Optical encoding of microbeads for gene screening: alternatives to microarrays

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Rapid access to genetic information is central to the revolution presently occurring in the pharmaceutical industry, particularly in relation to novel drug target identification and drug development. Genetic variation, gene expression, gene function and gene structure are just some of the important research areas requiring efficient methods of DNA screening. Here, we highlight state-of-the-art techniques and devices for gene screening that promise cheaper and higher-throughput yields than currently achieved with DNA microarrays. We include an overview of existing and proposed bead-based strategies designed to dramatically increase the number of probes that can be interrogated in one assay. We focus, in particular, on the issue of encoding and/or decoding (bar-coding) large bead-based libraries for HTS.

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▼ We are just beginning to see how radically gene-based science can change the ways in which new drugs are discovered and developed. In the future, new generations of drugs will increasingly be tailored to individual patients and will target not only disease treatment but also disease prevention. Within the next decade, doctors will be able to remove much of the guesswork from diagnosis and drug prescription by sequencing a patient's DNA (Ref. 1). Tests will be available that will either guide preventative therapy, signal a need for lifestyle changes, or enable early intervention with medicines. However, it is not just healthcare that will benefit. To understand how genes work, scientists will compare them between many individuals at different stages of life and under different conditions. By studying the genetic activity of many individuals, the secrets of human diseases and traits will be unlocked and the normal variants that make each of us unique will be identified[2]. Eventually, the scientific community will determine how the human genome differs from other species and then establish how that makes humans distinct at the biochemical level.

DNA microarrays – why search for alternatives?

DNA microarrays (Fig. 1) are sophisticated tools for applications such as rapid detection of known genetic disorders; polymorphic variation within a species or subgroup; the time and place of expression of RNA during development; and primary DNA sequence in coding and regulatory regions[3]. Production of microarray devices is relatively well-defined and automated, thereby ensuring that they remain attractive to those involved in biopharmaceutical research[4]. However, although microarrays have already had a major impact on high-throughput gene screening, they must become more economical in terms of capital equipment outlays, operating expenses, sample preparation, analysis times, and array and reagent costs. A major disadvantage of microarrays is that they are currently unable to carry libraries of greater than a few hundred thousand compounds because of their 2D geometry and limitations in detection capabilities. For example, DNA microarrays that analyze genetic variability currently exist, but are limited to identification of a few types of genetic variation and they cannot contend with the entire human genome[5].

Microarrays have been shown to be suitable for monitoring gene expression quantitatively by addressing questions concerning individual gene

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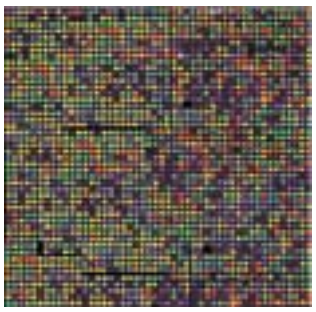


Figure 1. Comparison of DNA microarrays with microbead-based libraries. (a) Currently, DNA microarrays can carry a maximum of 5×10^5 oligonucleotide probes, immobilized at spatially resolved sites on a 2D solid support. (b) By comparison, microbeads exist in three dimensions and can contain oligonucleotide libraries of $>10^{10}$ compounds.

function, functional pathways and how cellular components work together to regulate and perform cellular processes[6]. Although microarrays have the capability of detecting a wide range of gene expression levels, such measurements are subject to variability relating to probe hybridization differences and cross-reactivity, differences between elements within microarrays, and differences from one array to another[7–10]. The ability to perform such analyses for extremely large numbers of gene probes ($>10^{10}$) on an inexpensive and well-defined high-throughput platform is highly desirable and will be the major challenge over the next few years.

Microbead-based libraries as alternatives to microarrays

Microbead-based libraries are emerging as a very attractive alternative to DNA microarrays because they promise a breakthrough in miniaturization by using significantly smaller probe sites (i.e. microbeads instead of spatially resolved sites on a microarray) and a 3D configuration (Fig. 1). Rather than preparing oligonucleotide (short, single-stranded DNA) probes in pixels on a microarray, the probes can be attached to (or synthesized on) either polymer or ceramic beads that are 2–300 μm in diameter (Table 1). Some of the most impressive advantages of microbeads are that (1) they are inexpensive to produce in large numbers, (2) they can be conveniently stored in small volumes of fluid, and (3) they can be easily optically bar-coded and screened using a variety of detection technologies[11]. Another important advantage, which has not been fully utilized to date, is the ability to generate large random libraries of gene probes via combinatorial split-and-mix processes[12,13].

