# Multiplexed Spectroscopic Detections

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arrays, fluorescence, surface plasmon resonance, surface-enhanced Raman spectroscopy, microparticles

## Abstract

This review describes various platforms used for multiplexed spectroscopic analysis. We highlight the use of different types of spectroscopy for multiplexed detections, including Raman spectroscopy, surface-enhanced Raman spectroscopy, surface plasmon resonance, and fluorescence. This review also explores the use of cross-reactive sensors in combination with pattern-recognition algorithms to monitor multiple analytes in aqueous and vapor matrices. It also discusses applications of these techniques, paying special attention to their use in the detection of biologically relevant analytes.

## 1. INTRODUCTION

Multianalyte detection schemes have become an important analytical tool and have been applied in fields ranging from genetics (1, 2) to environmental monitoring (3, 4). In particular, many spectroscopic techniques have been developed that enable multi-analyte detection. Compared with other analytical methods (such as magnetic resonance, chromatography, or electrochemistry), spectroscopy lends itself to multiplexing because different colors of light can be easily created, separated, and detected simultaneously. Advances in photonics technologies have led to the development of handheld fluorimeters (5), handheld Raman spectrometers (6), and portable total reflection X-ray fluorescence spectrometers (7), and additional miniaturization and capability are expected. To empower these devices further, investigators are developing multiplexed detection schemes that enable essentially thousands of experiments to be conducted concurrently.

Multiplexed spectroscopic detection is performed in one of two modalities. First and simplest is the use of spectroscopy in which each analyte possesses a different spectroscopic signature. This method is a direct one as the intrinsic properties of the analytes give rise to different spectroscopic signatures. Second, different indicators can be used to bind analytes and report their presence. This approach is indirect in that binding or reaction between the analyte and the indicator provides the signal. In either scheme, the detection of multiple analytes simultaneously requires that light of different wavelengths be distinguishable from one another. This distinction can be made by resolving the color or other property (e.g., fluorescence lifetime) of the different analytes or indicators. In addition to these two schemes (direct and indirect), we can further categorize multiplexed spectroscopic analysis according to how the different analytes are resolved. In the first approach, resolution is accomplished spectrally. In this scheme, the analytes or the indicators are identified by their distinct spectroscopic signatures. Another approach is to use spatial resolution whereby different sensing regions are spatially separated. For example, different indicators can be placed at different regions of a substrate, and a color change at a particular position correlates with the presence and/or concentration of the analyte. Spatially resolved multiplexed substrates are called arrays.

This review covers recent advances in various spectroscopic methods used for multiplexed detection schemes. We discuss the platforms that make multiplexed detection possible, such as arrays and encoded microcarriers. Some applications of systems that use fluorescence, surface-enhanced Raman spectroscopy (SERS), and surface plasmon resonance (SPR) are discussed in terms of both commercial and research uses. Other reviews have covered the basic operating principles of the different types of spectroscopy used in these schemes, so we do not cover them here (8–13).

## 2. DIRECT MULTIPLEXED DETECTIONS

### 2.1. Spectral Separation

The direct measurement of multiple analytes by optical methods requires that the analytes of interest be distinguishable from the background as well as from each

other. These methods typically involve collecting spectra and then applying deconvolution methods to separate the spectral signals arising from the different components in the sample. Fluorescence and absorption peaks tend to be broad, and the identification and quantification of multiple compounds are difficult in complex mixtures. For this reason, detection methods using Raman and X-ray fluorescence spectroscopy, whose peaks are narrow in comparison, have been developed for multiplexed detections. In addition, X-ray spectra are unique to specific elements.

Raman spectroscopy can directly detect many different analytes on the same platform, such as a variety of dyes used on ancient artifacts (14, 15), but it has not yet led to the development of a system capable of detecting multiple analytes simultaneously. Recently the Van Duyne group (16) proposed a method using SERS that may make it possible to simultaneously monitor both lactose and glucose at physiologically relevant concentrations. SERS has also been shown to be useful for the detection of bio- and chemical warfare agents (13, 17, 18). Although no multiplexing was performed, the technique was able to detect and identify *Bacillus anthracis*, *Yersinia pestis*, *Burkholderia mallei*, *Francisella tularensis*, *Brucella abortus*, and ricin on the same platform (19). The researchers first obtained Raman spectra for all live species and then employed pattern-recognition algorithms to identify all pathogens at the species level in blind trial evaluations (19). This Raman study shows great promise for the quick and accurate detection of multiple pathogens in real-world situations.

The detection of trace metals has become popular in the X-ray spectroscopic field (20–23). The detection of platinum, palladium, and rhodium, for example, is important in the recycling of complex materials, such as automotive catalysts. Van Meel et al. (23) used X-ray fluorescence to detect all three metals simultaneously with detection limits below 5 ppm for all metals. This technique was much faster than the typically used inductively coupled plasma optical emission spectrometry with a much easier sample preparation process (23). The simultaneous detection of all three elements is possible because each metal has unique emission peak locations.

These multiplexed detections of trace metals have also been applied to the study of various diseases, such as Parkinson's (24), amylotrophic lateral sclerosis (24), and prostate cancer (25). Through use of X-ray fluorescence, researchers were able to simultaneously detect potassium, calcium, iron, copper, zinc, and selenium in brain tissue (24). By using cluster and discriminant analysis, they classified samples as positive or negative for both Parkinson's and amylotrophic lateral sclerosis (24). Through similar data analysis (using X-ray fluorescence detection of iron, zinc, copper, and manganese), the investigators successfully classified prostate cancer in 97.7% of samples (25). The speed and accuracy with which these experiments can be performed may lead to novel diagnostic capabilities, as well as a better understanding of disease, especially if the concentrations of the metals can be correlated to biochemical changes within the cells (26).

## 3. INDIRECT MULTIPLEX DETECTIONS

### 3.1. Spectral Separation

The methods described above all employ an intrinsic spectroscopic property of the analyte of interest to carry out detection. In multiplexed detections, however, it is more common to use an indicator that interacts with the analyte of interest. These detection schemes are indirect because the signal does not come directly from the analyte of interest.

**3.1.1. Dyes as indicators.** To detect multiple analytes at a single location, one must have a spectral separation of the indicators. An example of multicolor detection is the Sanger method for DNA sequencing (27). In this method, each of the four bases (ACGT) is labeled with a different dye, and the incorporation of each labeled base into the growing DNA strand results in a different color that can be easily distinguished to identify the base at that position. Similarly, most multianalyte sensors use different color indicators to make the analysis easier. One- and two-layer sensors make use of a single sensing region to detect multiple species. These layered sensors contain all the sensing chemistry in a single material but, to date, have only achieved duplexed detection. These sensors are made of a porous solid support, such as ethyl cellulose (28) or silica sol-gel (29), which houses fluorescent dyes that are sensitive to the analytes of interest. These materials are typically suspended in a polymer that does not interfere with the excitation or emission spectral properties of the sensing dyes. When the polymer is cast, it forms a layer that is a few micrometers thick. The dyes must undergo reversible reactions with the analyte of interest to express a change in a fluorescent property, such as lifetime (30), intensity (31), or emission wavelength.

Dual sensors detect two analytes by positioning the sensing chemistries for both targeted analytes at a single location. The first dual-layer configuration was reported in 1988 and consisted of two different sensing layers (31). One layer housed a dye sensitive to $CO_2$, whereas the other layer housed an $O_2$-sensitive dye. Signal differentiation was accomplished by using two fluorescent dyes with different emission spectra. Variations in the thickness of the sensing layers resulting from the production process led to errors in fluorescence intensity.

Since these initial results, researchers have made various efforts to remove this dependence on layer thickness. One method involved the use of a reference dye that does not participate in any analyte-dependent reaction and does not interfere with the sensing dye. This approach compares the fluorescence intensity from the sensing dye with that of a reference dye housed in the same layer (**Figure 1**). Carbon-dioxide sensing chemistry is based on dyes that exhibit changes in fluorescence intensity resulting from changes in pH due to the formation of carbonic acid within the sensing layer. Tetraoctylammonium and tetraoctylammonium hydroxide were used to monitor $CO_2$ at low concentrations (high pH), whereas 8-hydroxypyrene-1,3,6-trisulfonate was used to monitor high concentrations of $CO_2$ (low pH). All dyes were dissolved in ethyl cellulose and ground into microparticles. The reference dye, $Ir_2(C_{30})Cl_2$, was dissolved in the gas-impermeable

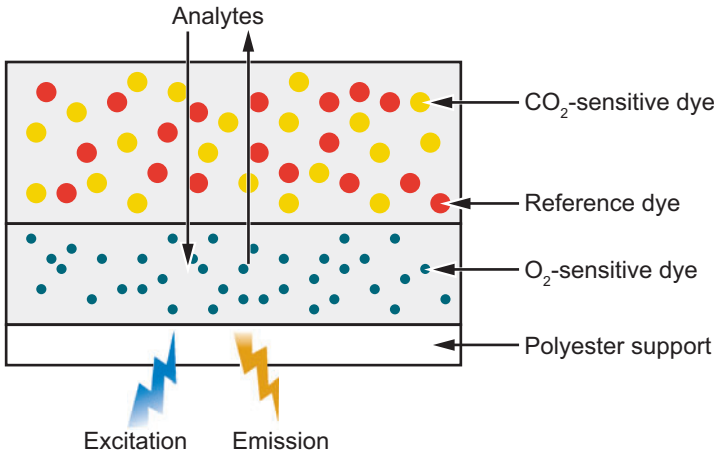



**Figure 1**

Schematic of a two-layer dual sensor used to monitor $CO_2$ and $O_2$. Analytes are allowed to diffuse from top to bottom, whereas excitation and emission collection are performed from the bottom. Figure reproduced from Reference 32.

