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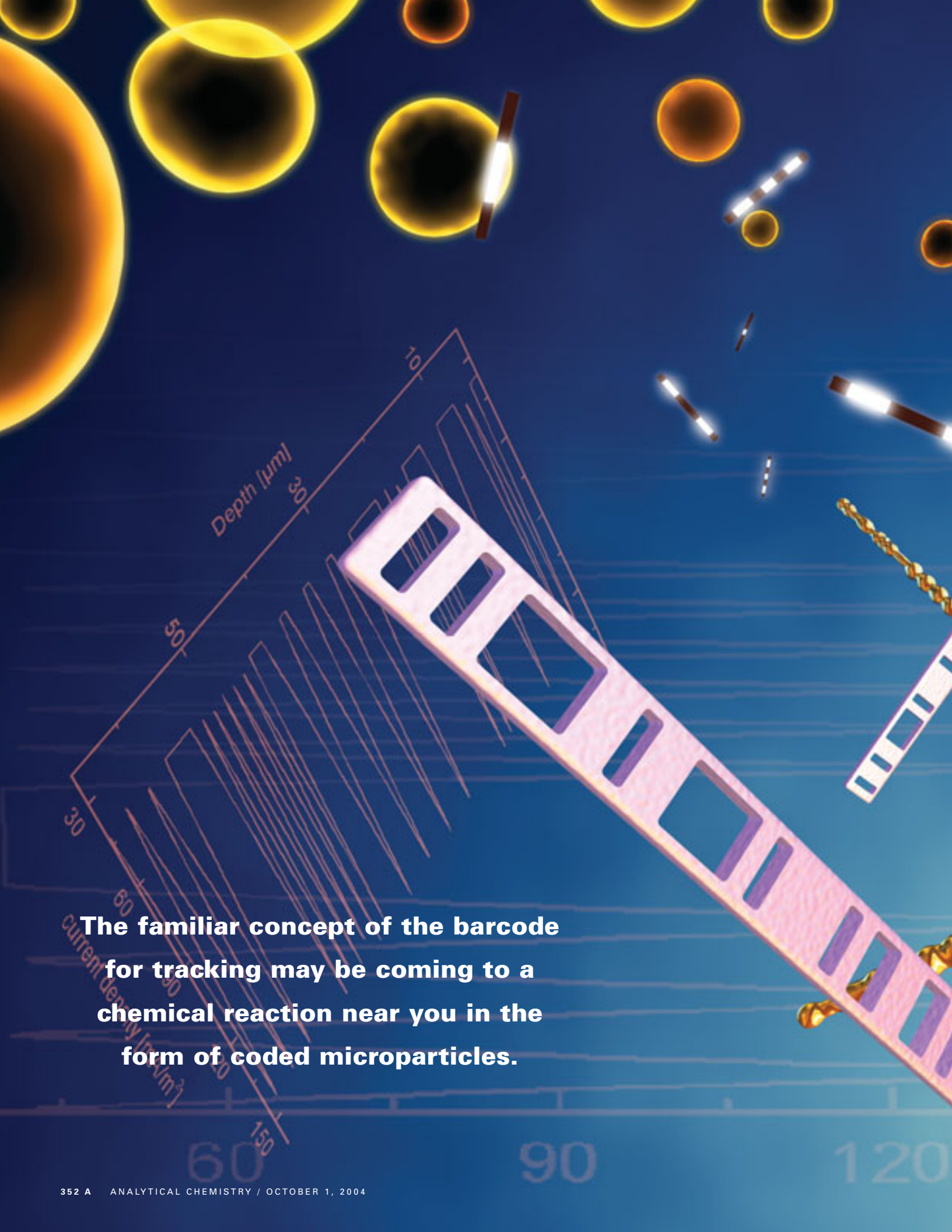


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**The familiar concept of the barcode
for tracking may be coming to a
chemical reaction near you in the
form of coded microparticles.**

BARCODING THE MICROWORLD

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arcodes are a part of our everyday life. You see them in the grocery store, on letters from the post office, and on DVDs you check out from the video store. Fast, simple, and accurate, barcoding has become the most popular data-entry method to track the ever-exploding amount of information in the macroscopic world in the past 15 years. In the meantime, an increasing demand for tracking smaller items and for direct monitoring of individual chemical interactions has driven the exploration for novel methods of barcoding at much smaller scales (1–4). Successful demonstrations of micro- or nano-sized barcodes in molecular interaction studies (4, 5), combinatorial screening (6), and convert tracking (7) have opened up new opportunities for research.

