single-stranded M13 viral DNA composed of 7249 bases, with more than 200 short synthetic DNA strands as helper (Figure 15(a)). There are two kinds of features on the tiles. One is the bar-code at the top left corner used to index the tiles and orient them for the AFM imaging. The other is probes dangling out of the tiles and used to detect multiplex RNA targets. Since probes at the edges of the rectangle tiles have the highest binding efficiency compared with at the inner positions, the probes are optimized to be immobilized on the edge and then are encoded by their position on the tile and the index of the tile.

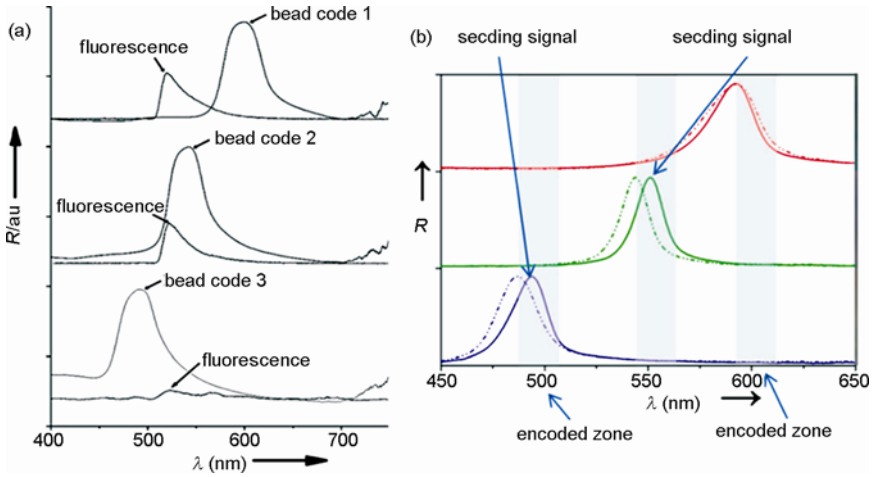

Figure 14 (a) Fluorescence and reflection spectra of three encoded photonic beads after multiplex detection FITC-tagged goat anti-human IgG and goat anti-rabbit IgG. Beads with code 1, 2 and 3 were immobilized with human, mouse, and rabbit IgG, respectively; (b) reflection spectra of three encoded photonic beads with molecular imprint of bovine Hb (hemoglobin), HRP (horseradish peroxidase), and BSA (bovine serum albumin), respectively. The cyan, green, and red dashed lines and solid lines are the spectra of the imprinted photonic beads before and after multiplex detection of Hb, and HRP. The gray areas are the encoding zone of the beads.

