Optical Biosensors: Today and Tomorrow (2nd Edition)
Frances S. Ligler and Chris Rowe Taitt (Editors)

Chapter 1

OPTRODE-BASED FIBER OPTIC BIOSENSORS (BIO-OPTRODE)

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Optrode-based fiber optic biosensors (bio-optrodes) are analytical devices incorporating optical fibers and biological recognition molecules. Optical fibers are small and flexible "wires" made out of glass or plastic that can transmit light signals, with minimal loss, over long distances. The light signals are generated by a sensing layer, which is usually composed of biorecognition molecules and dyes, coupled to the fiber end. Light is transmitted through the optical fibers to the sensing layer where different optical phenomena such as absorption or luminescence are used to measure the interactions between the analyte and the sensing layer. Bio-optrodes can be used for remote analytical applications including clinical, environmental, and industrial process monitoring. In the last decade, due to the rapidly growing use of fiber optics for telecommunication applications, new fiber optic technologies have been developed resulting in high-quality and inexpensive optical fibers that can be used for bio-optrode applications. Recent advancements in bio-optrode technologies include the development of nanoscale bio-optrodes, enabling measurements inside single living cells, and the development of multianalyte and reagentless bio-optrodes. Although currently there are only a very limited

number of bio-optrodes commercially available, it is expected that the development of advanced bio-optrode technologies will lead to many commercially available devices for various analytical applications.

1.1. Principle of operation

The word "optrode" is a combination of the words "optical" and "electrode" and refers to a fiber optic-based analytical device that can measure the concentration of a specific chemical or a group of chemicals in a sample of interest. The basic design of an optrode system is shown in Figure 1.1. The main components of an optrode are (a) a light source; (b) an optical fiber to both transmit the light and act as the substrate; (c) the sensing material, which is usually immobilized to the surface of the end face of the fiber; and (d) a detector to measure the output light signal. Computers or microprocessors are used to control the optrode instrumentation and are employed to analyze the output signals.

The "heart" of the optrode is the sensing element. When the sensing element interacts with the analyte, it undergoes physicochemical

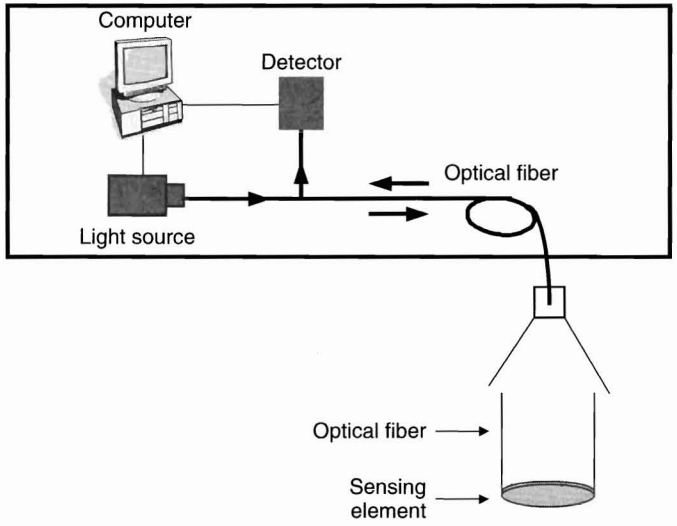


Figure 1.1 Schematic diagram of optrode system.

transformations that change its optical properties. This transduction mechanism generates optical signals that can be correlated to the analyte concentration. The optical signals are measured by launching light from the light source through the optical fiber to the fiber end, where the sensing element is immobilized. The same fiber (Figure 1.1), or a different fiber, is used to guide the output light to the detector (e.g., spectrophotometer, fluorometer) where the reflected, emitted, or absorbed light is measured. Optrode biosensors or bio-optrodes are optrodes in which the sensing elements are of biological origin. Biological sensing elements, such as enzymes, nucleic acids, antibodies, and cells, are immobilized on optical fibers and used for specific recognition of many different analytes (Cunningham, 1998; Mehrvar *et al.*, 2000; Wolfbeis, 2000a, 2000b, 2000c, 2000d, 2002, 2004, 2006; Kuswandi *et al.*, 2001; Lopez-Higuera, 2002; Narayanaswamy *et al.*, 2003; Monk and Walt, 2004a, 2004b; Brogan and Walt, 2005). Since most biological sensing elements and most analytes do not possess intrinsic spectral properties, the biorecognition events are transduced to optical signals (e.g., changes in fluorescence or absorbance) by coupling optically responsive reagents to the sensing elements. For example, fluorescent dyes are used to label nucleic acids and convert the biorecognition interaction between two complementary DNA strands into a fluorescence signal. In another example, an indicator dye, which is optically sensitive to changes in H^+ concentrations, is used to transduce enzymatic activity that consumes or releases H^+ into an optical signal. The signals are generated on the fiber optic face and transmitted by the optical fiber to a remote measurement device. The small dimensions of bio-optrodes allow measurement in very small sample volumes, which make them suitable for various clinical applications (Meadows, 1996; Vo-Dinh and Cullum, 2000; Vo-Dinh *et al.*, 2005). Bio-optrodes are useful for different sensing applications in the industrial, clinical, and environmental fields (Mulchandani and Bassi, 1995; Rogers and Poziomek, 1996; Scheper *et al.*, 1996; Rogers and Mascini, 1998; Marose *et al.*, 1999; Merhrvar, *et al.*, 2000; Kumagai and Kajioka, 2002; Berthold and Lopushansky, 2004).

In this section, the basic characteristics of optical fibers and the optical methods used to transduce a biorecognition event to an optical signal are described. The instrumentation employed in optrode biosensors, the

biological sensing elements, and the methods to immobilize them on the fiber optic surfaces are summarized.

1.1.1. Optical fiber characteristics and use in bio-optrodes

Optical fibers are small and flexible "wires" made out of glass or plastic that can transmit light signals, with minimal loss, for long distances. Optical fibers are remarkably strong, flexible, and durable and therefore can be used in harsh and hazardous environments. Optical fibers are non-electrical, which make them highly suitable for applications where the presence of electric current is detrimental (e.g., *in vivo* monitoring inside a patient body). In the last decade, due to the rapidly growing use of fiber optics for telecommunication applications, new fiber optic technologies have been developed, resulting in high-quality and inexpensive optical fibers that can also be used for sensing applications. Optical fibers can transmit multiple optical signals simultaneously, thereby offering multiplexing capabilities for sensing.

Optical fibers consist of a core with a refractive index, n_1, surrounded by a cladding with a lower refractive index, n_2 (Figure 1.2). The difference in the refractive indices between the core and the cladding enables the core–clad interface to effectively act as a mirror such that a series of internal reflections transmits the light from one end of the fiber to the other as shown in Figure 1.3a. Light undergoes total internal reflection (TIR) at the core–clad interface if two basic conditions are fulfilled: (a) The light strikes the cladding at an angle greater than the *critical*

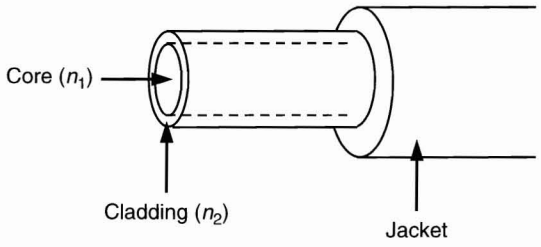


Figure 1.2 Schematic diagram of an optical fiber shows core and clad structure.