This article will introduce recent developments in the field of encoded micro- or nano-sized freestanding particles and various applications. Detailed discussions of other encoding methods, such as molecular tags (8, 9) and non-particle-based platforms (10, 11), are beyond the scope of our discussion. Readers who are interested in gaining an in-depth understanding of the field are referred to more comprehensive review papers.

A conventional macroscopic barcode is a series of vertical lines (“the bars”) and spaces of different widths. Various combinations of bars and spaces represent different characters incorporated in the code. Theoretically, varying the widths, sequential order, and total number of bars and spaces in the barcode can generate an unlimited number of codes. To achieve similar coding capacity at the nanoscale, the right elements to make up the codes (i.e., the bars and spaces) must first be identified. Two criteria are often used in the search for proper elements with distinctive attributes: They must be mixable at

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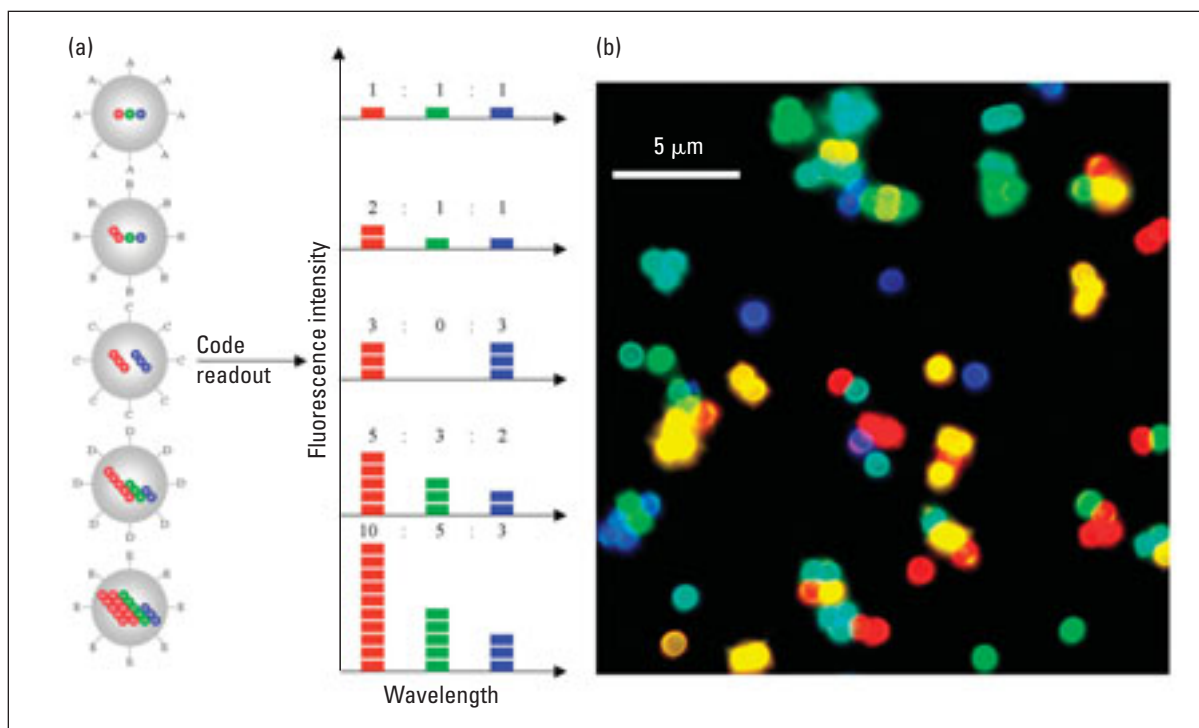


FIGURE 1. (a) Encoding scheme of quantum nanocrystal-infused microparticles using three different nanocrystals mixed at five different ratios and (b) the corresponding fluorescence image of encoded microparticles. (Adapted with permission from Ref. 2.)

different ratios with negligible signal crossover and be readily distinguishable by a simple readout method. Numerous molecules and nanomaterials meet these requirements and have been used in two general microscopic barcode fabrication processes (12, 13).