Flow cytometers are well-suited to the task of hybridization detection and classification and/or decoding of microbead-based DNA libraries. A flow cytometer can rapidly screen complex fluorescence and light scattering signals, and the technique has been adopted by many groups in their microbead-based assay processes. The Luminex Corporation (Austin, TX, USA) has developed a multiplexed microbead-based system that enables the assay of up to 100 different probes in one sample tube or well[14,15]. The microbeads are internally dyed with two fluorophores of varying concentrations, and a flow cytometer is used to distinguish separate sets of microspheres by analyzing the fluorophore ratio. Surface adsorbed proteins, such as streptavidin, facilitate attachment of probes to the microbeads to create a library containing up to 100 different probes. A third fluorophore, also detectable in the flow cytometer, is used to label the target molecules. The multiple analyte profiling (MAP) technique has been demonstrated for a range of assays involving immunoglobulins, human cytokines, human immunodeficiency virus (HIV) and hepatitis B seroconversion, single-nucleotide polymorphism (SNP) analysis, and DNA-based tissue typing.

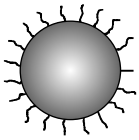
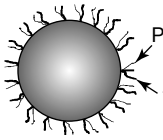
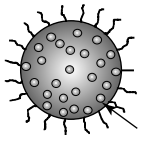
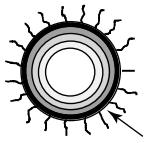
Several studies have demonstrated the utility of microbeads for hybridization analyses in both uniplex and multiplex fashions[15–19]. Most of these have used indirect or sandwich-type hybridization formats. Recently, the feasibility of multiplexed genotyping with microbead suspensions using direct hybridization analysis was demonstrated[20]. Using 32 different types of optically encoded microspheres and a flow cytometer, a 32-plex assay was performed, where the genotypes of eight different polymorphic genes were simultaneously determined.

Another example of hybridization analyses using microbeads includes the direct quantitation of HIV using flow cytometry[21]. In this assay, microbeads are derivatized with nucleic acid capture probes. The derivatized beads are used to capture single nucleic acid targets, which then capture fluorescent reporter probes via branched DNA. This assay format lends itself to multiplexing in the future by using microbeads of different sizes or colors in which the different beads are derivatized with capture probes for different targets.

Lynx Therapeutics (Hayward, CA, USA) has developed a DNA sequencing method termed 'massively parallel signature sequencing' (MPSS) analysis[7,22]. Typically, one MPSS machine can generate around 500,000 of 17 base-pair signature sequences per week. The potential for gene expression analysis was demonstrated by successfully comparing expression levels of genes of the human acute monocytic leukemia cell-line, THP-1, with the levels found by conventional sequencing[7].

An approach for mutational scanning of microbead-immobilized polymerase chain reaction (PCR) products by

Table 1. Encoding/decoding strategies for gene libraries

	Encoding	Decoding	Library size
(a)  Probe attached to bead	Polymer microbeads are internally and uniformly dyed with 2–3 fluorescent dyes	Flow cytometry analysis, digital imaging, optical fibers	100–270,000 probes (Refs 14–26)
(b)  Probe synthesized on bead; Tag synthesized on bead	Molecular tags are covalently synthesized in parallel with combinatorial synthesis of probe	Tag is cleaved from the microbead and analyzed further using mass spectrometry	$<10^6$ probes (Ref. 29)
(c)  Probes immobilized on spatially resolved sites on a 2D solid support	Positional encoding	Decoding via position in the microarray	$<10^6$ probes (Refs 1–3)
(d)  Probe synthesized on bead; Reporter	Encoded fluorescent 'reporter' particles are permanently attached to microbeads during split-and-mix synthesis (active encoding)	The color combination bundled into each reporter particle is read in parallel via automated detection instruments	$>10^{10}$ probes (Refs 12,13)
(e)  Probe synthesized on bead; Fluorescent layers	The unique 'optical signature' of each multi-fluorescent microbead is tracked via flow cytometry during the combinatorial synthesis of the probe. The microbead is composed of fluorescent dye layers around core particles	The optical signature is analyzed by flow cytometry and reaction history of the bead is determined by recalling data stored by the flow cytometer software during probe synthesis	$>10^{10}$ probes (Ref. 27)