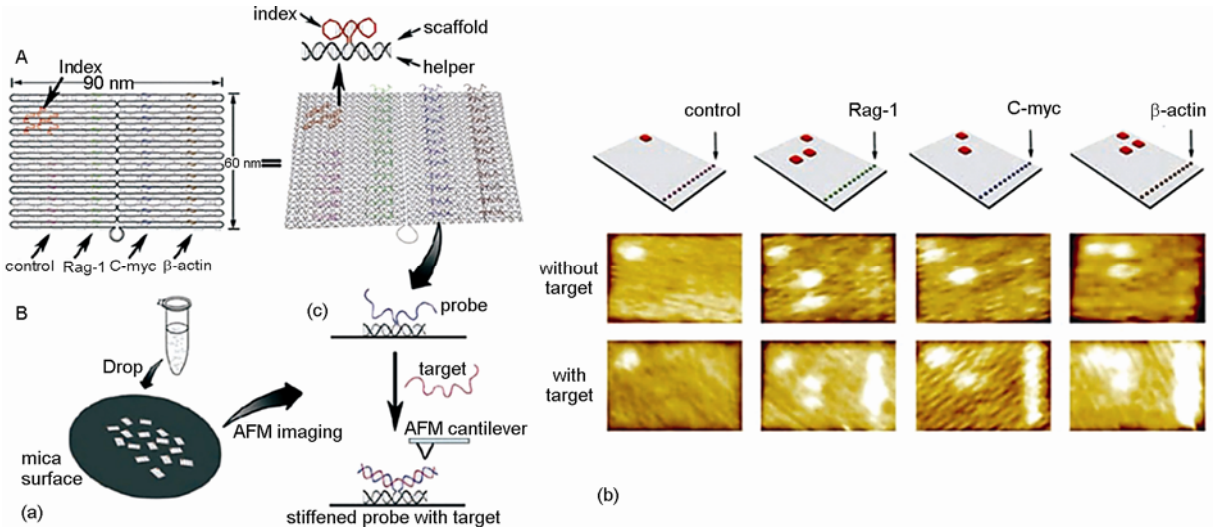

Figure 15 (a) Scheme of assay principle based on bar-coded DNA tiles. A, A rectangular-shaped indexed DNA tile (90 nm × 60 nm) bearing three different probes (for targets Rag-1, C-myc, and b-actin) and a control probe. Twelve copies of the specific probes are spaced at 5-nm intervals in a line, and lines of probes are separated by 20 nm. An index spot at the top left corner is use to orient the AFM image of each individual tile. B, Illustration of the operation process: Probe tiles hybridized with targets are dropped onto the mica surface for AFM imaging. C, Probe design and detection mechanism. A probe sequence is immobilized on a pair of neighboring helper strands extended out of the tile plane. Upon target hybridization, the double helix of the stiffer DNA-RNA duplex is readily detected with the AFM cantilever; (b) designs and usages of the bar-coded tiles. The DNA tiles are barcoded with one to three groups of topological features that distinguish them from each other. Each tile carries a line of probes for Rag-1, C-myc and β–actin gene, positioned near the right edge of the tile to optimize target-binding efficiency. Middle and bottom show AFM images of the bar-coded tiles without targets and with targets respectively.

Upon hybridization of the target to a pair of half-probes, the strands form a stiff V-shaped structure (Figure 15(a)). This local stiffening is readily sensed mechanically with an AFM cantilever and appears in the image as local high-spot (Figure 15(b)). By retrieving the index of the tiles, the probe is identified. A multiplex RNA detection demonstrated its applications (Figure 15(b)), featuring ultrahigh sensitivity and minimum sample volume owing to the single molecule detection ability of AFM, which made it appeal to single cell analysis. At the same time, the reaction is homogenous and can be completed within 30 min without washing steps. Therefore, the method appeals to the clinical applications by virtue of the very simple assay procedure. However, the signal readout by AFM takes a long time, and hence it's hard to be used for high-level multiplex detection although the bar-code has a high encoding capacity.

3.4 Nanodisk barcoding

Another method to simplify the detection process is to combine decoding and detection signal acquisition into one process. For example, Mirkin et al. used "on-wire lithography" to fabricate gold or silver nanodisk encoded microcarriers [62, 63]. First, nanowires of nickel with pairs of gold segments (Figure 16(a)) are synthesized by electroplating and then deposited randomly on a surface and covered with evaporated silica. After nickel is etched away, arrays of gold nanodisk pairs fixed along a silica 'backbone' are gained. The nanodisk pairs then acted as encodings bit of a binary barcodes. When they are used in binding detection, the capture probes are immobiolized on the gold nanodisks and the signal of the Raman labels from the captured targets will be enhanced owing to the SERS of gold nanodisk pairs. And hence, a visible code will be seen under the Raman microscope. Figure 16(C) shows a 5-bit binary barcode, and nanodisk pairs can be removed from the sting or not to create 0 or 1 digits. The encoding capacity of these nano-disk arrays is then $2^5 = 32$ codes. The encoding capacity can potentially be increased by using more disks in the array, and using a number of different Raman-active dyes, which can

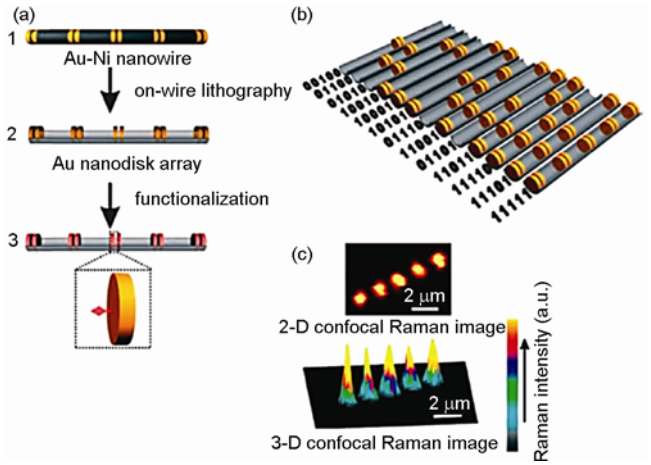

Figure 16 (a) Scheme of preparation of gold nanodisk arrays; (b) binary codes arranging sequences of '0' (nanodisk absent) and '1' (nanodisk present); (c) the binary code is read by observing the arrays using confocal Raman spectroscopy.

be identified by filtering the Raman images according to the unique Raman properties of each dye. In this method, since the codes will be seen only when there are target captured, the decoding and target signal acquisition are at the same time like the process on microarray. And because of the high sensitivity of SERS, a multiplex DNA detection with concentration as low as 100 fM was demonstrated. However, when larger numbers of codes are used, the decoding interference due to the aggregation or stacking of the nanodisk array will be unavoidable.