Microparticles host coding elements

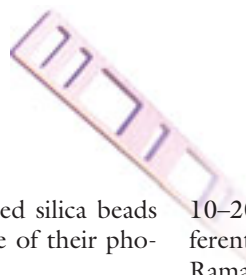
In one fabrication process, microparticles are used as “cargo” to host the coding elements—molecules or nanoparticles with identifiable features. Two approaches are typically adopted to incorporate these coding elements. In the first approach, the coding elements get encapsulated inside the microparticles when these microparticles swell open in organic solvents. When subsequently placed in an aqueous solution, these particles rapidly shrink and close the previously opened channels, locking in the coding elements (2, 14). The encapsulating process provides a chemically inert shield to protect the coding elements from possible structural degradation and performance deterioration during an unexpected exposure to the surrounding environment. The coatings also isolate individual elements and keep them from aggregating with their neighbors, which might alter the signal output.

Alternatively, the coding elements can be directly attached to the external surface of micro-sized beads through electrostatic interactions (15). The cargo particles used in either approach are often made of polymer materials because of their chemical resistivity. Sometimes silica beads are used instead. These microparticles occasionally also serve as solid supports in assay-based applications to attach capture reagents and participate in molecular interactions in solution. Thus, the particle sizes are typically large enough to accommodate sufficient

coding elements for detection and provide ample binding sites for the capture reagents, yet they are still small enough to stay suspended in solution during the assay.

The coding elements housed inside these particles are typically molecules or nanoparticles with unique optical or electrical properties (5, 16, 17). Fluorescent organic dyes are by far the most popular choice because of their structure-specific emission profiles and good sensitivity (1, 3, 18). As with the bars and spaces used in macroscopic barcodes, dye molecules of different emission profiles are mixed at various ratios. Upon excitation, these dyes emit distinct fluorescent signals readily detectable through the use of a simple spectrometer. The absolute intensity of each dye is then quantitated to reveal the identities of the particles. The most prominent example in this category is the fluorophore-impregnated microspheres manufactured by Luminex (14, 19, 20). In this platform, two organic dyes are encapsulated in micro-sized polystyrene beads at 10 different concentrations of each, resulting in a set of 100 uniquely encoded microspheres. A further increase in the coding capacity is theoretically feasible if additional dyes of various concentrations were to be infused into the host beads.

The quantum confinement effect experienced by semiconductor nanocrystals brings about the unique optical properties not observed for larger crystallites (2, 5, 17). Consequently, as a new class of fluorescent medium, these semiconductor nanocrystals have attracted considerable interest. Just as for organic dye molecules, a simple diffusion step is sufficient to upload these nanocrystals into large particles. Researchers can tune the fluorescence profiles by choosing a mixture of nanocrystals with different emission bands. In a similar way,



rare earth elements and organic-dye-incorporated silica beads have also been used as coding elements because of their photoluminescent properties (15, 21, 22).

Figure 1 illustrates three kinds of semiconductor nanocrystals with fluorescence emissions at red, blue, and green, which were mixed at five different ratios. Upon excitation, colored polystyrene beads that were well correlated to the loading of different amounts of each nanocrystal were clearly observable in the image. If the size of the incorporated nanocrystals is tuned from 2 to 7 nm, a fluorescence emission maximum from 400 nm to 2 μm can be obtained. With a fwhm of 10–20 nm in the typical fluorescence spectrum, a coding scheme using five or six colors with six intensity levels is considered feasible. This scheme corresponds to ~10,000–40,000 distinguishable codes, significantly more than what can be produced using organic dyes alone (2). The photostability and high quantum yield are additional benefits that make these nanocrystals excellent candidates for particle encoding. A single-wavelength excitation capability further simplifies the decoding instrument designs.