poly(acrylonitrile-coacrylic acid) and also ground into microparticles. Both the reference and sensing microparticles were held in polydimethylsiloxane to form the $CO_2$-sensing layer (**Figure 1**). The concentration of the analyte is calculated from the ratio of the two dyes and is therefore independent of the layer thickness (32). The two dyes have the same excitation and emission wavelengths but differ in their fluorescence lifetime. Dual-lifetime referencing is used to differentiate between the two signals. This technique works when the reference and sensing dyes have different (approximately three orders of magnitude) fluorescence lifetimes (33). For the oxygen-sensing layer, platinum(II)-5,10,15,20-tetrakis(2,3,4,5,6-pentafluorophenyl)porphyrin (Pt-TFPP), held in a polystyrene support, was used as the indicator (**Figure 1**) (32) as its fluorescence lifetime is dependent on oxygen concentration. The use of fluorescence lifetime enables the measurement to be independent of the sensing-layer thickness. Through the combination of dual-lifetime referencing and fluorescence-lifetime monitoring, researchers have produced sensors capable of monitoring bacterial growth by measuring both the consumption of $O_2$ and the production of $CO_2$ (32) and capable of monitoring both $CO_2$ and $O_2$ in aquatic systems (28).

Humidity (34) and temperature (35) changes result in large variations in the response of these indicators. Owing to the strong temperature dependence of many sensors, a method to monitor and compensate for temperature would be an important addition to the production of sensors capable of accurately monitoring multiple species in real-world situations (36). The dual-layer approach currently can monitor only two species simultaneously. For such sensors to find wider application in monitoring biological, clinical, or geological processes, they must be capable of monitoring more analytes simultaneously by using both spectral and temporal (dual-lifetime

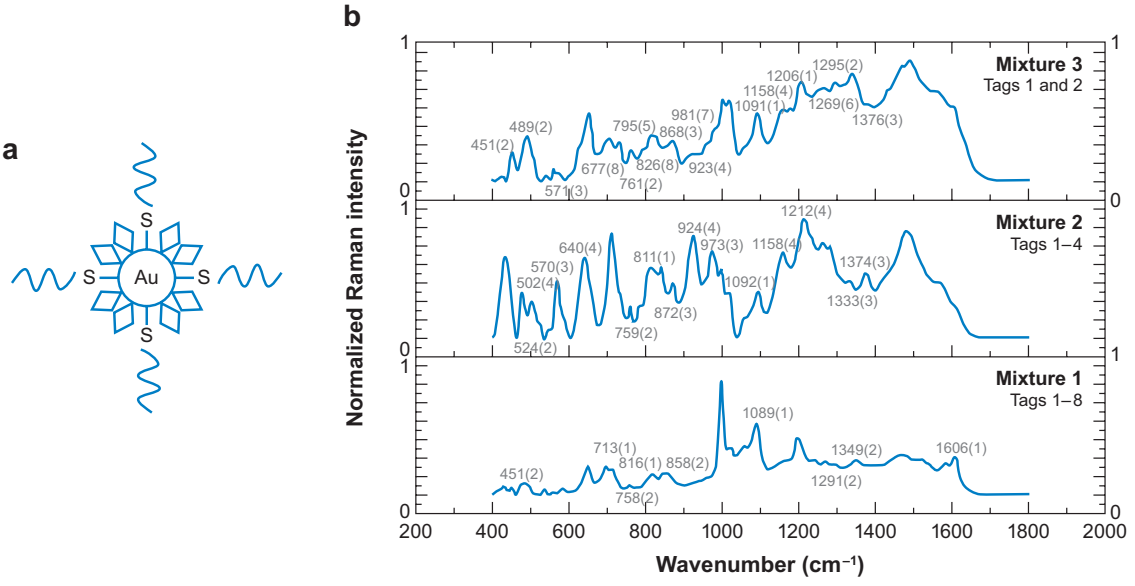
referencing) discrimination (37). The use of spectral separation alone will likely not be adequate for higher levels of multiplexing as fluorescence emission peaks tend to be broad and peak overlap is unavoidable.

**3.1.2. Dyes as labels.** Another approach to using spectral separation is to employ indicators as labels. This approach still separates the indicators spectrally, but they do not participate in a reaction with the analyte other than serving as labels. Multiplexing is accomplished by determining which indicators are bound, enabling a determination of the analytes present in the sample. Four different fluorescent dyes have been used for DNA sequencing as described above (38, 39); however, due to the broad peaks in fluorescence spectra, dyes with narrow bandwidths would be of significant utility. For example, multiplexed SERS detection can be performed effectively in this manner (40). SERS is well suited for multiplexing through the use of different labels because of the narrow (~1 nm, full width at half-maximum) bandwidths of Raman fingerprints (41). One can use most classes of chromophores as SERS labels; thus the number of possible Raman tags is extremely large (42). As shown by Irudayaraj and coworkers (43), the use of eight different nonfluorescent Raman tags could allow for a sensitive and selective simultaneous detection of eight different DNA sequences. These authors obtained SERS signals by attaching both the probe (a complementary DNA strand) and the label (a SERS-active tag) onto a gold nanoparticle (**Figure 2*a***). The nanoparticle probes each contained a unique DNA strand that corresponded to a unique Raman-active molecule. As an initial test, they allowed small aliquots of mixtures containing two, four, and eight different probes to dry on a gold-covered glass slide and obtained Raman signals (**Figure 2*b***). Many of the SERS tags had multiple overlapping peaks, thus reducing the number of unique identification peaks per label, and as the number of tags present increased, the peaks became less defined (**Figure 2*c***). Probes such as these will likely lead to the ability to perform DNA detections using spectral separation. Coupling both probe and label to a common structure (**Figure 2*a***) enables SERS to be utilized for in vitro or in vivo detections of various biological components. For example, breast cancer and floating leukemia cells were detected on the same platform through labeling receptors on the cell membranes with SERS tags (44).

The ability to employ Raman labels for multiplexed protein detections in human tissue was recently accomplished using composite organic-inorganic nanoparticles (COINs) (45). A COIN is formed when silver nanoparticles aggregate with the aid of Raman-active molecules (46). The Raman tags decrease the effective negative charge

**Figure 2**

(*a*) Illustration of a surface-enhanced Raman DNA probe, consisting of thiol-terminated DNA probes and Raman tags (*diamond shapes*) attached to a gold nanoparticle. (*b*) Normalized Raman intensity versus wavelength spectra for mixtures of tags 1 and 2 (*mixture 1*), tags 1–4 (*mixture 2*), and tags 1–8 (*mixture 3*). (*c*) Table of observed Raman peaks for each tag when examined alone and when multiplexed with the other seven tags. Figure reprinted with permission from Reference 43. Copyright 2007, American Chemical Society.




c

| Probe | Observed Raman peaks (cm-1) | Observed peaks in eight-component mixture ($cm^{-1}$) |
|---|---|---|
| Tag-1 | 416, 494, 646, 712, 813, 998, 1089, 1204, 1465, 1606 | 1091, 1206 |
| Tag-2 | 413, 450, 489, 524, 575, 593, 650, 691, 759, 821, 862, 1021, 1069, 1145, 1293, 1345, 1487 | 451, 489, 761, 1295 |
| Tag-3 | 441, 484, 569, 726, 747, 870, 977, 1000, 1098, 1186, 1234, 1334, 1378, 1518, 1579 | 571, 868, 1376 |
| Tag-4 | 438, 505, 565, 638, 710, 920, 1063, 1158, 1216, 1251, 1381, 1439, 1470, 1559, 1689 | 923, 1158 |
| Tag-5 | 540, 660, 733, 794, 880, 968, 1016, 1061, 1198, 1242, 1335, 1543, 1607, 1636 | 795 |
| Tag-6 | 483, 651, 728, 781, 1016, 1098, 1271, 1319, 1425, 1480, 1537 | 1269 |
| Tag-7 | 495, 651, 687, 728, 848, 981, 1072, 1173, 1320, 1477 | 981 |
| Tag-8 | 498, 540, 645, 675, 731, 784, 828, 853, 910, 1016, 1108, 1259, 1317, 1468, 1558 | 677, 826 |

on the surface of silver nanoparticles and reduce electrostatic repulsion, thus allowing aggregation to occur (**Figure 3*a***). The COINs can be coated with a ~10-nm-thick layer of cross-linked bovine serum albumin and then functionalized with the desired probe molecule, such as an antibody (**Figure 3*a***). The different COINs contain different Raman labels, enabling multiplexed detection. Each COIN's bovine serum albumin surface was functionalized with an antibody specific to a unique target protein through the use of pendant carboxylic acid groups (45). A two-COIN staining of a human prostate tissue sample was performed to detect the prostate-specific proteins PSA and CK18. The multiplexed spectrum in **Figure 3*b*** is an overlay of the signals from both COINs, in which individual signals are determined by a least-squares analysis. These studies demonstrate that it is possible to use SERS for multiplexed detection in cells and tissues, enabling their potential use in medical diagnostics and research.