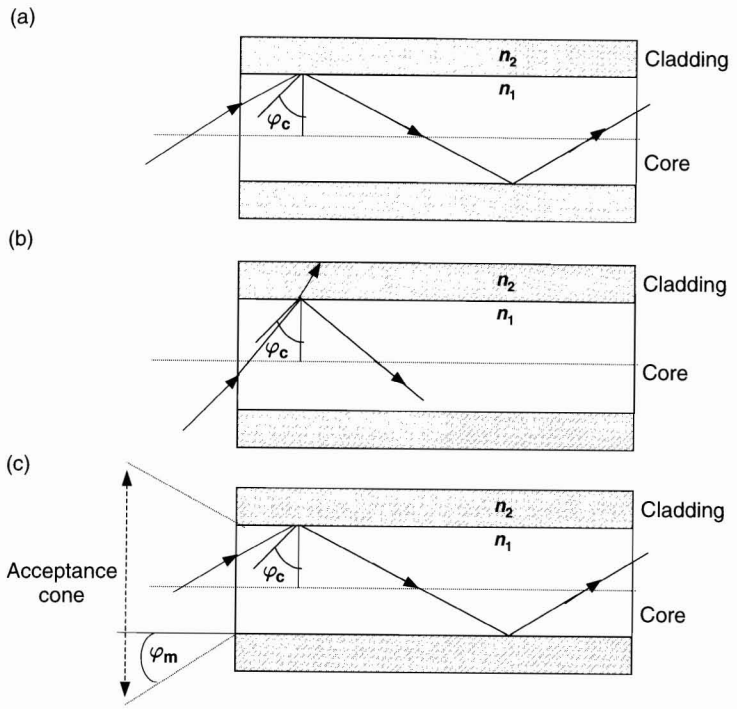


Figure 1.3 Propagation of light through the optical fiber occurs when the TIR condition exists at the interface between the core, (n_1), and clad, (n_2), such that $n_1 > n_2$. (a) Light entering the fiber will experience TIR, if the light angle is greater than the critical angle, φ_c. (b) Light will be partially reflected and partially refracted, if the light angle is less than the critical angle, φ_c. (c) Light will propagate by TIR only when the entering light angle is within the acceptance cone angle (φ_m) range.

angle, φ_c, (Figure 1.3a and b). The critical angle is defined by the ratio between the clad and the core refractive indices, as shown in Eqn (1.1):

$$\sin \varphi_c = \frac{n_2}{n_1} \tag{1.1}$$

(b) The angles of the light entering the fiber should be within the *acceptance cone* as shown in Figure 1.3c. The acceptance cone angle, φ_m, depends on the refractive indices of the core and the clad and also

on the refractive index of the medium from which the light enters the fiber, n_o:

$$\sin \varphi_m = \frac{\sqrt{(n_1^2 - n_2^2)}}{n_0} \tag{1.2}$$

Another important parameter that defines the fiber's light collection efficiency is the *numerical aperture* (NA). This parameter is related to the acceptance cone's angle and is given by:

$$\mathrm{NA} = n_o \sin \varphi_m \tag{1.3}$$

A high NA indicates a wide acceptance cone and better light-gathering capabilities of the fiber. A typical NA value for a high-quality glass fiber is 0.55, but fiber NAs as high as 0.66 or as low as 0.12 (Masson *et al.*, 2006) have been used for sensing.

Optical fibers are usually made out of plastic and glass and have many different configurations, formats, shapes, and sizes. Glass fibers are the most commonly used fibers in optrode biosensors. Glass optical fibers can transmit light in the visible and near-infrared regions of the optical spectrum (400 nm $< \lambda <$ 700 nm) and are therefore suitable for measuring fluorescence signals generated by most fluorescent dyes. For applications in which light in the UV region is required, quartz (pure silica) is used as the fiber's core material and doped silica (with a lower refractive index) is used as the cladding material. For most fiber optic-based biosensors, optical fibers with diameters ranging from 50 to 500 μm are employed.

Recently, fiber optic bundles (Figure 1.4a) comprising thousands of identical single fibers, each with a diameter of a few micrometers, were employed for bio-optrodes. The fibers can be bundled in a coherent or random fashion. In coherent fiber bundles, the position of each fiber on one end is identical to its position on the other end. These fibers were originally designed for imaging applications as shown in Figure 1.4b and are also often called "optical imaging fibers". Imaging fibers are suitable for multianalyte optrode biosensor design (Healey and Walt,

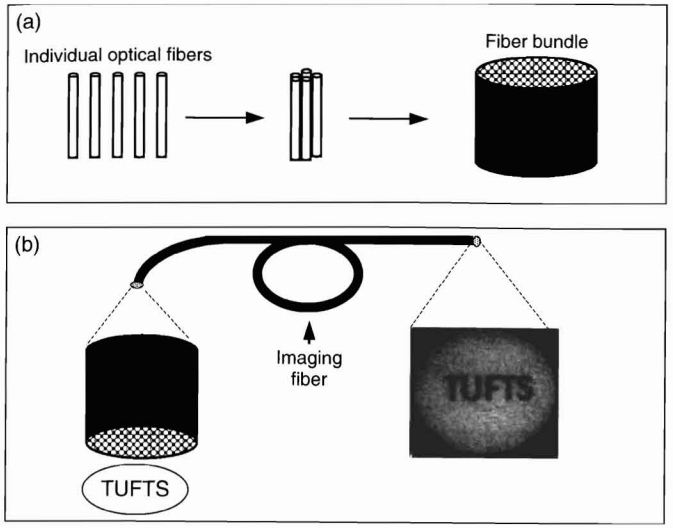


Figure 1.4 Optical fiber bundle fabrication and its use for imaging. (a) Fiber bundles are constructed from thousands of individual single fibers that are fused together. (b) Coherent bundles can be used for imaging (Pantano and Walt, 1995). Reprinted with permission from the American Chemical Society.

1995; Healey *et al.*, 1997a; Michael *et al.*, 1998; Steemers and Walt, 1999; Walt, 2000, 2002; Epstein and Walt, 2003; Wygladacz and Bakker, 2005), since each small individual fiber in the bundle can carry its own light signal from one end of the bundle to the other. Moreover, optical imaging fiber-based biosensors can be used for sensing and imaging simultaneously, providing remote spatial sensing capabilities (Walt, 1998; Issberner *et al.*, 2002).

1.1.2. Optical phenomena employed for biosensing in bio-optrodes

In bio-optrodes, dyes are coupled to the biological sensing element and transduce the biorecognition events to an optically detectable signal. Different optical phenomena—including fluorescence, luminescence, absorption, evanescent wave, and surface plasmon resonance (SPR)—are employed for monitoring these optical changes. In this

section, the basic principles of these phenomena and their use in bio-optrodes are described.

Fluorescence is commonly used in bio-optrodes. Fluorescence occurs when molecules are excited at a specific wavelength and re-emit radiation at a lower energy, i.e., a longer wavelength. The absorption of the excitation light promotes the molecule's energy from its ground state to a higher energy state. The molecule emits fluorescent light when it returns to the ground state. Each fluorescent molecule has a unique fluorescence spectrum since the excitation and emission occur only at distinct energy levels corresponding to particular wavelengths. The characteristic fluorescence spectrum of particular molecules allows multiple fluorescent dyes to be used simultaneously in a single analytical assay. In fluorescence-based bio-optrodes, the fluorescence signals are measured by transmitting the excitation light through an optical fiber and measuring the light emission using a detector. Usually, the increase or decrease in fluorescence intensity is measured and then correlated to the analyte concentration. For example, when a fluorescent-labeled antibody is used as the sensing element, the fluorescence intensity is proportional to the amount of antigen (analyte) bound to the optical fiber. Both time- and frequency-domain methods could be used to measure fluorescence lifetime (Chandler *et al.*, 2006). In the time-domain approach, a short pulse of light (the impulse) is used to excite the fluorophore and the statistical decay curve (the impulse response) at a sequence of time steps is observed. In the frequency-domain method, sinusoidally modulated light is used to excite the fluorescent molecule. The resulting emission light also oscillates at the same frequency. The emission light is phase shifted (delayed) and demodulated with respect to the excitation light because of the finite lifetime of fluorescence. The phase shift is expressed as a phase angle from which the lifetime can be determined using simple relationships between the modulation frequency and the degree of demodulation. The concentration of analyte that induces changes in the molecule's fluorescence lifetime can be determined by measuring phase angle values (Thompson *et al.*, 1996).