In addition to photoluminescent properties, IR and Raman spectral features of various molecules have also been used for coding (16, 23, 24). Fenniri et al. mixed six styrene derivatives with distinguished Raman and IR signatures at different ratios during the polymerization of carrier resins. The final spectrum of each resin showed a clear combination of IR and Raman bands of different intensities, similar to bars with different widths in macroscopic barcodes. Because all 6 reported derivatives had unique vibrational fingerprints, the random mixing yielded 24 unique codes, with more expected (24). It is worth noting that the presence of the multiple absorption bands from the same molecule ensures highly accurate decoding by redundantly providing structure-specific information. The trade-off, however, is a spectrum that is sometimes too crowded, which can complicate signal deconvolution and limit the maximum number of compounds that can be used without spectral overlap occurring.

Although optically encoded particles are widely used and commercially successful, applications beyond bioanalysis have been limited, mainly because of the small pool of uniquely coded beads. To circumvent this limitation, two approaches have been examined to increase the coding pools. The first approach increases the number of elements that can be differentiated using the same detection system by decreasing the fwhm of the emission bands of these coding elements. For example, the fluorescence emission bands of most conventional dye molecules have fwhm values of 50–200 nm. Consequently, only 4–6 different fluorescent dyes can be accommodated in the visible region (400–800 nm) with acceptable spectral overlaps. Semiconducting nanocrystals, on the other hand, have much smaller fwhm values of

10–20 nm in their fluorescence emissions; therefore, 10–12 different nanocrystals can be used for the same detection window. Raman scattering bands have further reduced fwhm values that suggest, theoretically, that a higher number of total codes are feasible. In reality, the presence of multiple Raman bands and relatively weak signals has limited the obtainable number of codes to <100.

The other approach to increasing the total number of codes is to increase the quantitative resolution of the amount of dye encapsulated. For example, for a system in which 4 fluorophores are used and 10 different concentrations are resolvable for each dye, a total of 10^4 unique codes are possible in the visible region. If the dynamic range and the resolving power of the detector are improved so that it can distinguish subtle differences between 100 instead of 10 different dye concentrations, the total number of codes would reach 100^4 . Keep in mind, though, that the increased cost of a detector with higher resolving power will limit the general applicability of this coding approach. Also note that the size of the host particle also has an effect on the ultimate coding capability of each method, because it sets a limit on the maximum number of molecules or nanoparticles that can be accommodated. In addition, the more concentration levels that need to be resolved, the more stringent the requirements on dye loading accuracy at each concentration level. Quality control is thus necessary to ensure a consistent yield of the desired code combinations.

Microparticles that are part of the coding elements

The second type of fabrication process uses the particles' physical properties as the coding elements and is considered "graphic" because most code deciphering is accomplished by pattern recognition. One distinguished example is using the unique optical properties of noble metals to encode particles (4, 25). These submicro-sized metallic barcodes are readily prepared by sequential electrochemical deposition of different metal ions (e.g., Ag^+ , Pt^{2+} , or Au^+) into a porous membrane template (4, 26, 27). The nanoparticles are then released by dissolution of the silver backing and the alumina membrane. The particles have a typical length of a few micrometers and a diameter of 0.3 μm . The intrinsic electron density of each metal used determines its light reflectivity at a particular illuminant wavelength. For example, at 430 nm, gold reflects only half the light that silver does.

As a result, striped metal nanoparticles can be directly observed at 430 nm from silver–gold mixed particles—the bright sections are from the silver stripes and the dark sections from the gold stripes. These distinct bar-and-space patterns are identical

to those of conventional barcodes (Figure 2a). By simply varying the electrochemical plating conditions, such as plating solution, current, and plating time, researchers can freely program the codes along the long axis to obtain almost unlimited combinations. For example, with 2 metals (silver and gold) and 8 segments of equal-length stripes in the particle, 136 unique barcodes were produced, whereas 4160 codes can be expected by a simple increase of the total segments in the particle to 13 stripes. A total of 8×10^5 distinctive coding patterns are theoretically achievable using three metals (4, 27). This coding method has the potential of creating a library with a massive number of unique codes. However, researchers must address the following problems: rapid settling of particles during the reaction because of the high density of the metals and the possible breakage of particles at the stripe interface after extensive processing, which is one of the main sources of false identifications.