Metallic nanoparticles are also capable of acting as optical labels. Metallic nanoparticles can exhibit strong SPR absorption peaks that are characteristic of their size and shape. When the environment surrounding such nanoparticles is altered, the spectral peak locations change. The development of simple methods for synthesizing gold nanostructures (47) with highly controllable surface plasmon wavelengths (48–51) has been crucial for enabling the use of SPR in multiplexed sensing platforms. Consequently, SPR is a convenient method for multiplexed detection because it is possible to produce gold nanorods that have well-defined and unique SPR absorption bands (47), which can be used for both encoding the particles and the analytical measurement step (52). The gold nanorods can be made into optical biosensors by attaching antibodies via thiol-gold binding. Obtaining an absorption spectrum of a solution of functionalized gold nanoparticles before and after sample introduction (**Figure 4**) enables the simultaneous detection of multiple analytes. Each absorption peak in the spectrum results from a different subpopulation of gold nanorods; the degree of red shift between the control and sample spectra is proportional to analyte binding and therefore to analyte concentration. The gold SPR spectral position is dependent on the solvent and the extent of equilibrium between free and bound analyte, and equilibrium must be reached prior to obtaining absorption spectra. Gold nanostructures can be made insensitive to solvent effects while still maintaining sensitivity to binding by coating them with a 1.5-nm layer of silica (53). This lab-in-a-tube concept may be useful for performing multiplexed in vitro and in vivo detections.

## 3.2. Spatial Separation

Researchers can perform multiplexed detection using spatial resolution by anchoring the sensing chemistry for different analytes at different locations on a solid support or by using many different solid supports, each unique for a single analyte. The solid support can be an encoded microparticle, containing a specific sensing molecule, or the different sensing molecules may share a support by being deposited or printed onto a substrate, such as plastic or glass. For the latter type of arrays, the position of a sensing element identifies its specificity.

Microparticle supports must be encoded to determine which molecules are attached to each microparticle. These encoding schemes all use light as a stimulus and

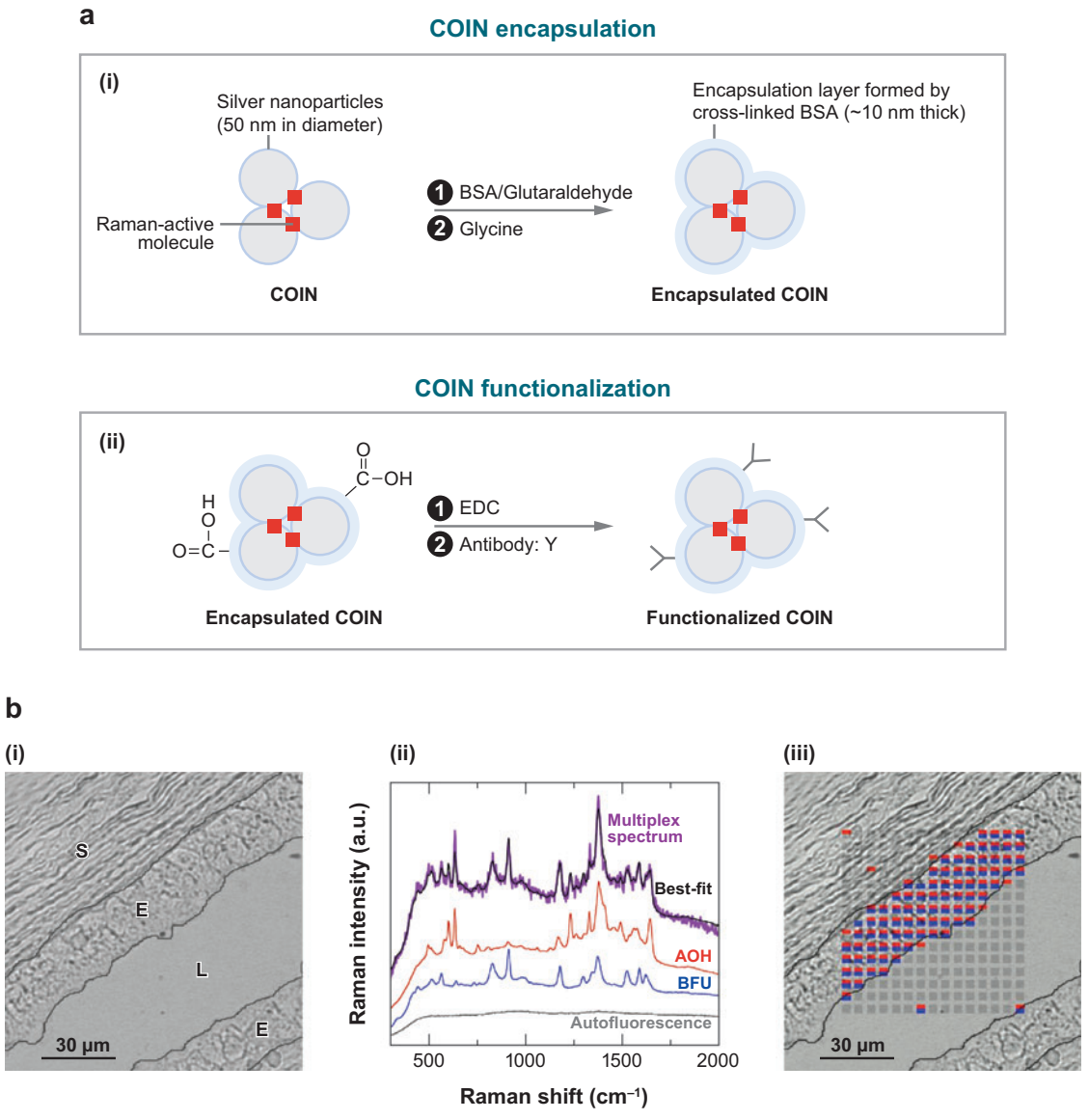



**Figure 3**

(*a*) Diagram describing composite organic-inorganic nanoparticle (COIN) production (*i*) and surface functionalization with biological probes (*ii*). (*b*, *i*) Light microscopy images of human tissue samples showing stroma (S), epithelium (E), and lumen (L). (*ii*) The background (autofluorescence), raw Raman spectrum when both COINs are present, and the resulting deconvoluted spectra of the two probes, each with a unique Raman-active molecule, acridine orange (AOH) or basic fuchsin (BFU). (*iii*) Same image as in panel *b*, part *i*, with each individual square representing where a Raman spectrum was obtained. The red squares indicate the detection of AOH, whereas the blue squares represent the detection of BFU. Figure reprinted from Reference 45. Copyright 2007, American Chemical Society.

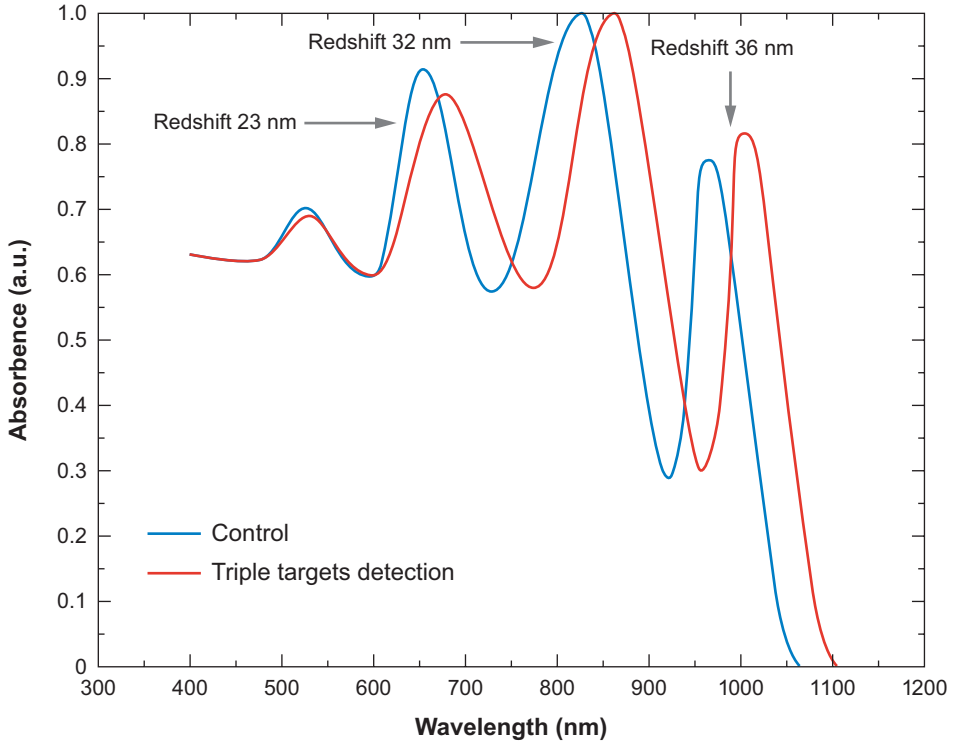



**Figure 4**

Absorption profile of a solution containing three gold nanorods before (control) and after (triplex targets detection) sample binding. Figure reprinted from Reference 52. Copyright 2007, American Chemical Society.

then read an optical response, such as fluorescence intensity, fluorescence lifetime, fluorescence emission location, SERS, or SPR. There are two types of optical responses from such encoded materials. First, the encoding signals provide information about which sensing chemistry is attached to each particle. Second, an orthogonal optical signal reports on the presence and concentration of the analyte that has bound to or reacted with the particle. Just like the encoding schemes, the analyte detection can be performed using the same set of spectroscopies. Although it is possible to use different types of spectroscopy for encoding and detecting, it is more typical to use the same spectroscopic technique for both functions.