3.5 Pattern encoded multifunctional microparticles

It is crucial to avoid decoding interference especially for large numbers of microcarrier codes, such as barcodes, graphic or pattern codes on anisotropic microcarriers like bar or flakes. Unlike the above mentioned methods batch decoding on planar substrate using imaging or spectrometry, Doyle *et al*. transferred the encoding and decoding processes into microfluidic chip in a high throughput way [64]. As illustrated in Figure 17(a), two laminar stream pass through a microfluidic channel together. Blue stream contains fluorescent labeled PEG monomers for the code section of the particle and red stream contains PEG monomers with oligonucleotide probes attached for the analyte section of the particle (Figure 17(b, c)). By irradiating with patterned high intensity UV light through a mask and lens, the monomers are polymerized into hydrogel microparticles with dotted pattern as code adjacent to the target capturing part. In detection, a microfluidic chip is used to flow-focus the microparticles through a channel only slightly larger than the particle width and the image of it is captured by an inverted fluorescence microscope. At last, all image sequences are analyzed to determine the particle code and quantify targets (Figure 17(f)).

In this method, the encoding capacity of the pattern can be infinity if it is not limit by the size of the encoding section and the resolution of photo-polymerization. For a pattern of 20 dots used here, the number is 2^{20}. And multiple probes or probe gradients can be incorporated into the probe section (Figure 17(d, e)), which not only multiplying the encoding number but also allowing for comparison of several targets on a single particle or broadening the detection range of targets with fixed detection sensitivity. Another feature of this method is the direct incorporation of probes into the encoded particles by simply copolymerizing the acrylate modified probes with hydrogel monomers, which makes the assays more convenient as it is applicable both for DNA and protein [64–66]. What's more, since the pattern codes and target label share the same fluorescence dye, the decoding process and target signal acquisition are completed simultaneous by imaging in a flow-focus microfluidic channel, which efficiently avoids the stacking of microcarriers. It was also shown that the method can detect DNA oligomers of 500 attomoles without biotin-avidin- aided signal amplification, mostly owing to the high surface to volume ratio nature of the hydrogel polymer used herein. However, the size of multifunctional particles is relatively large (as can be seen from Figure 17(d, e)), which will cost sample volume as large as sub-milliliter especially in high level multiplex and affect the reaction kinetics severely.

Mixing the reaction solution is a key step to improve the reaction kinetics in multiplex assays based on microparticles and enables the facile separation and washing. Kwon et al. also manufactured pattern encoded microparticles for multiplex detection of DNA in a similar way [67]. However, in their method, structure color encoding and magnetic function also are incorporated. In order to enlarge the encoding capacity, the dots in the encoding pattern were dyed with a single M-Ink material [68]. The M-Ink color comes from the photonic crystal structure formed by monodispersed superparamagnetic colloidal nanocrystal clusters (CNCs) aligned in magnetic field. The structure color can be easily tuned by different magnetic intensity, which will change the lattice plane distances. Like Doyle's work, the colored dot pattern (Figure 18(a)) was formed color-by-

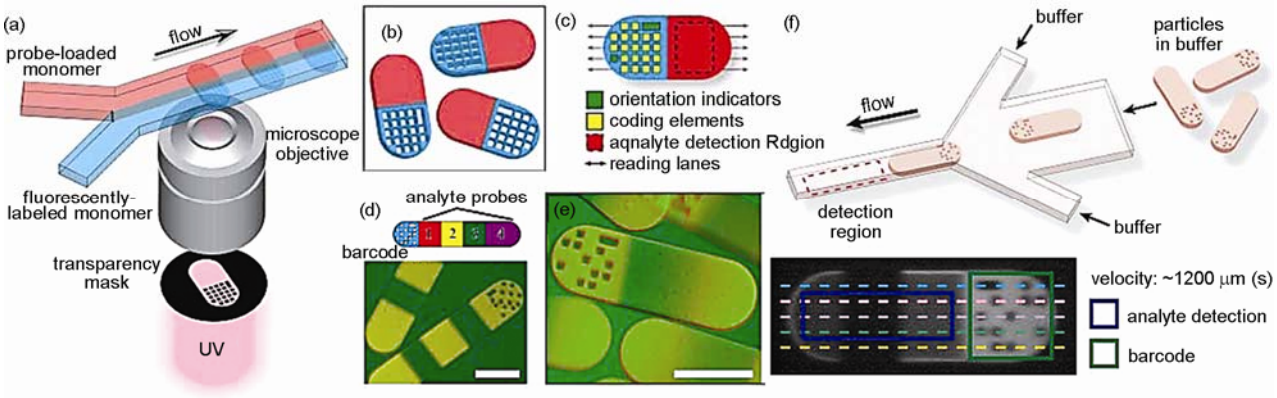

Figure 17 (a) A two laminar flow stream microchannel setup for manufacturing multifunctional microparticles with PEG photopolymer; (b, c) the structure of the multifunctional microparticles; (d, e) overlap of DIC and fluorescence image of microparticles with multiprobe and gradient probe; (f) decoding microfluidic setup for the multifunctional microparticles and a typical image of a microparticle flowing through the microfluidic channel, five dash lines indicate the fluorescence intensity measurement position used for decoding and target signal acquisition. Scale bars in (d) and (e) are both 100 μm.