A decrease in fluorescence intensity due to quenching can also be used for sensing. In this case, the biorecognition event causes a decrease in

fluorescence (quenching) of the fluorescent reporter molecule. The fluorescence decrease is related to the analyte concentration. For example, a dye that undergoes fluorescence quenching when the pH decreases can be coupled to an enzymatic reaction that converts a substrate into an acidic product and results in a pH drop. Thus, the decrease in fluorescence can be correlated to the analyte concentration (see also Section 1.1.4.1). Fluorescence quenching is also a manifestation of another fluorescence phenomenon used for sensing in bio-optrodes – *fluorescence resonance energy transfer* (FRET). This phenomenon occurs when two distinct fluorophores are present. If the emission spectrum of one fluorophore overlaps with the excitation spectrum of a second fluorophore, and the two fluorophores are in proximity (<100 Å), then the excited fluorophore (donor) can transfer energy non-radiatively to the second fluorophore (acceptor). There are two types of acceptors. Quenchers are acceptors that are not fluorescent and therefore cause the donor simply to decrease its fluorescence emission intensity. Acceptors can also be fluorescent dyes that accept the energy non-radiatively from the donor, and then re-emit the energy at specific emission wavelength. This energy transfer results in an increase in light emission by the acceptor and a decrease in light emission from the donor. When an energy transfer pair of fluorophores is used to label two interacting molecules (e.g., antibody–antigen, enzyme–substrate), they can be used for sensing. Recently, both the donor and the acceptor molecules have been incorporated into single biological molecules such as proteins (Hellinga and Marvin, 1998; Grant *et al.*, 2005; Komatsu *et al.*, 2006) and nucleic acids (e.g., molecular beacons) (Tyagi and Kramer, 1996; Tyagi *et al.*, 2000; Wabuyele *et al.*, 2003). When these sensing molecules are in their native conformation, the donor and the acceptor are in proximity and therefore low-fluorescence signals from the donor are obtained. When the molecule interacts with the analyte, conformational changes occur, which separate the donor and the acceptor molecules and cause an increase in the fluorescence from the donor (see Section 1.3.3).

The most commonly used fluorescent molecules in bio-optrodes are organic dyes. Recently, self-fluorescent proteins have also been used. The sources of these proteins are marine organisms such as the jellyfish *Aequorea victoria* that produce the green fluorescent protein (GFP)

(Chalfie *et al.*, 1994). When GFP is excited, it emits light at a lower energy and therefore at a longer wavelength. GFP is highly fluorescent, with a quantum efficiency of approximately 80% and is very stable to heat and pH (5.5–12). GFP has been expressed in different cell types (bacteria, yeast, mammalian, and plant) and used as a reporter gene for different cellular events (Naylor, 1999; Serganova and Blasberg, 2005). In order to allow monitoring of several cellular events simultaneously, several GFP mutants have been developed, each with unique excitation and emission wavelengths. Cells expressing fluorescent proteins and purified proteins have been used to construct different bio-optrodes (see Sections 1.1.4.1 and 1.3.3).

Time-resolved fluorescence is another phenomenon used in bio-optrodes. This method is based on the fluorescent molecule's excited state lifetime. The light intensity emitted from a molecule excited by a short pulse of light decays exponentially with time. The decay time pattern is unique for each molecule and can be used for analytical purposes. Barker *et al.* (1999) used this method to improve the performance of a bio-optrode for nitric oxide detection. Fang *et al.* (2004) recently reported the design and development of a compact optical fiber-based apparatus for *in situ* time-resolved, laser-induced, fluorescence spectroscopy of biological systems.

Chemiluminescence is another light emission phenomenon used in bio-optrodes. In contrast to fluorescence, chemiluminescence is produced when a chemical reaction yields an excited species that emits light as it returns to its ground state. The use of chemiluminescence in biosensors, including fiber optic-based biosensors, was recently reviewed (Aboul-Enein *et al.*, 2000; Gubitz *et al.*, 2001; Zhang *et al.*, 2005; Marquette and Blum, 2006). In many bio-optrodes, the chemiluminescence of luminol is used to generate the light signal. The reaction between luminol and H_2O_2 produces a luminescence signal and is also catalyzed by certain ions or molecules (e.g., MnO_4^{2-}, I_2, Cu^{2+}). This reaction can be used, e.g., in enzyme-based bio-optrodes in which the enzymatic reaction generates H_2O_2 (see Section 1.3.3). Enzymes such as horseradish peroxidase can also catalyze or induce a chemiluminescence reaction by producing H_2O_2. In addition, alkaline phosphatase (AP) and β-galactosidase

can be used to label biological sensing elements such as antibodies or nucleic acids. In the presence of a 1,2-dioxetane substrate (Bronstein *et al.*, 1996), these enzymes catalyze light formation proportional to the analyte concentration. Most recently, a fiber optic array biosensor was developed by Magrisso *et al.* (2006), in which chemiluminescence generated from both the complex cellular activity of granulocytes and the oxidative species involved in the bactericidal activity of granulocytes was used to monitor circulating phagocyte activity from multiple samples simultaneously. Chemiluminescence-based bio-optrodes are usually used in conjunction with flow cells. An optical fiber with an immobilized sensing element is placed inside the flow cell and transmits the light signals to the detector (Figure 1.5).

Bioluminescence is a biological chemiluminescent reaction. Many organisms produce bioluminescence for cell–cell signaling, self-protection, mating, attracting prey, and finding food (Campbell and Sala-Newby, 1993). The bioluminescence reaction is catalyzed by the

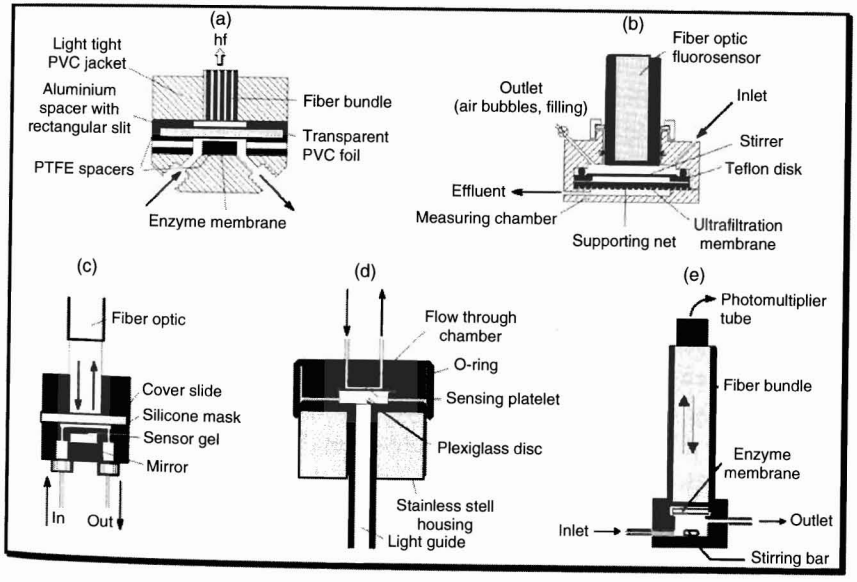


Figure 1.5 Design of flow cells incorporating bio-optrodes (Kuswandi *et al.*, 2001). Reproduced with permission of the Royal Society of Chemistry.

enzyme luciferase and requires the presence of oxygen. The bioluminescent substrate used in this reaction is called luciferin. Different luciferin molecules are used by different organisms. For example, aldehydes and flavins are used by bacteria and imidazolopyrazines are employed by some fish and squid. Bioluminescence can be applied for analytical measurements in two ways: (1) One can detect cellular events inside living cells by fusing the luciferase gene (e.g., the *luc* gene coding for firefly luciferase or the *lux* gene coding for *Vibrio fischeri* luciferase) to the gene of interest. The *in vivo* activity of the selected gene can be detected by monitoring the luminescence signal (LaRossa, 1998). (2) Alternatively, one can use purified recombinant luciferase and synthetic luciferin substrates for *ex vivo* detection assays for analytes such as ATP, NADH, and FMN (Blum *et al.*, 1993). In bio-optrodes, the cells or the purified enzymes are immobilized on the fiber tip and the luminescence signals are transmitted through the fiber to the detector (Polyak *et al.*, 2001; Hakkila *et al.*, 2004; Fine *et al.*, 2006).