## 3.3. Types of Array Platforms

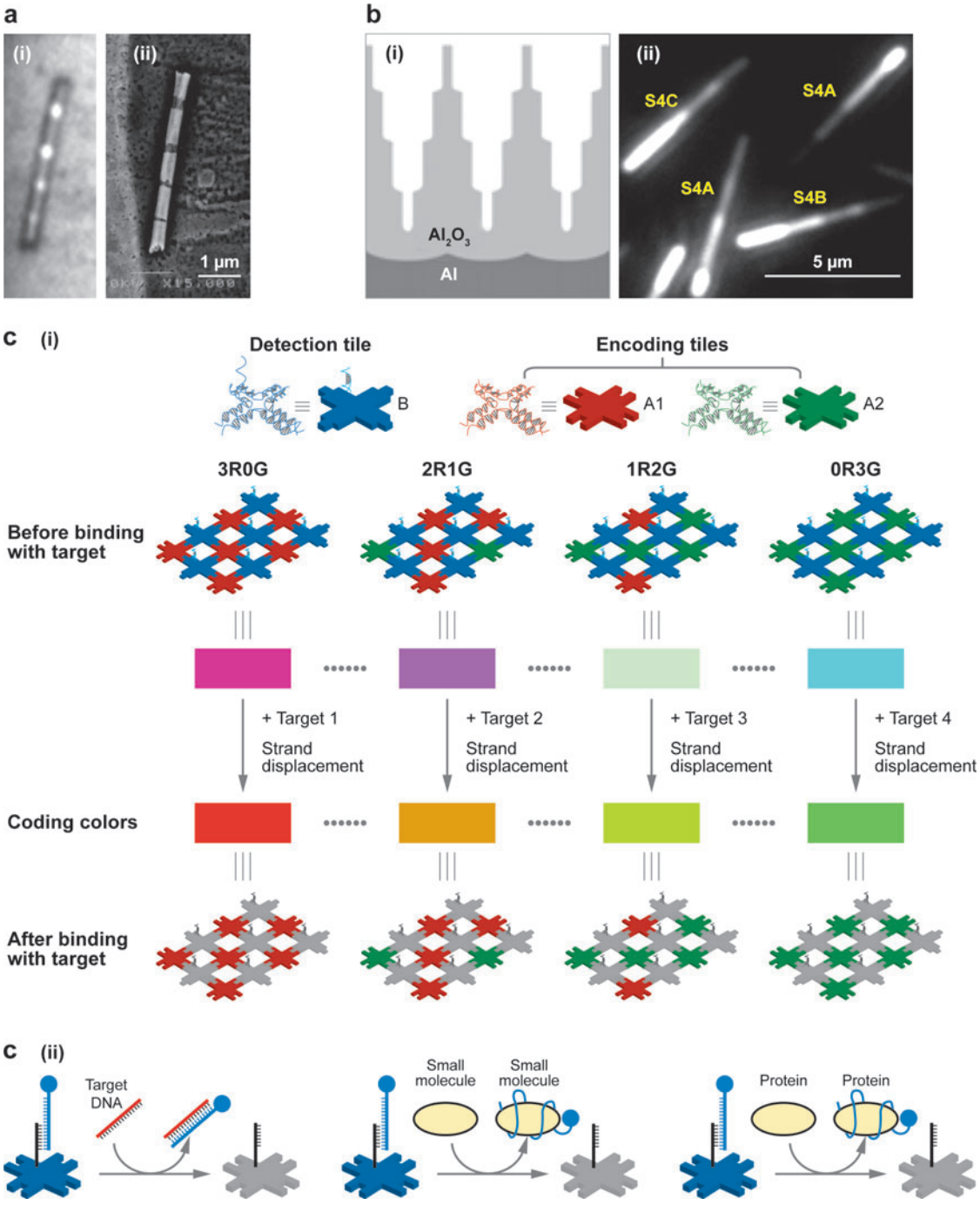
Arrays are defined by the method used to distinguish the different sensing chemistries from one another. Suspension arrays consist of small particles that contain the sensing chemistry, whereas fixed arrays possess all the sensing chemistry on a common solid substrate.

**3.3.1. Suspension arrays.** Mixtures of various sensing chemistries attached to different small solid supports (typically in the nano- to micrometer size range) are known as suspension arrays. The readout process of a suspension array is performed through one of three methods: by using a flow cytometer (54), by passing the solution through a microfluidic platform such that only one particle is interrogated at a time (55, 56), or by transferring an aliquot of the suspension to a microscope slide and imaging the sample using a two-dimensional imager such as a charge-coupled device (57). Each particle type contains sensing chemistry for a single analyte of interest and is encoded through the use of some type of optical signal that does not interfere with the detection scheme. Each particle type contains the same sensing chemistry with identical encoding but differs from particles that contain sensing chemistry for a different analyte. Through the use of optical bar codes, investigators can distinguish particles from one another. Various types of optical bar codes have been used to encode particles for multiplex suspension detections. One of the simplest approaches is to use polymer microspheres containing entrapped dyes. Polymer microspheres can be swelled in organic solvents, allowing fluorescent dyes to be entrapped inside the spheres once the solvent has evaporated and the microspheres have shrunk back to their original size (54, 58). By using different dyes and/or different concentrations of a dye, researchers can produce unique small particles containing the sensing chemistry.

In 2001, the Natan group (59) developed optical bar codes that resemble the thick and thin stripes seen on department store price tags. By individually electrodepositing gold and silver in a preformed porous $Al_2O_3$ membrane, the researchers produced metallic bar codes by varying the thickness of the gold and silver stripes (**Figure 5*a***). Silver reflects strongly at 405 nm compared with gold, which results in a reflection image showing dark and light areas on the rod that can be used for optical encoding. Thiol-terminated sensing molecules then can be easily attached to the metal surface to produce optical probes.

The shapes and composition of reflective solids have also been used to produce suspensions capable of multiplexed detections. Investigators used a two-step anodization process to produce an alumina template containing pores of various dimensions. They modified the pores in the template with silica and dissolved the alumina template to form silica nanotubes (SNTs) as seen in **Figure 5*b*** (60). The resulting size differences between the reflective SNTs allow them to be used for encoding. **Figure 5*b***(ii) shows a dark-field image of SNTs produced with four segments synthesized in the presence of templates created using four anodization steps. The total length of each SNT is held constant at 6.30 μm, whereas the lengths of the four segments composing the different nanotubes are varied. As seen in the image, each segment has a different diameter and can be used as an encoding bit. For example, in **Figure 5*b***, S4A comprises segments with lengths (in micrometers) of 0.8/2.4/1.6/1.6, whereas S4B has 1.6/1.6/1.6/1.6 and S4C has 2.4/0.8/1.6/1.6. From these ratios, it is possible to identify the different SNTs. Different sensing chemistries can be conjugated to the different SNTs by the use of well-established silica chemistry.

Both the gold and silver striped metal rods and SNTs provide an approach to optically encode particles containing sensing chemistry. Although time consuming, it is possible to produce large amounts of particles in these preparations. For example, in



the case of the SNTs, each preparation contains enough particles to perform 100,000 immunoassays (60).

Lin et al. (61) recently created a fluorescently encoded suspension array by self-assembly using DNA as building tiles. Each building tile possessed its own unique fluorescent dye. By using two building tiles in different ratios, along with a third detection tile, they formed a fluorescent suspension array (**Figure 5*c***). The detection tile housed a third fluorescent dye. This dye was attached to a DNA sequence that hybridized to a DNA strand on the detection tile. This hybridization was used in the detection scheme through a strand-displacement method. The binding of the dye-modified DNA to the target analyte, either DNA or a small molecule, is energetically favorable compared with its binding to the detection tile (61). Once the dye-labeled DNA is bound to the target, it is no longer associated with the detection tile (**Figure 5*c***). A positive detection is measured as the decrease in fluorescence of the detection nanotile. Owing to the decrease in the fluorescent signal with a positive detection, these types of probes are known as turn-off probes.

The Doyle group (56) recently developed a microfluidic platform for the production of polymer microparticles that house both sensing chemistry and optical bar codes. The particles have two or three different regions all produced at the same time by photopolymerization inside a microfluidic channel (**Figure 6*a***). The bar code region consists of orientation indicators (long stripes), as well as coding elements (squares) (**Figure 6*b***). The analyte detection region is polymerized such that it has sensing chemistry attached to the polymer backbone. The researchers used a flow-through particle reader to read the individual particles after incubation with a fluorescently labeled DNA target. In principle, the dot-coding system (**Figure 6*b***) can hold $2^{20}$ different bar codes. **Figure 6*c*** shows the results of a duplexed experiment using dye-labeled target DNA. This experiment demonstrated that the bar code region of the microparticles remains unchanged over a detection event, and only the region containing the complementary DNA strand increases in fluorescence when a positive detection event occurs (56).