Increasing library size ↓

subtractive oligonucleotide hybridization analysis[23] has led to the successful detection and approximate localization of single mutations in exons 6 and 7 of the human *p53* tumor-suppressor gene. In addition, IGEN (Gaithersburg, MD, USA) has engineered a system to screen for rarely transcribed differentially expressed genes[24]. This system is based on amplified detection using a ruthenium chelate, and uses streptavidin-coated magnetic microbeads as the solid-phase for an affinity reaction.

Bead-based optical fiber arrays are being developed by Illumina (San Diego, CA, USA) for applications such as SNP genotyping and high-throughput drug discovery[25,26]. These arrays are formed by creating microwells at the tips of optical fiber bundles, and filling the wells with probe-labeled beads. Although the arrays are easy to prepare, the probe-labeled beads are randomly self-assembled in each array and strategies must be devised for decoding each array. One strategy is to use optically encoded microbeads so that each bead in the array can be independently identified[23].

Towards extremely large microbead-based libraries

Most of the microbead-based screening technologies already described are still to reach maturity, and although they show the potential of eventually being able to deal with large libraries, they cannot yet compete with DNA microarray devices in terms of the number of probes that can be used for each assay. Furthermore, it is worth bearing in mind that 1 ml of a typical microbead suspension (containing 3 μm diameter microbeads at 20% solids) might contain $>10^{10}$ beads (Ref. 25). Creating libraries of 10^{10} compounds on microbeads is clearly a challenge, but if this can be accomplished, these libraries would greatly surpass microarrays in terms of library numbers, cost-efficiency and value.

Our group at the University of Queensland (Brisbane, Australia) is developing strategies for producing extremely large encoded libraries that can be used for applications such as gene screening and drug discovery[12,13,27]. The methods center on the use of the combinatorial split-and-mix process[28–31] for producing large chemical libraries ($>10^{10}$ compounds) in a

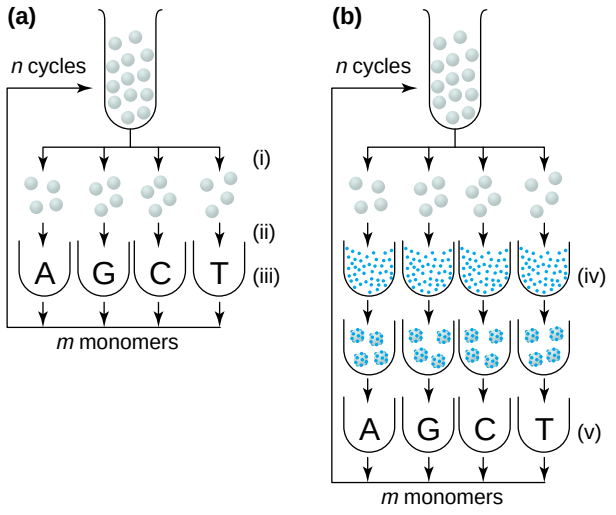


Figure 2. Split-and-mix DNA library synthesis on microbeads. **(a)** In the split-and-mix cycle, a large number of beads is equally partitioned into vessels (m = 4) (i); a different monomer (i.e. one of four nucleic acids, A, G, C or T) is reacted with each portion (ii); and (iii) the beads are recombined to complete the cycle. The split-and-mix process is repeated for a chosen number of cycles (n) resulting in a large DNA library comprising all combinations of oligonucleotides of length 'n'. The number of oligonucleotides in the library is given by m^n. **(b)** Bar-coding with fluorescent reporter particles is an efficient method of actively encoding each microbead during library synthesis[12,13]. In this modified split-and-mix process, smaller (color-coded) reporter particles are permanently physically attached to the microbeads (iv) before a monomer reaction (v). The color code within each reporter particle uniquely identifies the cycle number and monomer type for one individual reaction in the split-and-mix reaction pathway. After the split-and-mix process is completed, each microbead will contain n different varieties of reporter beads, the set of which reveals the precise reaction history of the microbead. Decoding can occur in parallel with the use of typically 3–4 optical filters.