Electrochemiluminescence (ECL) refers to the light emitted from species generated at electrode surfaces that undergo electron-transfer reactions to form excited states. ECL involves the production of reactive intermediates from stable precursors, such as $Ru(bpy)_3^{2+}$, at the surface of an electrode through application of a voltage. These intermediates then react under a variety of conditions to form excited states that emit light (Bard, 2004). ECL-active species have been used as labels on biological molecules and have found various applications in both immunoassays and DNA analysis with detection limits as low as 10^{-11} M. Commercial systems have also been developed that use ECL to detect many clinically important analytes such as R-fetoprotein and steroid hormones with high sensitivity and selectivity (Leland and Powell, 1991; Bard *et al.*, 2000). The principle of ECL and its biosensing applications are reviewed in Chapter 7 and elsewhere (Knight, 1999; Kuboka, *et al.*, 2000; Richter, 2004).

Using ECL detection in bio-optrodes is a relatively new approach and several such devices have recently been described (Jin *et al.*, 2001; Szunerits *et al.*, 2003; Szunerits and Walt, 2003; Monk and Walt, 2004a, 2004b). In one report (Szunerits *et al.*, 2003), an *optoelectrochemical*

sensor was fabricated by chemically etching an optical fiber bundle using NH_4F and HF solution. Since the core and cladding etched at different rates, a nanotip array was generated; the array was then coated with Au (Figure 1.6a), followed by resin insulation at its base (Figure 1.6b).

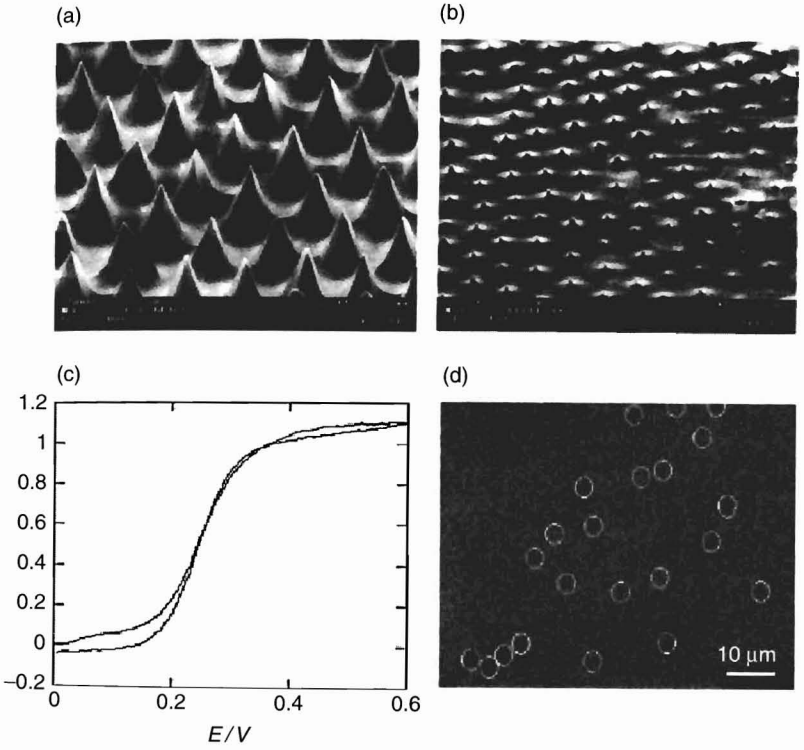


Figure 1.6 (a) SEM images of an etched and gold-coated fiber array and (b) an etched, gold-coated, and resin-insulated electrode array; (c) Cyclic voltammogram of the resin-insulated electrode array in 10 mM $Fe(CN)_6$ at 0.1 V s^{-1}; (d) ECL image monitored with an applied potential at selected areas of an etched, gold-deposited, and partially polymer-insulated electrode array. White circles indicate identified ECL spots. 1.2 V versus Ag/AgCl applied for 60 s, acquisition time 2 s, in 2 mM $Ru(bpy)_3^{2+}$/100 mM TPrA/pH 7 phosphate buffer. The red color corresponds to the most intense ECL signal; green and blue corresponds to little or no ECL signal. Reprinted with permission from the American Chemical Society. (see Plate 1)

Near-ideal microelectrode behavior was observed by measuring a cyclic voltammogram of potassium ferrocyanide (Figure 1.6c). ECL generated from the tips of the electrode array in an aqueous solution containing $Ru(bpy)_3^{2+}$ and tri-*n*-propylamine (TPrA) was detected with a high-resolution CCD camera (charge-coupled device; Figure 1.6d). Optoelectrochemical bio-optrodes are uniquely advantageous since they combine all the benefits of microelectrodes, such as fast response time and high signal-to-noise ratio, with the imaging properties offered by optical fiber bundles. This combination enables the simultaneous acquisition of both spatially resolved electrochemical and optical information from analytical samples.

Absorption is a simpler process than fluorescence and has also been used in bio-optrodes. Absorption is a process in which light energy is absorbed by an atom or a molecule, promoting the molecule from the ground energy state to a higher energy excited state. The resulting energy is dissipated non-radiatively (i.e., thermally) to the medium when the excited state relaxes to the ground state. The absorbance changes are related to the concentration $[C]$ via the Beer–Lambert relationship:

$$A = \log\left(\frac{I_0}{I}\right) = \varepsilon \cdot [C] \cdot l \tag{1.4}$$

where A is the optical absorbance, I_0 and I are the intensities of transmitted light in the absence and presence of the absorbing species, respectively, l is the effective path length, and ε is the molar absorption coefficient. In practice, optical fibers are used to measure absorbance by transmitting light through the fiber to the sensing layer and measuring changes in the scattered light. Alternatively, light is transmitted through one arm of bifurcated optical fiber to the sensing region, and reflected light signals are measured through the other arm of the fiber (Figure 1.7b). In a different configuration, two fibers are placed with one fiber facing the other, creating an optical cell in which the distance between excitation and collection fiber is the path length.