**3.3.2. Fixed arrays.** Other arrays have their sensing chemistries at specifically defined locations on a solid support; such arrays are known as fixed arrays. The different sensing chemistries can be physically placed at a desired spot on the solid support or

**Figure 5**

(*a*) Reflection (*i*) and field emission–scanning electron microscopy (*ii*) images of a gold-silver multistripe particle. The narrow, dark stripes in part *ii* are silver stripes of (*from top to bottom*) 240, 170, 110, and 60 nm. **Figure 5*a*** reprinted from Reference 59 with permission from AAAS. (*b*) $Al_2O_3$ template after third pore widening and fourth anodization (*i*) along with the resulting silica nanotubes under dark-field illumination (*ii*). **Figure 5*b*** reprinted from Reference 60, copyright 2007, American Chemical Society. (*c*) Schematic production of self-assembled encoded DNA nanotile suspension array components (*i*) and the corresponding detection events for DNA, small molecules, and proteins (*ii, left to right*) showing the turn-off probe style of detection. **Figure 5*c*** reprinted from Reference 61. Copyright 2007, American Chemical Society.

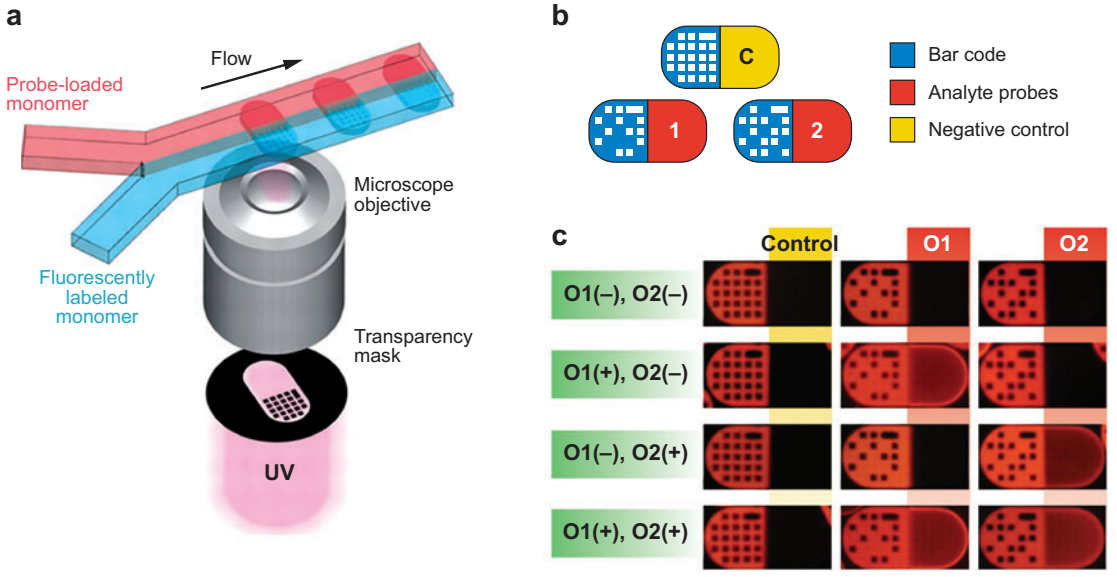



**Figure 6**

(*a*) Microfluidic platform for the production of encoded polymer microparticles that house both bar coding and detection regions synthesized by photopolymerization. (*b*) Schematic of control and sensing microparticles. (*c*) Fluorescent images of (*from top to bottom*) microparticles after hybridization with no target, the complement to 01, the complement to 02, and the complements to both 01 and 02. Figure taken from Reference 56. Reprinted with permission from AAAS.

by randomly distributing microparticles onto a preformed array of microwells. Arrays in which the researcher has control over the location of particular sensing molecules are known as directed arrays, whereas the arrays in which the location of sensing molecules must be determined after production of the array are known as randomly ordered arrays.

***3.3.2.1. Directed arrays.*** Directed arrays were first developed in the mid-1990s by the Fodor (62–64) and Brown (65) groups. These early arrays were developed primarily for gene screening (63, 65). The Brown group, for example, was able to utilize a two-color fluorescence readout of 45 different genes simultaneously (65). A directed array is produced when the various sensing molecules are placed in a specific location on a solid support. This placement (66) can be done by contact printing (65, 67), noncontact printing (67, 68), or photolithography (64). Each position on directed arrays is specific for a particular analyte. These positions are fixed, and signals therefore can be attributed to the proper analyte by the location at which they occur. An important parameter for array technologies is the density of sensing spots per area as it is a major factor for controlling the number of analytes that can be measured in a given sample volume. The density is largely controlled by the feature size of the sensing spots. A spot size of 50 μm has been realized using commercially available pin printers, whereas photolithography (69) and photomasking techniques (70) have

enabled the spot size to decrease to 10–18 μm. Recently, Bright and coworkers (71) demonstrated the ability to print array features of 9 μm by the use of a quartz pin printer, thus increasing the probe density possible for printed arrays.

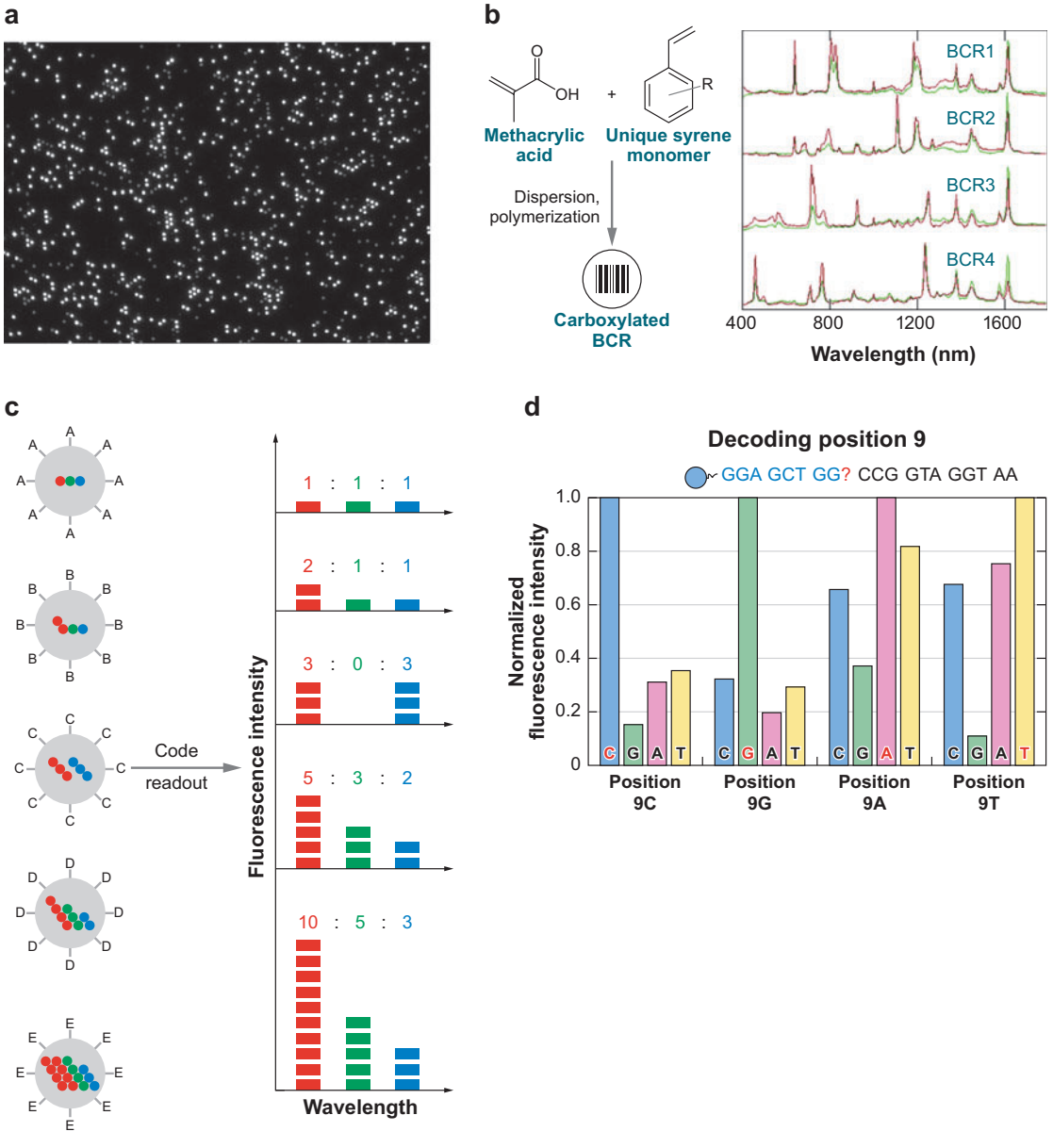
***3.3.2.2. Randomly ordered arrays.*** Randomly ordered arrays use self-assembly techniques to create arrays of microspheres (also called beads) in random locations in an ordered substrate. The substrate is prepared with micro- or nanosized wells that can be loaded with micro- or nanospheres matched to the well size and that contain the sensing chemistry for a particular analyte (72). One can produce the wells by etching a fiber-optic bundle in acidic solution (73) or by a breath-figure method (74). In the fiber-optic approach, a fiber-optic bundle is etched in acidic solution. The cladding of the optical fiber etches at a slower rate than the optical-fiber core. This differential etching produces wells at the end of the fiber bundle (73, 75, 76). The depth of the wells is dependent on the length of time and the concentration of the acid used. The breath-figure method forms an ordered array of pores by a three-step process (77). First, a solid support (such as a glass slide) is covered with a thin layer of a polymer dissolved in a hydrophobic solvent. This solvent layer is exposed to air with high humidity (~50%). As water droplets form on the surface of the hydrophobic solvent layer, the temperature of the surface and the water droplets equilibrates, and the water begins to sink into the hydrophobic solvent owing to its higher density. Ordered arrays are formed when both the water and hydrophobic solvent evaporate (74, 77).