Using a size-controlled catalyst instead of template-based synthesis, Lieber's group has prepared barcoded nanowires composed of GaAs and GaP (28). Gold clusters guide the growth of the superlattice structures of GaAs during a laser-assisted catalytic growth process. When a second reactant is introduced, GaP grows continuously at one end of the wire. The growth time and growth rate of different reactants can be controlled to produce nanowire superlattices with the desired striping patterns. Upon excitation at 488 nm, GaAs exhibits a unique photoluminescence response as a direct band gap semiconductor, whereas GaP sections do not respond to the light in a similar fashion. Therefore, a barcoded pattern is clearly observed under the microscope (Figure 2b). Although this concept has been feasibly demonstrated, its practical application is limited because of the sequential fabrication process.

The semiconductor industry has used photolithographic techniques to routinely generate highly reproducible patterns. They have also been adapted for coding purposes—optically encoded aluminum stripes were patterned on a silicon wafer with the aid of an etching technique (Figure 2c; SmartBead at www.smartbead.com). The photobleaching or photochroming of coding patterns onto fluorescently activated particles has also been reported as a low-cost and easy-to-implement method that may be able to generate large numbers of codes (29). However, because each pattern is directly “written” on the particle in a sequential fashion, the code-generation rate is rather limited. The need to orient each bead to the correct position for an accurate identification also restricts the application of this approach.

Silicon particles with multilayered porous films have also been fabricated through the use of a periodic electrochemical etching method (30). The key step in the fabrication is the applica-

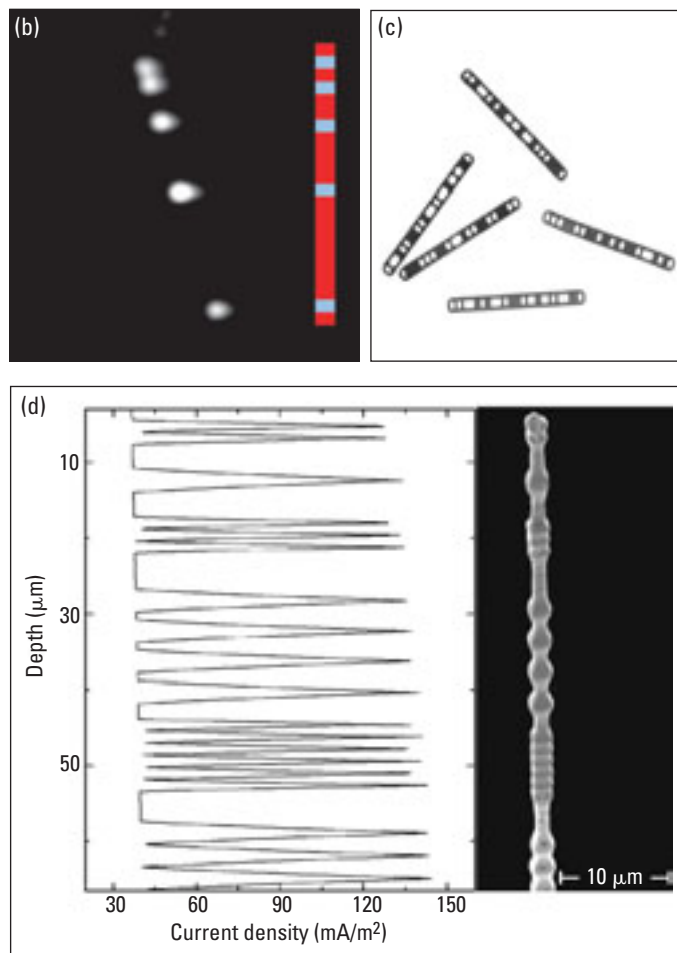


FIGURE 2. Optical images of encoded particles.