Surface plasmon resonance is another light phenomenon that has found wide use in bio-optrodes during the past decade. SPR refers to the

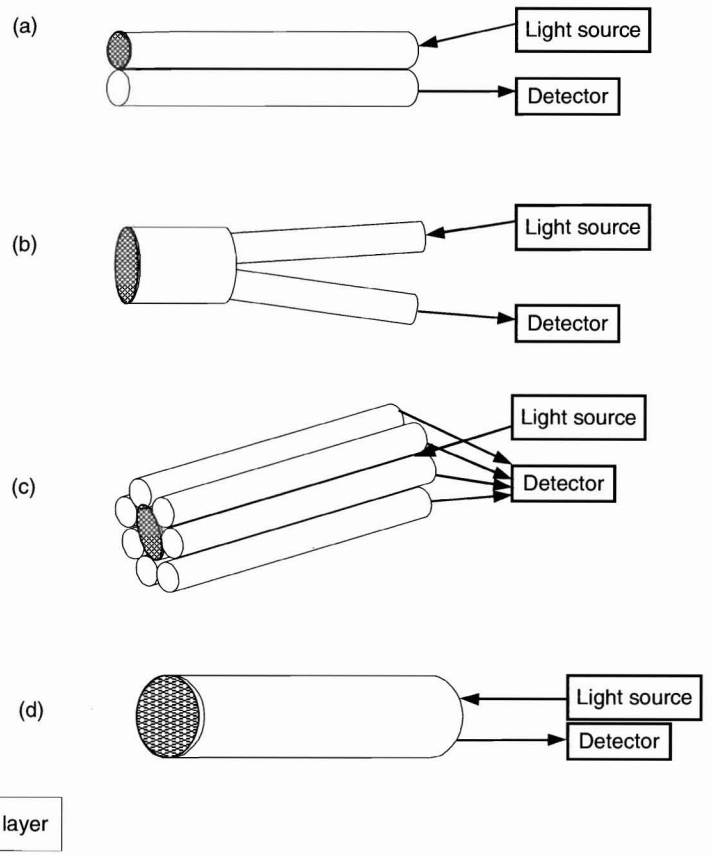


Figure 1.7 Design principle of a bio-optrode. (a) Two fibers: one carries light to the sensing layer and one carries the signal to the detector. (b) Bifurcated fiber: the biosensing layer is placed on the fused end of the fiber. (c) The biosensing layer is placed on the central fiber and the surrounding fibers are used to collect the light signals. (d) Single imaging fiber: the biosensing layer is placed on the distal end of the fiber, excitation and emission light are carried through the same fiber, and light signals are collected at the proximal end.

optical excitation of surface plasmons, which are collective oscillations of free electrons in a metallic film at the interface between a conductor (Au or Ag) and a dielectric (gas, liquid, or solid). The excitation of surface plasmons is accompanied by the transfer of optical energy

into the metal layer, which then results in a narrow dip in the spectrum of the reflected light. Since surface plasmon waves are extremely sensitive to small changes in the refractive index near the sensor surface, and these changes are proportional to the sample mass on the surface, SPR can be used in *label-free* biosensing (Liu *et al.*, 2005; Ince and Narayanaswamy, 2006; see also Chapter 4). Many optical coupling schemes has been used for SPR biosensors, such as the most widely used Kretschmann prism configuration (Otto, 1968), grating (Cowan and Arakawa, 1970), channel waveguide (Lambeck, 1991), light pipe (Homola and Yee, 1996), and optical fiber (Jorgensen and Yee, 1993) schemes.

The idea of the SPR fiber optic sensor was first proposed by Jorgensen and Yee (1993) to overcome the drawbacks associated with the traditional Kretschmann prism configuration, such as its high cost, large size, and incompatability for remote sensing. The SPR sensor developed by Jorgensen and Yee was fabricated by first removing part of the cladding of a conventional polymer–clad silica fiber and then symmetrically depositing a thin (300 nm) metal (Ag) layer around the exposed section of the fiber core. White light was used as the source of a range of optical wavelengths guided into the optical fiber and the detection limit of the sensor was as low as 5×10^{-5} refractive index units (RIU). Many fiber optic SPR sensors have been developed since then for various biosensing purposes, such as monitoring red blood cell–ligand interactions (Quinn *et al.*, 2000), detecting myoglobin and cardiac troponin I (Masson *et al.*, 2004a), and quantifying survival motor neuron protein (Masson *et al.*, 2004b).

1.1.3. Optrode biosensor (bio-optrode) design and instrumentation

The design and use of different bio-optrode systems have been recently reviewed (Mehrvar *et al.*, 2000; Wolfbeis, 2000a, 2000b, 2000c, 2000d, 2002, 2004, 2006; Kuswandi *et al.*, 2001). The design of bio-optrodes is similar to chemical optrode design and two basic configurations are used: (a) a single fiber is used to transmit the light from the light source to the sample region and back to the detector, as shown in Figure 1.1, or

(b) multiple fibers are used in which one fiber is employed to transmit the light to the sample region and the other fiber or fibers are used to transmit light from the sample region to the detector, as shown in Figure 1.7a. For the second configuration, the most common format is a bifurcated fiber. Bifurcated fibers are fabricated by fusing two fibers on one end leaving the other ends free. The sensing elements are immobilized on the fused side and the other ends of the bifurcated fiber are connected to the light source and to the detector as shown in Figure 1.7b. In a different configuration, multiple fibers comprising one central fiber surrounded by several fibers are employed. The central fiber carries the immobilized sensing elements and is connected to the light source; the surrounding fibers collect the output light signals and transmit them to the detector (Figure 1.7c). Another configuration made use of a single imaging fiber. The biosensing layer is placed on the distal end of the fiber, both the excitation and the emission light are carried through the same fiber, and light signals are collected at the proximal end (Figure 1.7d).

The light sources used for bio-optrodes should provide sufficient light intensity within the sensor wavelength operating range. In addition, the light output should be stable over long time periods since light fluctuations may add noise to the measurement and reduce the sensor sensitivity. The different light sources used in bio-optrodes and their characteristics are summarized in Table 1.1.

In most fiber optic biosensor systems, the light transmitted from the sensing element (output light) is measured by using photon detection devices, which absorb photons and convert them into electrical signals. Several photon detectors are available as shown in Table 1.2.

1.1.4. Biological sensing elements

Bio-optrodes are constructed by immobilizing biological recognition components, such as enzymes, antibodies, nucleic acids, or cells to optical fibers. In nature, interactions between biological molecules, such as receptor–ligand, antibody–antigen, or two complementary DNA strands, are highly specific. Some of these recognition molecules can be purified

Table 1.1 Light sources.

Type	Wavelength (nm)	Characteristics
Tungsten lamp	IR/NIR, visible	High power output, bulky, expensive, used together with wavelength selection device
Deuterium lamp	200–300	
Xenon lamp	200–1000	
LEDs	470–1300	Low power output, high stability, long life, robust, compact size, inexpensive
Laser (N_2, Ar^+, He-Ne) .	377, 488–568, 633	Monochromatic, very high power output, directional, bulky, expensive
Laser Diodes	800–904	High power output, long life, narrow spectral band, inexpensive, compact size

Table 1.2 Light detectors.

Detector type	Advantages	Limitations
Photomultipliers (PMT)	Sensitive, fast, low noise, internal amplification, compact	Need for high power voltage supply, destruction by over exposure
Photodiodes (PD)	Fast, robust, compact, inexpensive	High noise, no internal amplifier
Charge-coupled devices (CCD)	Very sensitive, can be used for imaging	Slow, expensive, need for a cooling system
Avalanche PDs	Lower noise than PD, fast, sensitive, can tolerate intense illumination	More expensive than PD
Complementary-metal-oxide semiconductor (CMOS) imaging sensors	Lower power consumption, lower cost, and higher system integration than CCD	Lower S/N than CCD

and used in fiber optic biosensors. Moreover, through genetic engineering, the original recognition element's structure can be modified and designed for a specific analytical application (Hellinga and Marvin, 1998; Looger, 2003; Kasper *et al.*, 2005). Biological sensing compounds can be divided into two main categories based on their bioactivity: biocatalysts (enzymes and cells) and bioaffinity molecules (antibodies, receptors, and nucleic acids).