To create a randomly ordered array, one needs to load microspheres or nanospheres into the well arrays. A typical procedure for loading microspheres into microwells involves a self-assembly method in which an aqueous solution containing microspheres is placed on the well array surface and allowed to evaporate. Capillary forces cause the beads to assemble into the wells, presumably to minimize surface free energy. Each bead contains sensing chemistry that is either specific to a particular analyte or reacts with many analytes. Because the array is assembled in a random fashion and contains beads with different specificities, it is necessary to determine the location of the different bead types. There are two general approaches for determining bead location—bead encoding and bead decoding. Encoding is accomplished using a number of approaches, including the incorporation of dyes in the beads by solvent swelling (72, 76), by attaching different ratios of different quantum dots (78), or by producing microspheres made from polymers containing Raman-active functional groups (known as bar coded resins) (79). In bead decoding, the different bead types are interrogated sequentially by exposing the beads to solutions containing molecules with attached labels that bind specifically to each bead type. For example, oligonucleotide arrays can be decoded by exposing them to pools of dye-labeled oligonucleotides (80) (**Figure 7**). All these encoding or decoding approaches result in the ability to distinguish the identity of each individual microsphere. Through this randomly ordered approach, investigators have realized array densities as high as $4.5 \times 10^6$ array elements $mm^{-2}$ using 300-nm-diameter spheres with 500-nm separation (81).

**3.3.3. Applications of arrays.** Arrays have been extensively used as detection tools for biologically relevant species. Arrays have been produced that are capable of

detecting cells, proteins, and nucleic acids. There has also been a considerable interest in the use of cross-reactive sensors for air and water monitoring.

***3.3.3.1. Detection of biologically relevant analytes.*** The area that has benefited the most from advances in fluorescence-based multiplexed detections has been the biological sciences (8, 82). Detection of whole cells, proteins, RNA, and DNA can all



be accomplished using a microarray platform with fluorescence detection as the predominant signal transduction scheme, although other schemes have been employed. This review does not cover the large body of work enabled by commercially available microarrays used for genotyping and gene-expression analysis even though most such platforms are based on fluorescence detection of tens of thousands to millions of array features. Instead, we focus on a number of diverse and lower-density applications of multiplexed detections using spectroscopic techniques.

Investigators have detected cells (83–85) and oligonucleotides (86) using a sandwich assay format, whereby the target analyte is bound by an immobilized capture probe on the array surface and detected using a fluorescently labeled secondary probe. For increased sensitivity, polymerase chain reaction amplification has been used to amplify and label target oligonucleotides (87); the products have then been hybridized to microarrays (88–90).

Antibodies have been used with array platforms to detect proteins (55, 91, 92) and cells (55, 91, 93–95) in a multiplexed format. Recently, Fernandez-Calvo et al. (95) developed a competitive immunoassay for use in astrobiology for the detection and identification of analytes ranging from small compounds, such as naphthalene, to whole cells. This assay is performed by creating a competition for binding sites between antigens and the contents of the sample of interest. First, researchers allowed antigens used as competitors to bind to antibody tracers. They then allowed these complexes to bind to the array. Upon sample introduction, the antigens in the sample compete for the antibody tracers. After a rinse step, a fluorescently labeled protein binds to the tracers that remain on the array. The presence of the analyte of interest in the sample results in a lower fluorescent signal on the array (95).

The detection of whole cells on an antibody array has been used to detect biological threat agents (93, 94, 96). For the detection of *Salmonella typhimurium* (96) in irrigation water used for sprouting seeds, investigators used an optical-fiber-based technique to accomplish a normally labor-intensive procedure much faster (20 min) than typical microbiological methods that require a 48-h or longer culturing. The speed, accuracy, and simplicity of optical array-based techniques may accomplish the goal of "detect to protect" human populations from biological attacks (55, 94).

The ability to identify and quantify pathogens by their specific DNA or RNA sequences has also been exploited to detect food-borne pathogens (88, 97), biowarfare

**Figure 7**

(*a*) Fluorescent image of europium (fluorescent-dye) encoded beads of 0.1 M (*dim spheres*) and 0.5 M (*bright spheres*). **Figure 7*a*** reprinted with permission from Reference 92. Copyright 2006, Elsevier. (*b*) Production of bar coded resins (BCRs) and corresponding Raman spectra of four different BCRs. **Figure 7*b*** reprinted from Reference 79, copyright 2007, American Chemical Society. (*c*) Theoretical code readout of nanoparticle-encoded beads showing the ability to multiplex by varying the ratio of different quantum dots. **Figure 7*c*** reprinted by permission from Reference 78. Copyright 2001, Macmillan Publishers Ltd. (*d*) Normalized experimental results for decoding a position along the DNA sequence of many microspheres by a combinatorial approach using short oligonucleotides. **Figure 7*d*** reprinted from Reference 80. Copyright 2003, American Chemical Society.

agents (90, 98), and respiratory pathogens (61); to monitor harmful algae blooms in coastal waters (89); and to compare and contrast biological communities in natural samples (soil, river sediments, and marine sediments) (4). The use of a sandwich assay for DNA detection allows an extra level of selectivity, as both the capture probe and the labeled probe must bind to the target, thereby reducing the amount of nonspecific binding that occurs. Multiplexed detection schemes afford the capability to simultaneously detect many different DNA strands and to use multiple probes per organism. Through the use of two oligonucleotide probes for *Salmonella* spp., Ahn & Walt (88) employed a fiber-optic-bundle array to obtain a high level of discrimination between the target organisms and other related organisms with high sequence similarity that could have caused false-positive signals. These experiments were performed using a sandwich assay with a fluorescently labeled signal probe. Using the same fiber-optic-bundle platform but with a single capture probe sequence per organism, Ahn et al. (89) simultaneously detected three toxin-producing algae species with a high degree of specificity. These two studies demonstrate the ability to use multiplexed detection schemes both to rule out false positives by monitoring multiple probes per organism and to detect multiple species simultaneously.

Recently, Heath and coworkers (99) described how spotted DNA microarrays could be used as a platform to simultaneously detect DNA, proteins, and cell populations (**Figure 8**). They employed a variety of transduction schemes, but fluorescence detection could be used for all analytes. DNA detection on this platform was accomplished using a sandwich assay as described above. For proteins, antibodies were attached to DNA complementary to oligonucleotides on the microarray, thus forming a nucleic acid tether. This tether enabled the antibodies to be immobilized on the

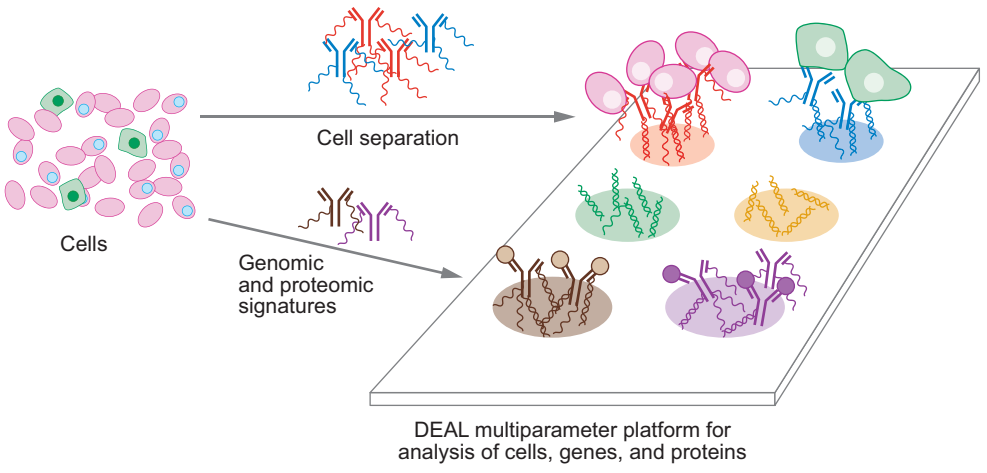



**Figure 8**

Illustration showing how a DNA microarray can be multiplexed to simultaneously detect cells, DNA, and proteins on the same platform. The immobilized oligonucleotides were used as both capture probes and tethers for antibodies. Figure reprinted from Reference 99. Copyright 2007, American Chemical Society.

DNA array, converting it to an antibody array. The antibodies are able to bind to the analytes of interest, such as proteins (99, 100) or cells (**Figure 8**) (99). In addition to the extremely high multiplexing capability, there are many advantages to producing arrays in this way. DNA microarrays are stable over much longer periods of time than protein and antibody arrays, and by keeping the antibodies in solution until use, one can maintain their binding activity. From a production perspective, the DNA surface can be used as a universal platform for any protein detection as it is only necessary to modify the proteins with the tethering oligonucleotide (100). This universal system capable of simultaneously detecting cells, DNA, RNA, and proteins is a major step forward toward using multiplexed analyte detection for diagnostics of cancer and cardiovascular disease (101).