(a) Metallic nanoparticles, (b) semiconducting nanowires, (c) aluminum-etched nanowires, and (d) shape-modulated gold wires. [Adapted with permission from Refs. (a) 4, (b) 28, and (d) 35. (c) Courtesy of SmartBead.]

tion of a computer-generated pseudo-sinusoidal-current waveform through the electrochemical cell during the etching. A combination of different waveforms, along with the etching current density, solution, and duration, yields layered stacks of silicon with varied refractive indexes. The apparent advantage of this approach is its robustness, because the codes are an integral part of the particle. Therefore, as for a piece of holographic film, partial damage of the particle does not affect the readout accuracy. However, the periodic electrochemical etching method does not seem capable of generating hundreds of uniquely coded particles without being combined with other methods.

Last but not least, the shapes of particles have been tailored to generate different codes in a most straightforward fashion. Examples include direct pattern writing on magnetic microstructures using a micromachining technique (31, 32), fabricating polymeric and metallic particles of different shapes (32, 33), and dry reactive ion etching to make silicon pieces of different notch patterns (34). A pulsed current has also been used to vary the inner diameters of the channels formed in a silicon wafer during anodic etching; subsequent deposition of gold or other noble metals into the channels has yielded wires of modulated periods that exhibit different reflective patterns during the readout (Figure 2d; 35).

Although a couple of the encoding methods described so far in this article have matured and are commercially viable, most are still under development. To be competitive with spatially encoded microarrays, not to mention macroscopic barcoding, a significant increase in code production at an industrial scale (low cost and high efficiency) is much needed. A careful re-

view of critical steps involved in the manufacturing process with regard to quality assurance and quality control is also desirable to meet the high demands of massive encoding.

Detection of encoded particles

When a scanner is passed over a barcode, a photocell detector in the scanner receives the reflected light and converts it into an electrical signal that corresponds to the bars and spaces of a barcode. A decoder is usually a separate box that takes the digitized bar-and-space patterns and translates them into the correct data. The data are then linked to the associated computer records that contain descriptive data and other pertinent information.

A detection system working in a similar fashion is designed to decipher microscopically encoded particles. A spectrometer (the decoder) coupled with a flow cytometer (the scanner) have been used for their speed (19). A conventional optical microscope has also been used for direct visualization of particle coding patterns in a static mode (27). A bundle of fibers with selectively etched pores to accommodate individually color-coded particles has also been used to directly capture coding patterns (18, 36). Note that the etched fiber bundles only act as a light-transmitting medium, whereas the coding is still accomplished by the dye-encapsulated beads.

Despite encouraging initial work, some decoding needs are unmet. The measurement speed with an optical microscope is at most several tens of particles per second because of the limited number of particles that can fit into one image and the time required to collect high-quality images and to process and transfer data. Thousands of experiments or tagged items with hundreds of copies of each code require on the order of 10^6 particles to be interrogated in one experiment for signal averaging to ensure identification fidelity. Consequently, obtaining a total measurement in a few minutes requires a throughput of $\sim 10^4$ particles/s—not a trivial rate in static-mode-based detection.

A flow-based detector may provide a better solution in this case because <0.01 s is typically needed for a particle to flow through a narrow channel. At this rate, 10,000 particles can be detected within a couple of minutes. Coupling detection with downstream sorting also makes the technique attractive for sample separation. Note that light of sufficient intensity must be collected at a high temporal resolution because the particle passes the detection window in the blink of an eye. Thus, the problem is obtaining suitable optics and a fast-response detector with a reasonable cost and size.

For small-scale analysis, experienced operators perform direct visual decoding. For high-throughput screening and objective data interpretation, a sophisticated software program that automatically converts collected signals to particle identities and that integrates additional information is highly desirable. The demand for automated data analysis has compelled this aspect into an independent research field of its own (27).

Multiplexed bioapplications

Multiplexing is a way to simultaneously investigate multiple independent reactions in the same solution. Biological multiplexing has become one of the fastest growing areas in life science for extracting the most information from the smallest volume of sample at the lowest possible cost. Two-dimensional microarrays that spatially define each capture reagent and accommo-



The chemical and geometric flexibility of these particles also makes them applicable to tagging unconventional objects, such as high-temperature parts, slippery surfaces, and items with complex shapes, sizes, or materials.

date thousands of reactions on the same surface have great potential. Yet, uncertainties related to slow diffusion at the solid/liquid interface and inflexible chip fabrication have limited their applications.