small number of reaction steps. By applying the split-and-mix parallel reaction process (Fig. 2a) to a large number of microbeads, an oligonucleotide library of $>10^{10}$ compounds can be produced in a facile and inexpensive manner within a few hours. [Each reaction cycle (n) takes about 15 min in an automatic DNA synthesizer and the number of compounds in the library is 4^n. After n = 17 cycles, more than 10^{10} different 17-mer oligonucleotide probes attached to microbeads can be prepared.]

Despite its significant advantage (in terms of compound numbers), split-and-mix synthesis of DNA libraries on microbeads has rarely been reported[30,31]. The major reason for this appears to be the inherent difficulty of encoding the library such that the sequence of each oligonucleotide probe can be identified without cleavage of the probe from the bead. As DNA does not have intrinsic properties that are useful for direct high-sensitivity detection, the DNA sequence on each bead cannot easily be determined. For microbead-based screening with extremely large oligonucleotide libraries, encoding becomes a significant challenge. The conventional strategies for encoding split-and-mix libraries, such as covalently linking molecular 'identifier' tags (e.g. nucleic acids, secondary amines, fluorophenyl ethers and fluorescent molecules)[32–34] to the beads in parallel with the compound synthesis [Table 1(b)], are far from optimized or are entirely unsuitable for oligonucleotide libraries. These encoding methods usually require laborious and expensive methods for covalently attaching and decoding the molecular tags.

Our approach involves elaborate optical encoding of microbeads used for split-and-mix synthesis of large libraries, as well as the use of novel bead-types to enable organic synthesis[12,13,27] [Table 1(d–e)]. The libraries produced differ radically from those already described. The important distinction is that the previous libraries are created by attaching prepared probes to pre-encoded microbeads in aqueous media, while synthesis of our libraries occurs on the microbeads in a random fashion in solvent conditions (a procedure we have termed 'active encoding'). Such an approach has several powerful advantages: (1) because information is encoded onto the microbeads during a split-and-mix synthesis, libraries of enormous complexity could be produced and bar-coded in a facile manner; (2) the parallel split-and-mix reaction procedure enables the production of large libraries in a low number of reaction cycles; and (3) the active encoding procedure produces large libraries in a format that is ready to read and decode via a variety of detectors in a facile and high-throughput manner.

Fig. 2b illustrates one of these methods. This method involves the active generation of a 'fluorescent barcode' on each microbead during split-and-mix compound synthesis through the attachment of silica 'reporter' particles (0.2–5.0 μm in diameter), which contain specific and identifiable combinations of fluorescent dyes[12,13] [Table 1(d)]. In this split-and-mix process, the microbeads are split into several portions and each portion is mixed with a different reporter suspension. At least 50 reporter particles are permanently attached to each microbead by manipulation of physical colloidal forces (Fig. 3a). The reporter suspensions are distinguishable by the reporters they contain; reporters within a suspension are identical (i.e. the same size, dye combination and intensity), but a different dye combination is contained within reporters from different suspensions. Each portion is then reacted with a different monomer (e.g. nucleic acid) and the portions are recombined to complete the split-and-mix cycle. These processes are repeated for a chosen number of cycles (n), using different reporter suspensions to encode each reaction. This results in an encoded chemical library consisting of all combinations of monomer of probe length n.

The different fluorescent barcode actively generated on each microbead during probe synthesis (Fig. 3) is a record of the reaction history of each bead. Deciphering the barcode on any individual microbead could be achieved very simply by using a fluorescence microscope or an automated detection instrument (Fig. 3c), which can detect both the location of each reporter on the microbead and the color combination within the reporter. Typically, this is accomplished by the use of multiple optical filters. As each reporter encodes for a known monomer in a known cycle in the split-and-mix synthesis, identifying the dyes located within each reporter readily elucidates the structure of the probe synthesized on the microbead.