Researchers have also found increased use for arrays in screening small molecules as inhibitors of RNA interactions. This screening is important in the search for new antibiotics and for probing the side effects of current ones, such as streptomycin. Typically the methods used for screening these interactions have been low throughput and have required large amounts of sample. Multiplexing technologies have been applied to simultaneously probe a large number of RNA sequences or small molecules, while taking advantage of the small sample requirement seen when using microarrays. Small-molecule microarrays (102) have been used to find new ligands (103) and protein inhibitors (104), as well as to determine the specificity of a protease (105) or to detect kinases (106). An important limitation of these arrays is the method used to immobilize the small molecules to the array as the attachment may alter their biochemical function. To remove this limitation, Liang et al. (107) designed an experiment to probe the ability of a single small molecule to inhibit RNA binding to many sequences on a DNA microarray. Although only one small molecule could be interrogated at a time, this method has the benefit of being able to screen multiple RNA hybridizations simultaneously (**Figure 9*a***). A decrease in fluorescence signal (**Figure 9*b***) of particular sequences on the array in the presence of neomycin or streptomycin when compared with a control experiment lacking these molecules indicated binding of the small molecule to the RNA strand, thereby inhibiting its binding to the DNA on the microarray. These array applications demonstrate the flexibility of DNA arrays because the signals from these arrays detect the inhibition of binding owing to small molecules as compared with the studies discussed above in which they were used to monitor the presence and concentration of DNA.

Although fluorescence-based arrays have dominated the literature, SPR (11) has also recently been utilized for multiplexed detections (108, 109). SPR arrays have been produced by the vapor deposition of thin layers of silver onto glass slides covered with a monolayer of silica nanospheres. Investigators followed silver deposition with sonication to remove the nanospheres, thus resulting in slides consisting of triangular silver nanoparticle arrays formed between the nanospheres prior to removal (**Figure 10**) (110). As proof of concept, the investigators printed a $2 \times 1$ carbohydrate-sensing array on this type of silver nanoparticle substrate to simultaneously monitor the binding of concanavalin A to mannose and galactose (110).

Endo et al. (111) accomplished higher levels of SPR multiplexing through the use of core-shell structured nanoparticle layers. As seen in **Figure 11**, they produced

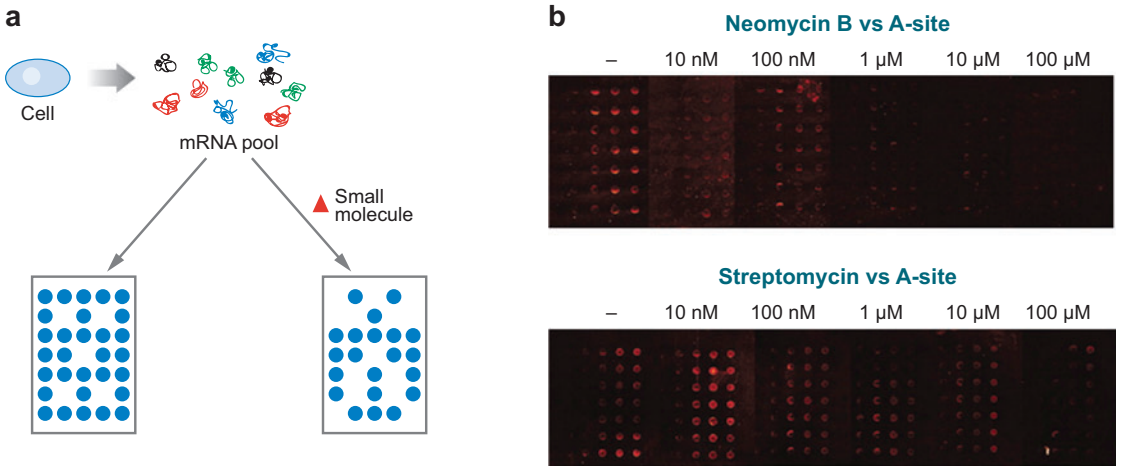



**Figure 9**

(*a*) Schematic of the method used for determining small-molecule interference of RNA binding and (*b*) experimental results from inhibition experiments performed with neomycin B and streptomycin ranging from 0 nM to 100 μM. Figure taken from Reference 107. Copyright 2006, National Academy of Sciences, U.S.A.

an array consisting of two layers of gold separated by silica nanoparticles, and the sensing chemistry was deposited by a nanoliter-dispensing instrument to form the printed array. White light was coupled down an optical fiber, and the reflected light was collected by the detection fiber in the same optical-fiber bundle. As shown in **Figure 11*a***, the absorbance of the array increased at ~550 nm as antigen binding occurred. Through this setup, the authors observed detection limits of 100 pg $ml^{-1}$ for all targeted proteins with a range of detection spanning between 6 and 12 orders of magnitude (111). SPR does not require a secondary probe, but the signal can be amplified if one is used. For example, through use of an enzyme-labeled indicator, an RNA microarray demonstrated a picomolar detection limit (112).

***3.3.3.2. Analyte detection using cross-reactive arrays.*** Another approach to performing multi-analyte sensing is to employ cross-reactive sensor arrays. In this approach, the different sensors in the array are not specific to a particular analyte but react with multiple analytes. The response pattern is unique to each analyte and provides the requisite specificity (113). This approach is based loosely on principles of the olfactory system and has been referred to as electronic or artificial noses for vapor sensing (75, 114) or electronic tongues for solution sensing (115). Both systems work by using cross-reactive sensors that respond differentially to various analytes (75). The sensor-array responses provide a combinatorial code that is analyzed with pattern-recognition algorithms, enabling the discrimination of more analytes than there are sensors. Both the electronic tongue and nose elucidate the identity of an unknown analyte or mixture by comparing the array responses to those from a library of responses obtained from known samples.

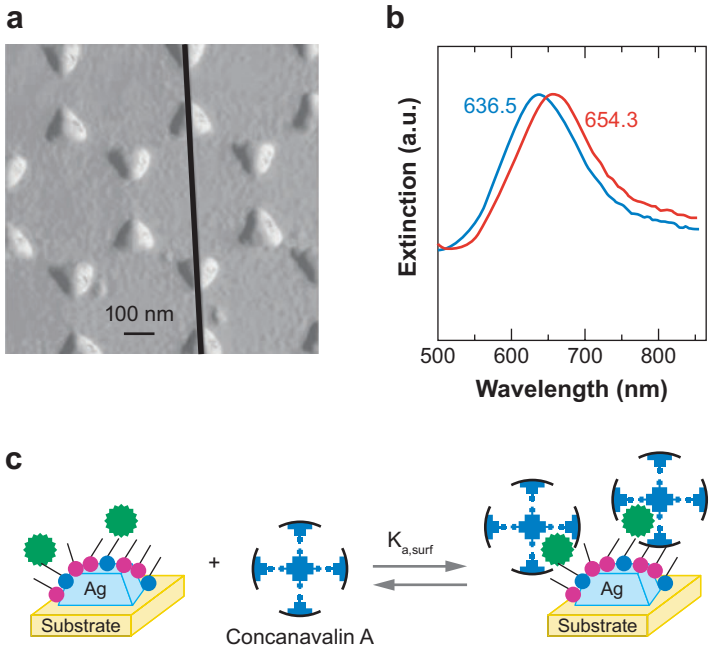



**Figure 10**

(*a*) Tapping-mode atomic force microscopy image of a silver biosensor produced by the vapor deposition of silver onto a glass slide after the removal of the nanosphere monolayer. (*b*) Surface plasmon resonance spectra of silver nanoparticles after mannose modification ($\lambda_{max} = 636.5$ nm) and after binding of concanavalin A ($\lambda_{max} = 654.3$ nm) under nitrogen. (*c*) Scheme for concanavalin A detection. The green circles represent mannose, the pink circles represent alcohol-terminated self-assembled monolayers, and the blue circles represent self-assembled monolayers terminating in mannose. Figure reprinted from Reference 110. Copyright 2004, American Chemical Society.

There are two aspects of these types of sensors that directly affect their ability to provide accurate identification of analytes. The first is the reproducibility of a response from the sensors to a given analyte; the second lies in the interpretation of the obtained responses. Many types of pattern recognition techniques, such as principal component analysis (116, 117), K-nearest neighbor (118), and discriminant analysis (119, 120) are widely used for interpreting data. However, this list is far from exhaustive and there are ongoing efforts to create pattern recognition methods that will result in better classification (121). Although there are many different electronic-nose formats (10), only a few are based on multiplexed spectral detection.