1.1.4.1. Biocatalyst-based optrodes

Enzymes are proteins that selectively bind and catalyze the conversion of a substrate to product. Enzymes are used as sensing elements in bio-optrodes based on their ability to bind specific substrates (e.g., the analyte) and catalyze their conversion into an optically detectable product. Many novel enzyme-based bio-optrodes have been developed (Kulp *et al.*, 1987; Luo and Walt, 1989; Healey and Walt, 1995; Li and Walt, 1995; Zhang *et al.*, 1997; Michel *et al.*, 1998a, 1998b; Marquette *et al.*, 2000; Choi *et al.*, 2001; Wu *et al.*, 2004) since the first one was reported by Arnold in 1985. This approach has been reviewed in detail by Kuswandi *et al.* (2001) and in other recent reviews (Marazuela and Moreno-Bondi, 2002; Monk and Walt, 2004a, 2004b; Wolfbeis, 2004). The optical signal obtained – absorbance, fluorescence, reflectance, or chemiluminescence – is proportional to the product concentration and, consequently, to the analyte concentration.

Products that possess intrinsic optical properties can be measured directly, but the most common enzymatic reactions products, such as H^+, ammonia, oxygen, carbon dioxide, and hydrogen peroxide, do not possess intrinsic optical properties and are therefore measured indirectly using indicators (Wolfbeis, 1997). Indicators change their optical properties when interacting with these products. For example, fluorescein is a pH indicator and its emission intensity can be correlated to changes in H^+ concentration. Other indicators employed in enzyme bio-optrodes were recently reviewed by Kuswandi *et al.* (2001).

An interesting example that demonstrates the simple fabrication and function of enzyme-optrodes is the one used for glucose detection based

on the enzyme glucose oxidase. Glucose oxidase catalyzes the oxidation of glucose with oxygen to produce gluconolactone and H_2O_2.

$$\text{Glucose} + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

Several approaches have been employed to determine glucose concentration with glucose oxidase: (1) monitoring of the oxygen consumed in the enzymatic reaction using a ruthenium complex as an indicator (Rosenzweig and Kopelman, 1996a, 1996b; Endo *et al.*, 2006); (2) measurement of H_2O_2 produced using luminol chemiluminescence (Marquette *et al.*, 2000) or ECL (Marquette *et al.*, 2001; Zhu *et al.*, 2002); and (3) direct measurement of the amount of H_2O_2 using the fluorescent probe europium (III) tetracycline (EuTc). Weakly fluorescent by itself, EuTc and the enzymatically generated H_2O_2 form a strongly fluorescent complex that can be detected in the time-resolved mode (Wu *et al.*, 2004).

In many cases, a sequence of enzymatic reactions is required to detect a specific analyte. In order to fabricate bio-optrodes for detection of such analytes, two or three enzymes are co-immobilized on the optical fiber in such a way that sequential reactions can occur. The first enzyme catalyzes the conversion of the analyte to a product that serves as a substrate for subsequent enzymatic reactions that eventually convert the initial analyte to an optically detectable product (Michel *et al.*, 1998a, 1998b). Using this methodology, analytes that could not be detected in a single reaction step can be detected. In addition, co-immobilizing two enzymes can achieve signal amplification through recycling systems, as shown in Figure 1.8 (Zhang *et al.*, 1997).

Inhibition of enzymatic reactions can also be used as a sensing mechanism in bio-optrodes (Freeman and Bachas, 1992). In this approach, the inhibitor is the analyte, and the measured signal is the decrease in enzymatic activity. One example is the detection of organophosphate and carbamate pesticides using an enzyme inhibition-based optrode. The bio-optrode is based on the inhibition of acetylcholinesterase by organophosphate pesticides. The enzyme is co-immobilized together

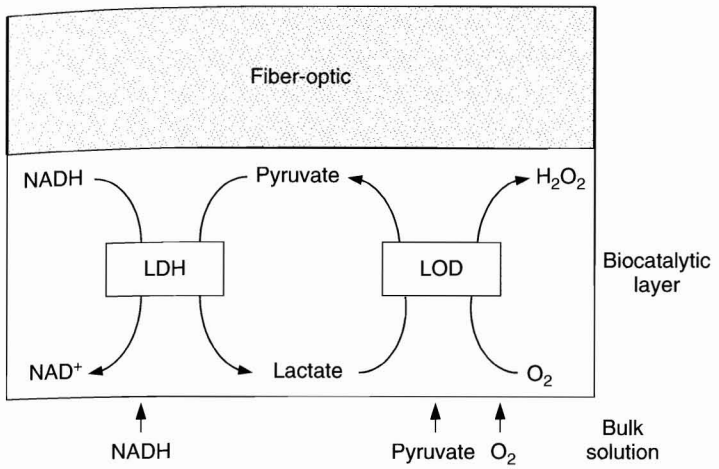


Figure 1.8 Schematic diagram of signal amplification using a dual-enzyme bio-optrode. Pyruvate is detected using lactate dehydrogenase (LDH) and lactate oxidase (LDO), which are co-immobilized on a fiber optic tip. Pyruvate concentration is determined by measuring NADH fluorescence. Pyruvate and NADH diffuse from the bulk solution into the enzyme layer, LDH catalyzes the formation of lactate and NAD^+ during the reduction of pyruvate. LDO then catalyzes the regeneration of pyruvate causing additional consumption of NADH by the LDH-catalyzed reaction. Thus, the signal obtained using a dual-enzyme system is higher than when a single enzyme is used (Zhang *et al.*, 1997). Reprinted with permission from Elsevier Science.

with a pH-sensitive dye at the fiber's distal end. The substrate acetylcholine is hydrolyzed by acetylcholinesterase, causing a change in the local pH and thereby the fluorescence signal. The inhibition of this reaction can be correlated to the pesticide concentration in the sample (Hobel and Polster, 1992; Choi *et al.*, 2001; Doong and Tsai, 2001).

In *living cells*, cellular functions are carried out by enzymes that simultaneously catalyze numerous biochemical reactions. Some enzymatic activities that occur in cells have been applied for bio-optrode fabrication. Although enzymes can be isolated and purified, their activity outside the cells is usually reduced compared to their activity within the cells where they function in an optimum environment containing all the

necessary cofactors. Whole cell biocatalysts are particularly advantageous when the detection is based on a sequence of multiple enzymatic reactions. These enzymatic cascade reactions are very difficult and complicated to accomplish *ex vivo* by co-immobilizing the enzymes, but are relatively straightforward when employing whole cells. In practice, whole cells possessing unique or enhanced enzymatic activities that transform the analyte into detectable products, or cells with specific cellular responses such as oxygen consumption or production, are immobilized on optical fibers (Preininger *et al.*, 1994). The methods for detecting the products in cell-based fiber optic biosensors are similar to those employed in enzyme optrodes.

In a more recent approach, cells are genetically engineered to overexpress specific enzymes involved in the analytical measurement. An example of this approach is the use of *Escherichia coli* cells engineered to overexpress the enzyme organophosphorus hydrolase (OPH) on their outer cell membrane (Mulchandani *et al.*, 1998). This enzyme catalyzes the hydrolysis of organophosphorus pesticides to form a chromophoric product that can absorb light at a specific wavelength. The cell optrode is fabricated by immobilizing the cells on a bifurcated fiber optic tip and using a photomultiplier detection system to measure the light signals. A fiber optic biosensor was recently reported by Durrieu and Tran-Minh (2002) for the detection of heavy metals by measuring the inhibition of AP present on the external membrane of *Chlorella vulgaris* microalgae cells. In general, although the specificity of whole cell optrodes is reduced compared to enzyme optrodes, cells are very simple to use and obtain (e.g., growing the cells for a few hours), and there is no need for purification steps, which makes cell bio-optrodes inexpensive to assemble (Campbell *et al.*, 2006; Kumar *et al.*, 2006).