The power of this encoding approach lies in the efficient use of a relatively few number of fluorescent dyes to record a large amount of information on each microbead. A simple combinatoric analysis of this process generates the following equation:

$$\text{Max compounds} = \left(\frac{2^c}{n}\right)^n \qquad (1)$$

where max compounds = maximum number of compounds uniquely encoded by this method; n = number of cycles in the split-and-mix synthesis; and c = total number of colors/dyes used in the encoding procedure.

Because Eqn (1) contains a power raised to a power, the number of compounds that can be encoded by this method becomes explosive with increasing number of fluorescent dyes used. Libraries containing 4.3×10^9 oligonucleotides (4^{16} probes) can therefore be encoded with just six fluorescent dyes (c = 6). Indeed, the power of this approach can be demonstrated by considering a combinatorial reaction matrix that involves all 20 known amino acids and 25 split-and-mix reaction cycles. Such a reaction matrix would produce 20^{25} ($\sim 10^{32}$) bead-based compounds (larger than the mass of the earth) and would only require nine colors to completely and uniquely encode the entire library[12,13]. This example is, of course, an absolute extreme. We therefore envisage that no more than seven or eight spectroscopically-distinct fluorescent dyes will ever be required in practice.

A second bar-coding and screening method that our group is developing is illustrated in Fig. 4 (Ref. 27). This concept is very different from the encoding strategies already described. Here, the strategy is to produce internally encoded, solvent-resistant microbeads with the view to tracking each microbead through the split-and-mix synthesis based on its 'optical signature' as determined by a flow cytometer. Novel particle synthesis techniques are being employed to produce microbead suspensions containing millions of particles with diverse fluorescent bar-codes [Table 1(e); Fig. 4a]. One technique we use is to synthesize various fluorescent silica layers around core