The first optical nose was developed in our laboratory using solvatochromic dyes entrapped in different polymers on the ends of different optical fibers (75, 122). The more modern platform for these arrays is based on the randomly ordered microsphere arrays discussed above, with the microspheres containing different vapor-sensitive chemistries. Researchers can form arrays by using thousands of cross-reactive fluorescent microspheres deposited in wells (113, 123) or by printing dyes on silica

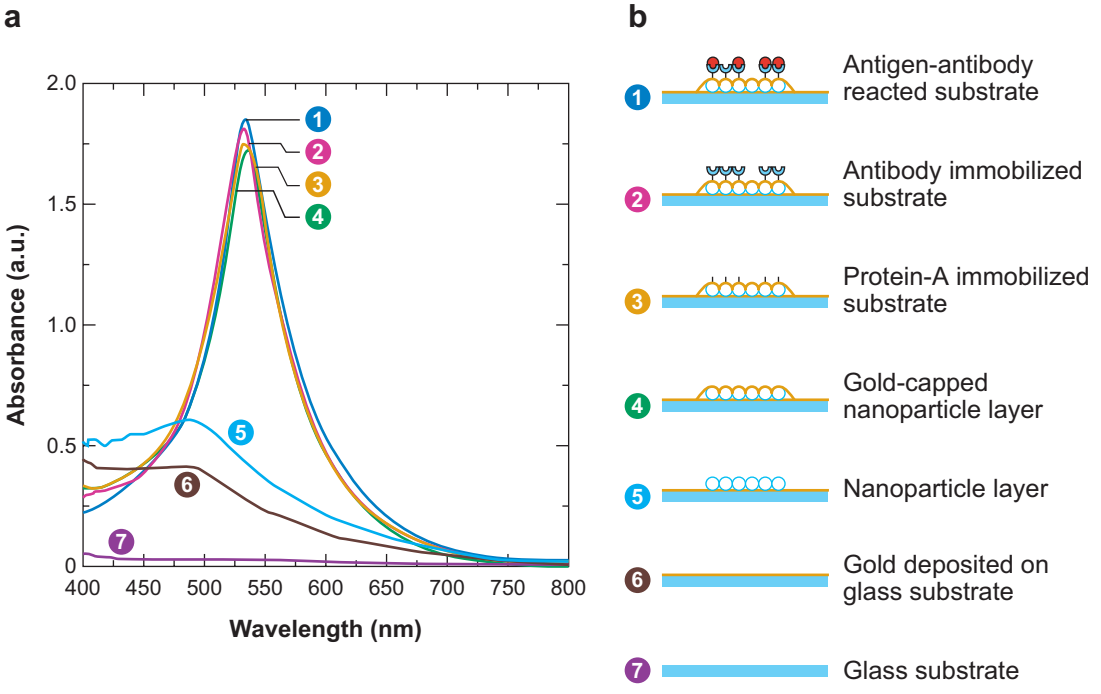



**Figure 11**

(*a*) Sequential absorbance spectra of the surface plasmon resonance biosensor as it is (*b*) formed from a glass substrate (*bottom panel*) to the binding of an analyte of interest (*top panel*). Figure reprinted from Reference 111. Copyright 2006, American Chemical Society.

thin-layer chromatography plates (124). Both these systems rely on the reproducible responses upon exposure to a particular vapor to identify analytes of interest. The printed arrays use a wide range of dyes, including metalloporphyrins, pH indicator dyes, and solvatochromic dyes (125). The diversity in the dye types enables these cross-reactive arrays to exhibit a high degree of sensitivity and selectivity to a wide range of analytes (125). The microsphere-based system monitors the change in fluorescence intensity over time and/or the emission wavelength maxima of each individual microsphere. These arrays have been able to identify complex vapor mixtures, such as various types of coffee-bean odors (126), as well as explosive vapors (127), with a high level of success. Perhaps the biggest advantage of these types of arrays is their ability to identify mixtures that they have been trained to detect (128). The level of confidence in a measurement is an important parameter for electronic noses as the number of false positives must be minimized if they are to be deployed for health and safety applications, such as for disease detection using breath samples or for explosives or chemical-agent detection in subway stations and airports. The flow environment, such as inside a model nasal cavity, can enhance vapor discrimination (129). Maintaining the long-term stability of electronic noses is also important if such systems are to find widespread applications for public safety. The longevity of a

fluorescent sensor is largely limited by photobleaching of the indicator dyes. By limiting the power of the excitation source and only illuminating one subsection of the entire array at any given time, one could use a system for, theoretically, over 170,000 exposures. This increase in longevity represents an increase of three orders of magnitude compared with a traditional system (130).

Electronic tongues work in a similar manner to electronic noses, except they are designed for monitoring liquid environments (131–134). To demonstrate the ability of such arrays to differentiate between complex mixtures, researchers used an array consisting of eight colorimetric anion sensors immobilized in polyurethane distributed in drilled wells on a microscope slide to measure the concentrations of multiple anions. Several sensors were selective for fluoride and pyrophosphate, whereas others showed cross-reactivity for other anions. The array responses were able to distinguish between eight different types of toothpastes in water (135). Responses from all eight toothpastes were first obtained to determine the characteristic responses from the electronic tongue unique to each toothpaste brand. By comparing these known responses with the responses obtained from unknown samples using pattern-recognition techniques (such as principal-component analysis), the electronic tongue identified the particular brands of toothpaste.

Cross-reactive optical sensors in conjunction with pattern-recognition techniques have also been used to detect and identify amino acids (136, 137). The amino acids were identified by indicator displacement assays. The receptors in the assay are noncovalently bound to fluorescent indicators. The addition of different amino acids results in different degrees of displacement of the indicators. Unique patterns for each amino acid can be established and used to identify an unknown sample through the use of multiple receptors and indicators. A solution-based system has been reported for the detection of proteins (138). In this system, six different gold nanoparticle surfaces are noncovalently coated with fluorescent polymer conjugates. When bound to the nanoparticles, the polymer fluorescence is quenched. The different nanoparticle preparations are placed in different solutions. When proteins are added to these solutions, the proteins compete for the different surfaces and displace the fluorescent polymers to different extents, causing the solutions to recover their fluorescence. Linear discriminant analysis was used to process the response patterns for the array of solutions to determine which protein was present. These polymer-nanoparticle hybrids were able to identify and quantify seven different proteins individually and in mixtures using the fluorescent response patterns.

## 4. CONCLUSION

In this review, we discuss the present state-of-the-art techniques for producing platforms capable of multiplexed spectroscopic detection. We highlight the production and uses of directed, randomly ordered, and suspension arrays, especially for biologically relevant analytes. For suspension and random arrays, we discuss the various methods of particle encoding, such as fluorescent dyes, Raman-active microspheres, size and shape variations, and bar codes on microparticles. Finally, multiplexing techniques involving newer spectroscopies, such as SERS and SPR, are summarized.

The ability to continually monitor two analytes at the same location has been accomplished by dual-layer sensors, whereas the need to increase the number of analytes these systems can monitor has been made apparent. The use of cross-reactive sensors in an array format is a promising tool for monitoring air and water samples. Fluorescence-based DNA microarrays have been used for a wide range of applications, including detecting and identifying cell types by their DNA or RNA, producing antibody arrays, and probing small-molecule interactions with RNA. Multiplexed SERS and SPR detections, although not as prevalent as fluorescence systems, show promise toward becoming useful tools in medical diagnostics. As the development of new methods for producing multiplexed systems is realized and as more analytes are able to be detected, the use of multiplexed detection systems will become more useful both in the laboratory setting and in a variety of commercial applications.

## SUMMARY POINTS

1. Multiplexed spectroscopic detection platforms have been used to measure thousands of analytes simultaneously.
2. Detections can be direct, in which spectroscopic signatures of the analytes are measured, or indirect, in which the measured spectral response results from the analyte reacting with an indicator molecule.
3. Dual-layer sensors continually monitor gases, both in solution and air, using fluorescent dyes that undergo a reversible reaction with the analyte gases. These reactions change the fluorescence intensity, lifetime, or wavelength of the dye that can be correlated to analyte concentration.
4. Surface-enhanced Raman tags can be employed to simultaneously detect multiple analytes in the same location owing to the tags' narrow spectral bandwidths.
5. Multiple particles, such as microspheres carrying different sensing chemistries, must be encoded to identify the sensing chemistry on each particle.
6. SPR peaks of metallic nanoparticles can be used for both encoding and detecting lab-in-a-tube type multiplexed analysis.
7. Arrays have been used extensively for biologically relevant analytes, including DNA, RNA, proteins, and cells. The ability to detect all these analytes on the same platform simultaneously has recently been demonstrated.
8. Cross-reactive sensors can be employed along with pattern-recognition software to detect multiple analytes. These types of cross-reactive sensors are known as artificial noses and artificial tongues. These sensors must first be calibrated with known samples to obtain the characteristic pattern for each analyte of interest.

### FUTURE ISSUES

1. There is a need to create higher-density arrays to enable smaller sample sizes and increase the number of analytes that can be detected.
2. There is a need to produce integrated systems for nonexperts, including arrays, sample and fluidic handling, and readout instruments that can perform analyses quickly and accurately.
3. The degree of multiplexing of dual-layer sensors must be increased to enable wider application of these systems.
4. The level of confidence in the identification of harmful vapors should be increased—as false positives and false negatives are highly disruptive and costly—before these systems can be used in airports, subway stations, or other public spaces where large numbers of people could be affected.
5. SPR and SERS probes should be further developed for in vivo and in vitro detections.

## DISCLOSURE STATEMENT

David R. Walt is the Scientific Founder and a Director of Illumina, Inc., a manufacturer of microarrays using the random array technology discussed in this paper. All of the work reported in this paper is the result of research carried out in the investigator's laboratory at Tufts University.

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