A different approach for sensing with whole cells that does not directly involve biocatalysis is based on utilizing genetic responses and signal transduction mechanisms in living cells (Naylor, 1999; Daunert *et al.*, 2000; Kohler *et al.*, 2000; Leth *et al.*, 2002; Brian *et al.*, 2003; Kuang *et al.*, 2004a, 2004b). Cells may express a specific gene or set of genes when a specific molecule (e.g., analyte) is present in the cell's environment. By fusing reporter genes, coding for optically detectable enzymes

or proteins (e.g., luciferase, β-galactosidase, GFP) to the responsive gene, the genetic response is measured and correlated to the analyte concentration.

Another interesting setup combining living cells and fiber optics was recently reported by DiCesare *et al.* (2005), in which an optical fiber-based technology was used to analyze whole cell migration; the ability of cells to migrate can predict the invasiveness and/or metastatic potential of tumor cells. Cells were labeled with a membrane-bound fluorescent dye, Vybrant DiO, and distributed onto a polished optical fiber bundle. When a cell passed over one of the individual fibers in the bundle, the membrane-bound dye caused a large intensity increase, which remained high for a given "residence time" until the cell moved to an adjacent fiber. A significant decrease in cell migration was observed when cells were exposed to an antimigratory drug.

1.1.4.2. Bioaffinity-based optrodes

The natural high selectivity of antibodies, receptors, and nucleic acids make them very powerful sensing elements for recognizing their binding partners. Such bioaffinity optrodes are used as probes because the recognition reaction is essentially irreversible. The bio-optrode sensing elements must be regenerated or recharged before the probe can be used to make another measurement. In many cases, a probe-based bio-optrode configuration involves the use of a permanent fiber optic and a disposable sensing layer that can be placed on the fiber optic's distal end (Figure 1.9).

Immuno/receptor optrodes are a major group of bioaffinity fiber optic biosensors based on transducing antibody–antigen (analyte) interactions into an optical signal that is proportional to the antigen concentration. Monoclonal antibodies that can recognize a specific antigenic epitope region (i.e., a specific spatial structure on the antigen molecule) or polyclonal antibodies that recognize different antigenic epitopes are used in immuno-optrodes. Several detection schemes are employed. The simplest scheme involves the detection of intrinsically fluorescent analytes such as polynuclear aromatic hydrocarbons(PAHs) (Vo-Dinh *et al.*, 2000). Antibodies are immobilized on the fiber surface and a

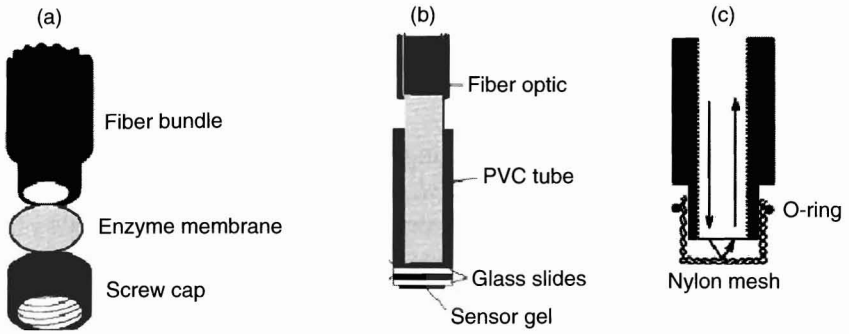


Figure 1.9 Configuration of probe-based bio-optrodes with disposable biosensing elements. (a) Biorecognition sensing molecules immobilized on a membrane, which is held by a screw cap on the optical fiber tip. (b) Disposable glass slide with gel-entrapped enzyme. (c) Nylon membrane with immobilized sensing molecules attached to the fiber using an O-ring (Kuswandi *et al.*, 2001). Reproduced with permission of the Royal Society of Chemistry.

fluorescence signal is obtained when the analyte (antigen) binds to the optrode's surface as shown in Figure 1.10a. One example of a direct assay where analytes are not intrinsically fluorescent was reported by Battaglia *et al.* (2005). Their fiber optic SPR biosensor quantified cytokines involved in wound healing by measuring the shift of SPR wavelength when analytes bound to antibodies immobilized on the fiber. The detection limits for interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α) were reported to be 1 ng/ml in buffered saline solution and in spiked cell culture medium.

A competition assay is a more generalized detection scheme that can be applied to any antibody–antigen pair. The detection is based on competition for the antibody-binding site between the antigen present in the sample (analyte) and an externally added fluorescent-labeled antigen as shown in Figure 1.10b. A known concentration of fluorescent-labeled antigen is added and captured by an antibody, which is immobilized on the optical fiber surface. The fluorescence signal obtained is measured and set as the initial signal. To perform an analysis, the same fluorescent-labeled antigen concentration is mixed with a sample containing an unknown antigen concentration. When this

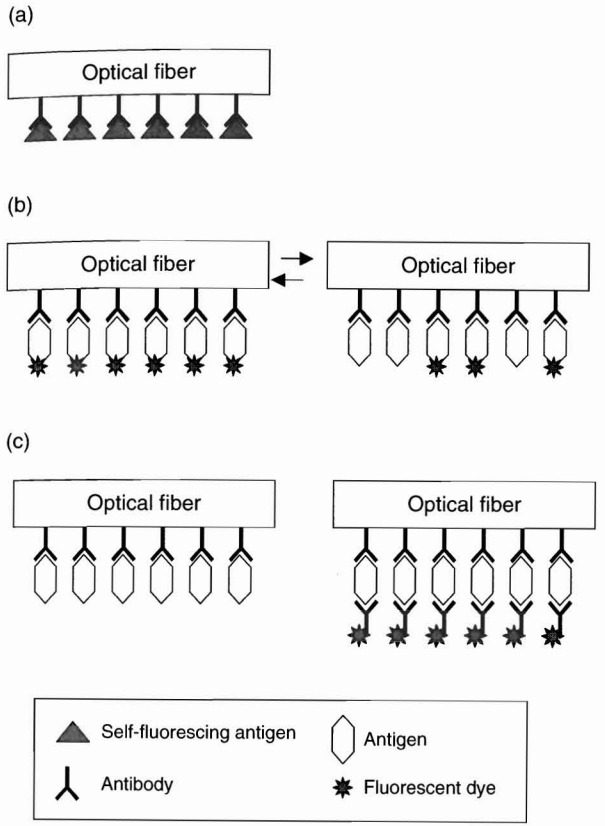


Figure 1.10 Schematic principle of immuno bio-optrodes. (a) Detection of intrinsically fluorescent molecules using immobilized antibodies. (b) Competition assay using a fluorescent-labeled antigen. (c) Sandwich immunoassay using an immobilized antibody and a fluorescent-labeled antibody.

mixture is analyzed using the bio-optrode, the resulting fluorescence signal obtained is lower than the initial signal because of competition with the labeled antigen in the sample. The relative decrease in the initial signal is proportional to the analyte concentration in the sample. Using this detection scheme, bio-optrodes for the detection of different analytes have been developed (Zhao *et al.*, 1995; Wittmann *et al.*, 1996; Maragos and Thompson, 1999).

The preferred detection scheme is the sandwich immunoassay, which involves the use of two antibodies. The first antibody is immobilized to the fiber and used to capture the antigen while the second antibody, which is labeled by a fluorescent dye or enzyme, is used to generate the signal (Figure 1.10c) (Szurdoki *et al.*, 2001; Balcer *et al.* 2002).