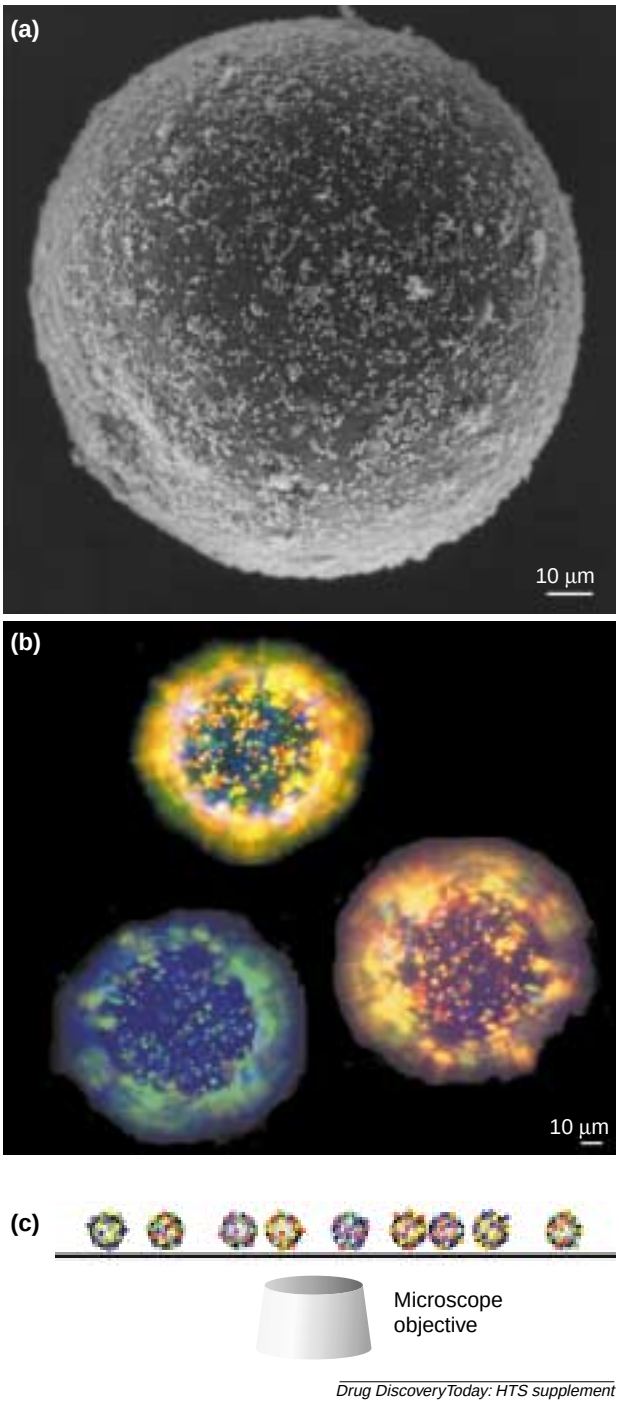
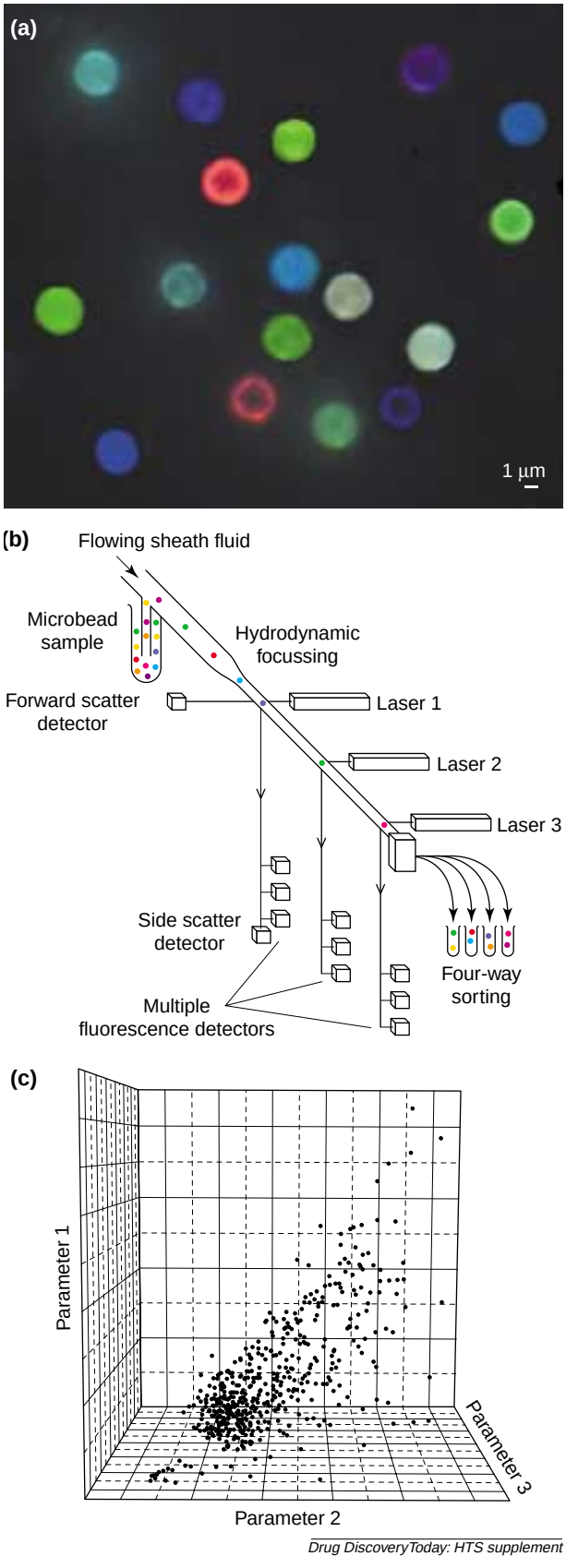


Figure 3. Bar-coding microbeads with multi-fluorescent particles. **(a)** Scanning electron micrograph of a polystyrene/divinylbenzene microbead (110 μm diameter) actively encoded with silica reporter particles (0.2 μm diameter) during a split-and-mix process (see Fig. 2). **(b)** Composite fluorescence microscopy image of three microbeads from a split-and-mix library, each bearing a different fluorescent bar-code. **(c)** The bar-code is a record of the reaction history of the bead and reading the bar-code through various optical filters in a fluorescence microscope reveals the identity of the compound synthesized on the bead.