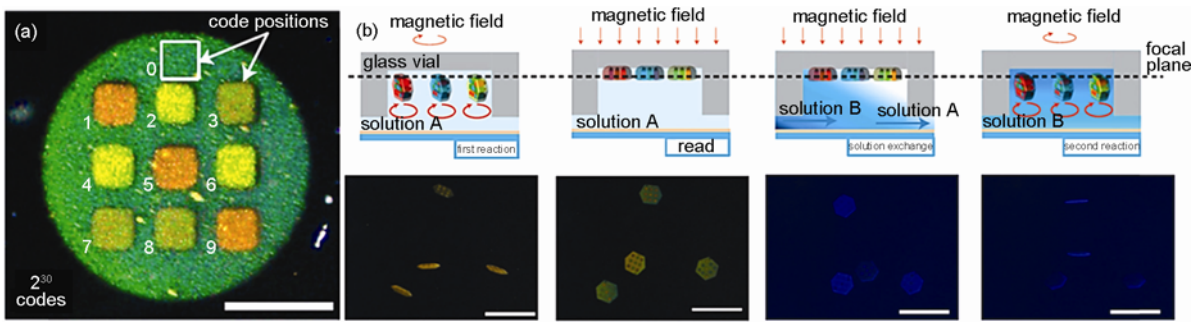

Figure 18 (a) A colored pattern encoded magnetic microparticle; (b) 3D reaction and 2D reading scheme for a multistep reaction: first 3D reaction, 2D reading, solution exchange and second 3D reaction in a different solution. The scale bar is 100 μm in (a) and 500 μm in (b).

color in a microfluidic channel by consecutive modulation the UV light with a digital micromirror device (DMD) and the magnetic field by an electromagnet. Periphery to the encoding pattern region is the hexagonal probe region also polymerized under UV light, avoiding spectral overlap between the fluorescence signal for detection of hybridization and the structural colour signal for colour encoding. The encoding number in this method amplified to $8^{10} = 2^{30}$ for a ten dot pattern with 8 colors. While in multiplex detection, the microparticles can be easily oriented or moved instead of using flow-focus like in Doyle's method. And the best feature is that during the assays (Figure 18(b)), the microparticles can be rotated at a fast rate to create microscale rotating stirrers (3D reaction) depending on the direction of the external magnetic-field lines, which will overcome the kinetic problems associated with big flake particles. Compared with assays in Doyle's method without rotation operation, the oligonucleotide hybridization time was reduced to 10 min. Also, the particles can be rotated to lie horizontally with respect to the vial surface, enabling code identification or solution exchange. Yet, the sample volume need is still high due to the big size of the microparticles. And, in high level of multiplexing, the time cost is a problem for the complex microparticle manufacture, no mention that in high throughput screening. In addition, the color code reading and target label reading are separated here, which means more time-consuming and high detection complexity.

4 Remarks and conclusion

As the rapid developments of genomics and proteomics continue, there is an increasing demand in assays for multiplex and high throughput in order to save the sample, time, labor and cost. And in the last decades, many kinds of technologies emerged taking advantages of encoding microparticles to release the biomolecular binding events from the fixed planary substrates. Some of them have been commercialized, such as fluorescence dye encoded microspheres of Luminex, Nanotags of Oxonica, Nanostring, Nanosphere, holography barcodes of Illumina. While few of them are clinically used. And table 1 compares the aforementioned encoding methods from some practical points of view in multiplex and high throughput assays. The majority of them are still staying in the lab, facing a huge gap between the research results and the requirements of real applications. Two key points may contribute to this situation.

First, much emphasis is put on the encoding number while their applications and concomitant problems are neglected. Usually pattern encoding, graphic encoding and radio frequency encoding have higher encoding capacities than color encoding or spectra encoding. For example, although the pattern encoding can produce millions of codes, these numbers are of little meaning in biological applications. The main reason is that the largest multiplicity is hundreds and will be further limited by the non-specific reactions due to the cross reaction of antibodies. However in gene express or profiling assays, the advantages of pattern or graphic encoded microparticles need to be reconsidered. Besides the long time of producing so many encoded microparticles in a complex way, the size of them is relatively big since the resolution of code unit is usually limited to tens of micron. Take 100 μm×100 μm×10 μm microparticles for an example. The volume of one million microparticles is 0.1 mL, which is much larger than the usual sample volume. And it is laborious to detect so many particles by imaging process or scanning. In this respect, microarrays still have big advantages, since resolution of the position codes on a microarray is to some extent equal to that of the pattern or graphic on a single microparticle. Moreover, this problem is more obvious when high throughput samples are to be detected. Therefore, the command on the size of the microparticles should not be counteracted by increasing the encoding number. The smaller size will result in higher surface to volume ratio and endow the assay with features like rapid homogeneous reaction, higher sensitivity and broader dynamic range. In this way, the color encoding is more flexible than pattern or graphic encoding because of its versatility in complex encoding and feasibility in combining with functional molecules like aptamers or DNA tiles.