Encoded particles are an apparent solution. Just like each spot on a microarray, each particle in the solution can be considered an individual chemistry lab that independently detects and analyzes a unique compound or reaction. Particles have minimal impact on binding kinetics and can be mixed in any combination. When coupled with a powerful deconvolution method, these particles could be used in thousands of biological tests conducted simultaneously in a clinical lab, thus replacing the current tests, which are laborious, time-consuming, and costly.

Using encoded nanoparticles as microcarriers for multiplexed biological assays is rather straightforward. Specific receptors are immobilized onto particle surfaces through passive adsorption or active chemical binding. Nonspecific adsorption of receptors from electrostatic or hydrophobic interaction is a simple approach, but it provides minimal control of the final orientation of the receptors; this limitation sometimes leads to a severe reduction in receptor bioactivity and assay reproducibility. Site-specific covalent bonding provides a more stable, thus more controllable, surface assembly by cross-linking amine, thiol, or carboxylic acid groups of capture reagents.

Once a receptor–analyte recognition step enables immobilization of the target molecules, additional optical probes can be introduced for detection. These probes, which are often fluorophores of different emission profiles, indicate the occurrence of a specific reaction between analytes and substrate particles (2, 4, 14). An ideal detection system is designed so that the measurement of the amount of bound fluorophores can be taken sequentially or in parallel during decoding. Various groups have demonstrated the feasibility of using a simple detector to decode particle identities and quantify bound analytes.

For example, Figure 3a shows a reflectance image of two kinds of striped particles at 400-nm illumination. Two distinct patterns were clearly observed: gold–silver–gold and gold–nickel–gold. In Figure 3b, only gold–silver–gold particles coated with antihuman IgG attracted fluorescein isothiocyanate-tagged human IgG, which showed up brightly in the image, whereas gold–nickel–gold particles coated with antirabbit IgG remained dim. The opposite response is shown in Figure 3c, in which rabbit IgG was introduced. Cross-referencing the data-

specific targets, encoded particles have also been used simply as detection probes, in the place of conventional fluorescent tags, to indicate the occurrence of binding events on a surface. The benefits of using encoded particles rather than fluorescent tags include improved chemical and photostability and, because more tags are available, more flexibility. Mirkin and colleagues used six Raman-tagged microparticles to perform multiplexed detection of oligonucleotide targets (16, 41). The same concept is universally applicable wherever conventional probes are used as labels, such as sandwich immunoassays, single-nucleotide polymorphism genotyping, and tissue labeling (42, 43).

Combinatorial screening

The combinatorial method is an approach in which thousands of similarly designed experiments are conducted in parallel to search for an optimal reaction condition or a best-performing product. Its powerful potential has been amply demonstrated in locating drug candidates by systematically examining different molecular structures stemming from the same origin.

As the combinatorial approach expands to fields beyond drug screening, the ability to track each entity during a reaction and quickly filter through the massive amount of generated data to identify the most relevant information becomes paramount (6, 24).

Traditionally, combinatorial reactions are performed in a split-mix fashion. Each chemical

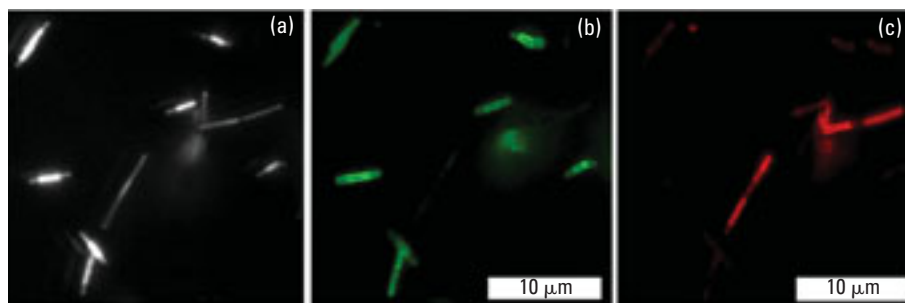


FIGURE 3. (a) Reflectance image of two different nanoparticles at 400 nm. Fluorescence images of sandwich immunoassays using (b) fluorescein isothiocyanate (human IgG) and (c) Texas red (rabbit IgG) as the detection probes. (Adapted with permission from Ref. 4.)

base for the nature of the capture reagents immobilized on different particles revealed the identities of the analytes present in the sample solution. The concentration of the analyte was subsequently quantitated on the basis of the average brightness of the fluorophores.