silica particles (data submitted for publication). A schematic of these particles is shown in Table 1(e). Attributes of each microbead that comprise the optical signature, such as size, refractive index profile, and the presence and concentration of fluorescence moieties, can be accurately detected at a very high rate (up to 100,000 particles sec^{-1}) using a high-performance flow cytometer (HPFC) (Fig. 4b). HPFCs such as Cytomation's MoFlo™ (Fort Collins, CO, USA), have capabilities such as multi-laser beam excitation; multi-parametric analysis; a modular format enabling expansion of detection capabilities; and the ability to physically separate microbeads into sub-populations based on any parameter or combination of parameters. In multi-parametric analysis, the data from several detectors can be stored as matrices, thereby preserving the association of events (i.e. the optical signature). A HPFC can be used to separate those microbeads with unique optical signatures from a population of optically diverse microbeads. The concept can be further understood from the data shown in Fig. 4c. These silica microbeads contain one or two fluorescent dyes (fluorescein isothiocyanate and/or quinolizino-substituted fluorescein isothiocyanate) and their fluorescence and side scatter values can be detected and stored as an optical signature. If the optical signature is examined in three parameters, about 30% of particles are found to have unique signatures. However, by incorporating more dyes into the microbeads and detecting the optical signature in eight parameters, the number of microbeads with unique optical signatures is increased to 99% (Ref. 27). The probability of detecting two microbeads with duplicate optical signatures is therefore relatively small and duplication can be completely eliminated by removal of one or both of the particles via a HPFC sort decision. After collection of a chosen number of uniquely encoded beads, the strategy is to perform split-and-mix library synthesis while tracking each microbead via its optical signature and recording its reaction history[27]. The structure of any chosen probe is then identified by analyzing

Figure 4. Exploiting the unique optical signature of internally bar-coded microbeads. (a) Novel techniques are used to synthesize multiple fluorescent layers around core silica particles (2.9 μm in diameter). This composite fluorescence microscopy image shows that our internally bar-coded microbeads exhibit a complex mixture of fluorescence emission characteristics. (b) High-performance flow cytometers possess multiple lasers set up in parallel, with accompanying detectors that can measure a wide variety of fluorescence wavelengths as well as forward and side (90°) light scattering. (c) A 3D flow cytometry plot of silica particles (10–12 μm in diameter) containing red, green or a red/green combination of fluorescent dyes. Each dot on the plot shows the optical signature of one microbead. Detection of the microbeads in three parameters (i.e. side scatter, red fluorescence and green fluorescence) shows that the optical signatures are reasonably well dispersed, and about 30% are unique. By increasing the number of dyes within each microbead and detecting the beads in eight parameters, 99% of microbeads are found to have unique optical signatures.

the optical signature of the microbead on which it resides. This is done automatically, using the data that was stored by the flow cytometer software during the probe synthesis.

Conclusions

Although DNA microarrays are sophisticated devices for rapid analysis in gene screening, they remain costly to set up and the experimental protocols necessary for sample preparation and analysis are relatively time consuming. Furthermore, their current 2D geometry imposes serious limitations in terms of being able to address and analyze extremely large compound libraries. The completion of the Human Genome Project has heralded a new era of genetic information and has increased the demand for more economical and flexible alternatives for gene screening.

Microbeads are becoming a viable alternative to microarrays, being versatile, inexpensive and able to be stored in large numbers in just a few milliliters. Many companies and research groups are recognizing the potential of increasing the size and complexity of the libraries of bead-based probe molecules. With flow cytometry beginning to play a major role in microbead-based gene screening, the material, size and optical properties of the microbeads are being exploited to achieve diversity. Encoding strategies are emerging that can produce combinatorial split-and-mix libraries of probe compounds on microbeads, with each microbead possessing a distinguishable optical barcode. Potentially, these strategies can result in extremely large libraries (>10^{10} compounds) and might be generically suitable for applications such as SNP genotyping, gene expression and drug discovery.

Here, we have highlighted existing and proposed strategies that promise cheaper and higher-throughput alternatives to DNA microarrays. With the intrinsic advantages that they possess, it is expected that microbead-based libraries will increasingly challenge microarrays in the future for a wide variety of high-throughput screening applications.

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