Second, the convenience of assays operation based on

Table 1 Comparison of different multiplex assays based on encoded methods

Encoding type	Encoding method	Microparticle dimension	Encoding capacity	Limit of detection (LOD)	Reaction type	Operation (separation and washing)	Reaction vessel and readout	Reference
Label Encoding	FRET encoding	~10 nm	<10	nM–PM for nucleotide acid	homogeneous	separation and washing free	microwell plate compatible and photometer	20–22
	DNA nanobarcodes	<30 nm	hundreds to thousands	aM for nucleotide acid	heterogeneous sandwich binding	separation and washing needed	tube and fluorescence microscope	23, 24
	Nanostring	~300 nm	thousands	fM for nucleotide acid	homogeneous sandwich binding	separation and washing needed	microchip and fluorescence microscope	25, 26
	Nanoplex	~90 nm	up to millions	pg/mL for protein	homogeneous sandwich binding	magnetic separation and washing-free	microwell plate compatible and Raman spectrometer	29
	Biobarcode	~30 nm	up to million	zM for nucleotide acid and pg-fg/mL for protein	homogeneous sandwich binding	magnetic separation and washing needed	microwell plate compatible and scanometer	34–36
	Electrochemical encoding	<10 nm	tens	pg/mL for protein	homogeneous sandwich binding	magnetic separation and washing needed	tube and stripping voltammeter	39, 40
	Fluorescence encoded nanoarray	tens of μm	<100	nM for nucleotide acid	homogeneous direct binding	separation and washing free	tube and fluorescence microscope	44
	structure color encoded microcarriers	tens of microns to hundreds of μm	tens−millions	ng/mL for protein and pM for nucleotide acid	heterogeneous sandwich or direct binding	separation and washing needed	microwell plate compatible and fluorescence microscope & spectrometer	46–60
Carrier Encoding	Bar-coded DNA tiles	~ tens of nm×tens of nm	millions	single molecule	homogeneous direct binding	centrifuge separation and washing free	tube and atomic force microscope	61
	Pattern encoded multifunctional microparticles	~hundreds of μm×tens or hundreds of μm×tens of μm	millions	aM for nucleotide acid	homogeneous direct binding	separation and washing needed	microfluidic chip and fluorescence microscope	64–66

encoding microparticles is usually overlooked. The most common situation is that higher throughput is more preferred than higher level multiplexing. And the higher throughput will benefit from easier operation and higher atomization. Therefore, multifunctional microparticles, for example those with magnetic property enabling facile separation and mixing, are being extensively explored [69–71]. And washing-free or label-free detection will have great prospects on the condition that the sensitivity or accuracy is not balanced. On the other hand, in bioassays based on encoding microparticles, the particle code and the biorecognition events occurring on each particle needed to be acquired. Hence, the decoding and detection signal acquisition should be included in the design of encoding strategy. As mentioned above, it will greatly reduce the complexity of assays to obtain the two kinds of data at the same time by only one readout. And microfluidic chips will be a good optional platform to integrate the assay operations especially when they have good compatibility with existing automatization instrument or detection platform, which is also a very promising direction in the development of future encoding technology.

In summary, the multiplex and high throughput detection technology is in a relatively early stage of development, and more novel methods and materials are expected to be explored in the encoding of multiplex bioassays not only *in vitro*, but also in cell and *in vivo*. Also, the encoding microparticles are anticipated to be more widely applied in a variety of practical assays with high speed, throughput, accuracy, and automation becoming increasingly achievable in the next few years.

We are grateful for the support of Grant for Teaching and Research of Excellent Young Teachers in Southeast University, 333 Talent Project Foundation and the Qing Lan Project of Jiangsu Province, Jiangsu Science and Technology Department (BE2009148 & Bk2008318), and the National Natural Science Foundation of China (50925309, 20703010 & 21073033), and Y. J. Z. thanks the Scientific Research Foundation of the Graduate School of Southeast University.

1 Hayden EC. Life is complicated. *Nature*, 2010, 464(7289): 664–667
2 Ekins RP. Ligand assays: from electrophoresis to miniaturized microarrays. *Clin Chem*, 1998, 44(9): 2015–2030
3 Nadon R, Shoemaker J. Statistical issues with microarrays: Processing and analysis. *Trends Genet*, 2002, 18(5): 265–271
4 Miklos GLG, Maleszka R. Microarray reality checks in the context of a complex disease. *Nat Biotechnol*, 2004, 22(5): 615–621
5 Canales RD, Luo YL, Willey JC, Austermiller B, Barbacioru CC, Boysen C, Hunkapiller K, Jensen RV, Knight CR, Lee KY, Ma YQ, Maqsodi B, Papallo A, Peters EH, Poulter K, Ruppel PL, Samaha RR, Shi LM, Yang W, Zhang L, Goodsaid FM. Evaluation of DNA microarray results with quantitative gene expression platforms. *Nat Biotechnol*, 2006, 24(9): 1115–1122
6 Jin SQ, Ye BC, Huo H, Zeng AJ, Xie CK, Ren BQ, Huang HJ. Multiplexed bead-based mesofluidic system for gene diagnosis and genotyping. *Anal Chem*, 2010, 82(23): 9925–9931