In a more sophisticated example, 15 cytokine secretions from stimulated peripheral blood mononuclear cells were comprehensively profiled using dye-encoded microparticles (37). For each cytokine, 500 microspheres of each code were immobilized with a specific primary capture antibody. After antibody immobilization, 15 different types of particles were mixed with a cocktail of the sample and the corresponding secondary detection antibodies. The overall performance was comparable to traditional ELISAs in sensitivity, accuracy, and reproducibility. The same platform has also been used successfully to detect HIV-1 antigens and muted gene sequences (38–40).

In addition to serving as reaction carriers to track

step involved in the generation of the product is identified by a thorough analysis of the structure of the final product, which is often time-consuming and technically challenging. Barcoding each reaction significantly simplifies the deciphering process by directly correlating each reaction route or specific product to a particular code. For example, Trau and colleagues developed an active optical barcoding method in which the particles were encoded dynamically during combinatorial library synthesis (22). Fluorescently encoded silica nanoparticles were used as reporters to attach to microcarrier beads at each split-mix step. After several cycles, each carrier bead was coated with different reporters at different ratios, depending on the preceding reaction steps. The decoded fluorescent profile of the microcarrier can elucidate the structure of the product of interest. It has been calculated that only 6 fluorescent dyes are needed to code $>4.3 \times 10^9$ library entities (20). With the help of a dual recursive deconvolution method, the tracking

process has been further simplified and can identify the first and last synthetic position of the product of interest (44).

Nonbiological applications

With the maturation of micro-sized barcode fabrication and decoding processes, most coding applications in the macroscopic world, such as assembly-line tracking, inventory, and library automation, can benefit from microparticle-based encoding. Especially for those applications requiring small, robust, and uniquely identifiable but inexpensive tags, encoded microparticles have shown unprecedented potential (7). The chemical and geometric flexibility of these particles also makes them applicable to tagging unconventional objects for which conventional barcoding often fails, such as high-temperature parts, slippery surfaces, and items with complex shapes, sizes, or materials. Microbarcodes are still in their infancy, and the potential applications outside of the field of life science have been the main driving force to further improve the fabrication, handling, and detection of large libraries of encoded particles.

Summary

With the rapid development of nanotechnology in the past few years, the use of micro- or nano-sized barcodes in various applications has attracted a lot of attention. Novel applications of bar-coded particles are reported every week as researchers find creative ways of utilizing the efficiency and flexibility made possible by encoded microparticles. The focus in biological multiplexing has shifted from massive multiplexing to detecting groups of specified analytes. In other words, the current multiplexing capability is limited by the number of assays that can be conducted simultaneously without significant compromise of sensitivity and specificity, and not by the number of particles available, as in the past. To ensure the economic soundness of adopting microparticle-based barcoding technologies, industrial pioneers are pushing the limits in low-cost, large-scale particle production and portable detection systems. Automated detection has become increasingly important to improving identification accuracy and efficiency.

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Nancy H. Finkel and Xinhui Lou are graduate research assistants, Cuiying Wang is a postdoctoral associate, and Lin He is an assistant professor at North Carolina State University. Finkel's research focuses on developing 2-D nanostructures for MALDI substrates and applying them to metabolite profiling. Lou's research interests include developing novel DNA detection probes and fabricating core-shell nanoparticles for gene delivery. Wang has been studying the spatial dislocation of membrane-bound peptides on lipid bilayers. He's research involves developing bio-

analytical tools by coupling nanomaterials with MS and surface plasmon resonance and using them to study transmembrane traffic. Address correspondence about this article to He at Department of Chemistry, 815 Dabney Hall, North Carolina State University, Raleigh, NC 27695 (lin_he@ncsu.edu).

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