7 Hu L, Zuo P, Ye BC. Multicomponent mesofluidic system for the detection of veterinary drug residues based on competitive immunoassay. *Anal Biochem*, 2010, 405(1): 89–95
8 Jin SQ, Yin BC, Ye BC. Multiplexed bead-based mesofluidic system for detection of food-borne pathogenic bacteria. Appl Environ Microb, 2009, 75(21): 6647–6654
9 Zhang D, Zuo P, Ye BC. Bead-based mesofluidic system for residue analysis of chloramphenicol. *J Agr Food Chem*, 2008, 56(21): 9862–9867
10 Mandy FF, Nakamura T, Bergeron M, Sekiguchi K. Overview and application of suspension array technology. *Clin Lab Med*, 2001, 21(4): 713–+
11 Pietz BC, Warden MB, DuChateau BK, Ellis TM. Multiplex genotyping of human minor histocompatibility antigens. *Hum Immunol*, 2005, 66(11): 1174–1182
12 Haab BB. Antibody Arrays in cancer research. *Mol Cell Proteomics*, 2005, 4(4): 377–383
13 Jaras K, Ressine A, Nilsson E, Malm J, Marko-Varga G, Lilja H, Laurell T. Reverse-phase versus sandwich antibody microarray, technical comparison from a clinical perspective. *Anal Chem*, 2007, 79(15): 5817–5825
14 Wang L, Zhao WJ, Tan WH. Bioconjugated silica nanoparticles: Development and applications. *Nano Res*, 2008, 1(2): 99–115
15 Lee J, Hernandez P, Lee J, Govorov AO, Kotov NA. Exciton-plasmon interactions inmolecular spring assemblies of nanowires and wavelength-based protein detection. *Nat Mater*, 2007, 6(4): 291–295
16 Dubertret B, Calame M, Libchaber AJ. Single-mismatch detection using gold-quenched fluorescent oligonucleotides. *Nat Biotechnol*, 2001, 19(7): 680–681
17 Seferos DS, Giljohann DA, Hill HD, Prigodich AE, Mirkin CA. Nano-flares: Probes for transfection and mRNA detection in living cells. *J Am Chem Soc*, 2007, 129(50): 15477–+
18 Maxwell DJ, Taylor JR, Nie SM. Self-assembled nanoparticle probes for recognition and detection of biomolecules. *J Am Chem Soc*, 2002, 124(32): 9606–9612
19 Yang RH, Jin JY, Chen Y, Shao N, Kang HZ, Xiao Z, Tang ZW, Wu YR, Zhu Z, Tan W H. Carbon nanotube-quenched fluorescent oligonucleotides: Probes that fluoresce upon hybridization. *J Am Chem Soc,* 2008, 130(26): 8351–8358
20 Song SP, Zhang J, Wang LH, Zhang H, Boey F, Fan CH. Aptamer-based multicolor fluorescent gold nanoprobes for multiplex detection in homogeneous solution. *Small,* 2010, 6(2): 201–204
21 Li GX, Song SP, Liang ZQ, Zhang J, Wang LH, Fan CH. Gold-nanoparticle-based multicolor nanobeacons for sequence-specific DNA analysis. *Angew Chem Int Ed*, 2009, 48(46): 8670– 8674
22 He SJ, Song B, Li D, Zhu CF, Qi WP, Wen YQ, Wang LH, Song SP, Fang HP, Fan CH. A graphene nanoprobe for rapid, sensitive, and multicolor fluorescent DNA analysis. *Adv Funct Mater*, 2010, 20(3): 453–459
23 Li YG, Tseng YD, Kwon SY, D'Espaux L, Bunch JS, Mceuen PL, Luo D. Controlled assembly of dendrimer-like DNA. *Nat Mater*, 2004, 3(1): 38–42
24 Li YG, Cu YTH, Luo D. Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes. *Nat Biotechnol*, 2005, 23(7): 885–889
25 Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, Fell HP, Ferree S, George RD, Grogan T, James JJ, Maysuria M, Mitton JD, Oliveri P, Osborn JL, Peng T, Ratcliffe AL, Webster PJ, Davidson EH, Hood L. Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nat Biotechnol*, 2008, 26(3): 317–325
26 Su YH, Li E, Geiss GK, Longabaugh WJR, Kramer A, Davidson EH. A perturbation model of the gene regulatory network for oral and aboral ectoderm specification in the sea urchin embryo. *Dev Biol*, 2009, 329(2): 410–421
27 Nicewarner-Pena SR, Freeman RG, Reiss BD, He L, Pena DJ, Walton ID, Cromer R, Keating CD, Natan MJ. Submicrometer metallic barcodes. *Science*, 2001, 294(5540): 137–141
28 Qian XM, Peng XH, Ansari DO, Yin-Goen Q, Chen GZ, Shin DM, Yang L, Young AN, Wang MD, Nie SM. *In vivo* tumor targeting and spectroscopic detection with surface-enhanced Raman nanoparticle tags. *Nat Biotechnol*, 2008, 26(1): 83–90
29 Doering WE, Piotti ME, Natan MJ, Freeman RG. SERS as a foundation for nanoscale, optically detected biological labels. *Adv Mater*, 2007, 19(20): 3100–3108
30 Xu HX, Aizpurua J, Kall M, Apell P. Electromagnetic contributions to single-molecule sensitivity in surface-enhanced Raman scattering. *Phys Rev E*, 2000, 62(3): 4318–4324
31 Jiang J, Bosnick K, Maillard M, Brus L. Single molecule Raman spectroscopy at the junctions of large Ag nanocrystals. *J Phys Chem B*, 2003, 107(37): 9964–9972
32 Cao YWC, Jin RC, Mirkin CA. Nanoparticles with Raman spectroscopic fingerprints for DNA and RNA detection. *Science*, 2002, 297(5586): 1536–1540
33 Chen Z, Tabakman SM, Goodwin AP, Kattah MG, Daranciang D, Wang XR, Zhang GY, Li XL, Liu Z, Utz PJ, Jiang KL, Fan SS, Dai HJ. Protein microarrays with carbon nanotubes as multicolor Raman labels. *Nat Biotechnol*, 2008, 26(11): 1285–1292
34 Nam JM, Thaxton CS, Mirkin CA. Nanoparticle-based bio-bar codes for the ultrasensitive detection of proteins. *Science*, 2003, 301(5641): 1884–1886
35 Nam JM, Park SJ, Mirkin CA. Bio-barcodes based on oligonucleotide-modified nanoparticles. *J Am Chem Soc,* 2002, 124(15): 3820–3821
36 Nam JM, Stoeva SI, Mirkin CA. Bio-bar-code-based DNA detection with PCR-like sensitivity. *J Am Chem Soc*, 2004, 126(19): 5932–5933
37 Goluch ED, Stoeva SI, Lee JS, Shaikh KA, Mirkin CA, Liu C. A microfluidic detection system based upon a surface immobilized biobarcode assay. *Biosens Bioelectron*, 2009, 24(8): 2397–2403
38 Goluch ED, Nam JM, Georganopoulou DG, Chiesl TN, Shaikh KA, Ryu KS, Barron AE, Mirkin CA, Liu C. A bio-barcode assay for on-chip attomolar-sensitivity protein detection. *Lab Chip*, 2006, 6(10): 1293–1299
39 Wang J, Liu GD, Merkoci A. Electrochemical coding technology for simultaneous detection of multiple DNA targets. *J Am Chem Soc*, 2003, 125(11): 3214–3215
40 Wang J, Liu GD, Munge B, Lin L, Zhu QY. DNA-based amplified bioelectronic detection and coding of proteins. *Angew Chem Int Ed*, 2004, 43(16): 2158–2161
41 Braeckmans K, De Smedt SC, Leblans M, Pauwels R, Demeester J. Encoding microcarriers: Present and future technologies. *Nat Rev Drug Discov*, 2002, 1(6): 447–456
42 Wilson R, Cossins AR, Spiller DG. Encoded microcarriers for high-throughput multiplexed detection. *Angew Chem Int Ed*, 2006, 45(37): 6104–6117
43 Han MY, Gao XH, Su JZ, Nie S. Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules. *Nat Biotechnol*, 2001, 19(7): 631–635
44 Lin CX, Liu Y, Yan H. Self-assembled combinatorial encoding nanoarrays for multiplexed biosensing. *Nano Lett*, 2007, 7(2): 507–512
45 Taubes G. Physics - Photonic crystal made to work at an optical wavelength. *Science*, 1997, 278(5344): 1709–1710
46 Cunin F, Schmedake TA, Link JR, Li YY, Koh J, Bhatia SN, Sailor MJ. Biomolecular screening with encoded porous-silicon photonic crystals. *Nat Mater*, 2002, 1(1): 39–41
47 Meade SO, Yoon MS, Ahn KH, Sailor MJ. Porous silicon photonic crystals as encoded microcarriers. *Adv Mater*, 2004, 16(20): 1811–+
48 Meade SO, Chen MY, Sailor MJ, Miskelly GM. Multiplexed DNA detection using spectrally encoded porous sio2 photonic crystal particles. *Anal Chem*, 2009, 81(7): 2618–2625
49 Zhao XW, Liu ZB, Yang H, Nagai K, Zhao YH, Gu ZZ. Uniformly colorized beads for multiplex immunoassay. *Chem Mater*, 2006, 18(9): 2443–2449
50 Zhao XW, Cao Y, Ito F, Chen HH, Nagai K, Zhao YH, Gu ZZ. Colloidal crystal beads as supports for biomolecular screening. *Angew Chem Int Ed*, 2006, 45(41): 6835–6838

51 Zhao YJ, Zhao XW, Sun C, Li J, Zhu R, Gu ZZ. Encoded silica colloidal crystal beads as supports for potential multiplex immunoassay. *Anal Chem*, 2008, 80(5): 1598–1605

52 Sun C, Zhao XW, Zhao YJ, Zhu R, Gu ZZ. Fabrication of colloidal crystal beads by a drop-breaking technique and their application as bioassays. *Small*, 2008, 4(5): 592–596

53 Zhao XW, Zhao YJ, Hu J, Xu M, Zhao WJ, Gu ZZ. Sintering photonic beads for multiplex biosensing. *J Nanosci Nanotechno*, 2010, 10(1): 588–594

54 Zhao YJ, Zhao XW, Hu J, Xu M, Zhao WJ, Sun LG, Zhu C, Xu H, Gu ZZ. Encoded porous beads for label-free multiplex detection of tumor markers. *Adv Mater*, 2009, 21(5): 569–+

55 Li J, Zhao XW, Zhao YJ, Gu ZZ. Quantum-dot-coated encoded silica colloidal crystals beads for multiplex coding. *Chem Commun*, 2009(17): 2329–2331

56 Li J, Zhao XW, Zhao Y , Hu J, Xu M, Gu ZZ. Colloidal crystal beads coated with multicolor CdTe quantum dots: microcarriers for optical encoding and fluorescence enhancement. *J Mater Chem*, 2009, 19(36): 6492–6497

57 Zhao YJ, Zhao XW, Pei XP, Hua J, Zhao WJ, Chen BA, Gu ZZ. Multiplex detection of tumor markers with photonic suspension array. *Anal Chim Acta*,2009, 633(1): 103–108

58 Ganesh N, Zhang W, Mathias PC, Chow E, Soares JANT, Malyarchuk V, Smith AD, nningham BT. Enhanced fluorescence emission from quantum dots on a photonic crystal surface. *Nat Nanotechnol*, 2007, 2(8): 515–520

59 Qian WP, Gu ZZ, Fujishima A, to O. Three-dimensionally ordered macroporous polymer materials: An approach for biosensor applications. L*angmuir*, 2002, 18(11): 4526–4529

60 Shamah SM, nningham BT. Label-free cell-based assays using photonic crystal optical biosensors. *Analyst*, 20, 136(6): 1090–1102

61 Ke YG, Lindsay S, Chang Y, Liu Y, Yan H. Self-assembled water-soluble nucleic acid probe tiles for label-free RNA hybridization assays. *Science*, 2008, 319(5860): 180–183

62 Qin L, Banholzer MJ, Millstone JE, Mirkin CA. Nanodisk codes. *Nano Lett*, 2007, 7(12): 3849–3853

63 Banholzer MJ, Osberg KD, Li SZ, Mangelson BF, Schatz GC, Mirkin CA. Silver-based nanodisk codes. *Acs Nano*, 2010, 4(9): 5446–5452

64 Pregibon DC, Toner M, Doyle PS. Multifunctional encoded particles for high-throughput biomolecule analysis. *Science*, 2007, 315(5817): 1393–1396

65 Appleyard DC, Chapin SC, Doyle PS. Multiplexed Protein Quantification with Barcoded Hydrogel Microparticles. *Anal Chem*, 2011, 83(1): 193–199

66 Pregibon DC, Doyle PS. Optimization of encoded hydrogel particles for nucleic acid quantification. *Anal Chem*, 2009, 81(12): 4873–4881

67 Lee H, Kim J, Kim H, Kim J, Kwon S. Colour-barcoded magnetic microparticles for multiplexed bioassays. *Nat Mater*, 2010, 9(9): 745–749

68 Kim H, Ge J, Kim J, Choi S, Lee H, Lee H, Park W, Yin Y, Kwon S. Structural colour printing using a magnetically tunable and lithographically fixable photonic crystal. *Nat Photonics*, 2009, 3(9): 534–540

69 Hu J, Xie M, Wen CY, Zhang ZL, Xie HY, Liu AA, Chen YY, Zhou SM, Pang DW. A multicomponent recognition and separation system established via fluorescent, magnetic, dualencoded multifunctional bioprobes. *Biomaterials*, 2011, 32(4): 1177–1184

70 Nair PR, Alam MA. Theoretical detection limits of magnetic biobarcode sensors and the phase space of nanobiosensing. *Analyst*, 2010, 135(11): 2798–2801

71 Peng QW, Cao ZJ, Lau CW, Kai M, Lu JZ. Aptamer-barcode based immunoassay for the instantaneous derivatization chemiluminescence detection of IgE coupled to magnetic beads. *Analyst*, 2011, 136(1): 140–147