Competition and sandwich assays require using labeled antigens or antibodies. Fluorescent molecules and enzymes are employed for labeling using different chemistries (Wortberg, 1997). Although the majority of published reports used fluorescent molecules as labels, enzyme labels are useful and powerful since they catalyze the production of many readily detectable product molecules. Some enzymes used for labeling, such as AP, catalyze the conversion of a non-fluorescent substrate to a fluorescent product and can be detected by monitoring the fluorescent signal generated (Michael *et al.*, 1998). Other enzymes, such as horseradish peroxidase, can catalyze chemiluminescence reactions and are detected by monitoring the emitted light signals (Spohn *et al.*, 1995; Diaz *et al.*, 1998; Aboul-Enein *et al.*, 2000; Xing *et al.*, 2000; Gubitz *et al.*, 2001; Leshem *et al.*, 2004). Enzyme labeling is more sensitive than fluorescent dye labeling since the signal is amplified by the enzymatic reaction. Another new technology to increase the labeling efficiency of biological molecules using quantum dots was proposed at the time of writing and is discussed in Chapter 15.

Nucleic acid-based optrodes are the second major group of bioaffinity-based optrodes. Nucleic acid base pairing is used as the sensing mechanism in bio-optrodes for nucleic acid detection. The presence of a specific DNA sequence, the "target," among millions of other different sequences is detected by hybridization to its complementary DNA sequence, the "probe," which is immobilized on the optical fiber. Hybridization with complementary target DNA can be detected through the use of fluorescently labeled target sequences or by intercalating dyes that exhibit enhanced fluorescence intensity upon hybridization (Ferguson *et al.*, 1996; Healey *et al.*, 1997; Monk and Walt, 2004a; Brogan and Walt, 2005; Massey *et al.*, 2005; Wang and Krull, 2005a, 2005b; Wolfbeis 2006). In a typical assay (Figure 1.11), the target DNA is first amplified and fluorescently labeled using fluorescent primers

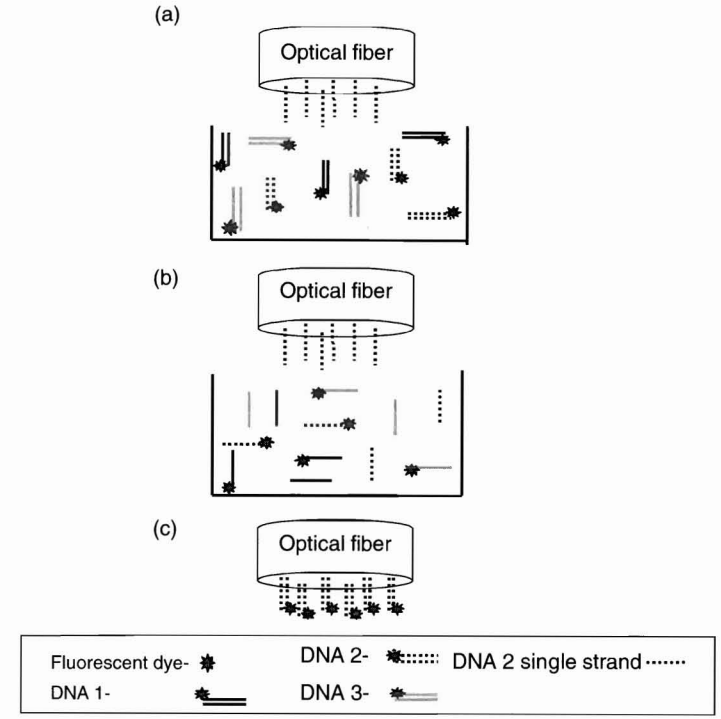


Figure 1.11 Principle of DNA fiber optic biosensors. (a) Single strand DNA probe molecules, with a sequence complementary to the target DNA sequence, are immobilized onto the fiber. (b) The fluorescent-labeled sample DNA molecules are first dehybridized and the fiber is dipped into the sample solution. (c) After hybridization, the complementary strands of the target DNA are attached to the probe DNA on the fiber and a fluorescence signal is obtained.

and the polymerase chain reaction (PCR). The resulting fluorescent double-stranded DNA molecules are dehybridized (usually by heating) (Figure 1.11b) and then allowed to rehybridize (by cooling) to the single-strand DNA probe molecules immobilized on the fiber surface (Figure 1.11c). The excess DNA molecules are washed away, and if the complementary target DNA sequence is present in the sample, a fluorescence signal is detected (Ferguson *et al.*, 1996).

Nucleic acid bio-optrodes have attracted considerable attention in the last few years, partially due to the tremendous amount of genetic information generated from various sequencing efforts but more importantly due to the unique characteristics of DNA oligonucleotides as sensing elements. These characteristics include high selectivity, the predictability and similarity in binding of all the different probe molecules, chemical stability, easy amplification through PCR, and readiness to be functionalized and immobilized on the surface of optical sensing substrates (Watterson *et al.*, 2001).

Nucleic acid bio-optrodes have been used in a variety of applications including the detection of pathogenic bacteria. The target pathogen DNA can be easily extracted from water, wastewater, or clinical samples, and the presence of pathogenic microorganisms can be determined by the bio-optrode (Pilevar *et al.*, 1998; Iqbal *et al.*, 2000; Chang *et al.*, 2001; Ahn and Walt, 2005). Such bio-optrodes have also been used to detect biological warfare agents (Song *et al.*, 2006).

Nucleic acid bio-optrodes also enable genetic analysis for detecting single nucleotide polymorphisms (SNPs) (Watteson *et al.*, 2003), high-throughput genotyping with SNP interrogation (Gunderson *et al.*, 2005), and potentially whole genome analysis. Epstein *et al.* (2003a) developed a novel optical fiber-based DNA microarray by randomly distributing oligonucleotide-functionalized microspheres on the face of an etched imaging fiber bundle. Batches of microspheres were modified with different oligonucleotide probes and then pooled to form bead libraries containing multiple sensor types. Beads with different probe types were encoded using different fluorescent dyes at various concentrations to create a unique signature for each bead type, enabling the positional registration of every bead in the array. In a continuation of this research, the same authors (Epstein *et al.*, 2003b) developed a novel method for decoding random arrays of every possible *n*-mer by performing consecutive hybridizations with fluorescently labeled combinatorial oligonucleotide decoding libraries.

New categories of nucleic acid molecules, such as aptamers (Lee and Walt, 2000) and molecular beacons (Liu *et al.*, 2000; Steemers *et al.*, 2000; Spiridonova and Kopylov, 2002), have been incorporated as sensing molecules into bio-optrodes. These different DNA-sensing schemes can be multiplexed by fabricating an array of hundreds to thousands of probes as will be described later in Section 1.3.2.

1.1.5. Sensing element immobilization

Immobilization of sensing biomolecules to the optical fiber is a key step in bio-optrode development. A good immobilization method should not only be simple, fast, and durable but, more importantly, gentle so that the biological molecule being immobilized can retain its biochemical activity. In addition, biological recognition elements are often co-immobilized with indicator dyes, so that ideally the immobilization method should be suitable for both molecules. In some cases, the recognition compounds are immobilized directly to the optical fiber surface. Alternatively, the molecules are first immobilized on membranes, such as cellulose acetate or polycarbonate, which are later physically attached to the optical fiber (Figure 1.9). There are three main methods for immobilizing a biological sensing compound: adsorption/electrostatic interaction, entrapment, and covalent attachment. A schematic representation of these methods is shown in Figure 1.12.

Adsorption immobilization methods involve adsorbing the sensing material onto a solid surface or polymer matrix through the formation of van der Waals and hydrogen bonds. Sensing materials can be adsorbed directly on the fiber optic end. This immobilization method is very simple; however, the adsorbed molecules tend to gradually leach from the solid support, decreasing sensing performance and/or lifetime. In order to overcome leaching problems, the solid support surface may first be modified with complementary functional groups. For example, a hydrophobic surface can be prepared to immobilize a hydrophobic species. Electrostatic interaction can also be employed for immobilization. This immobilization scheme is based on interaction between oppositely charged molecules. For example, an optical fiber surface can be coated with a positively charged layer (i.e., using poly-L-lysine) that interacts electrostatically with negatively charged recognition molecules (Figure 1.12a). The electrostatic immobilization method is very easy

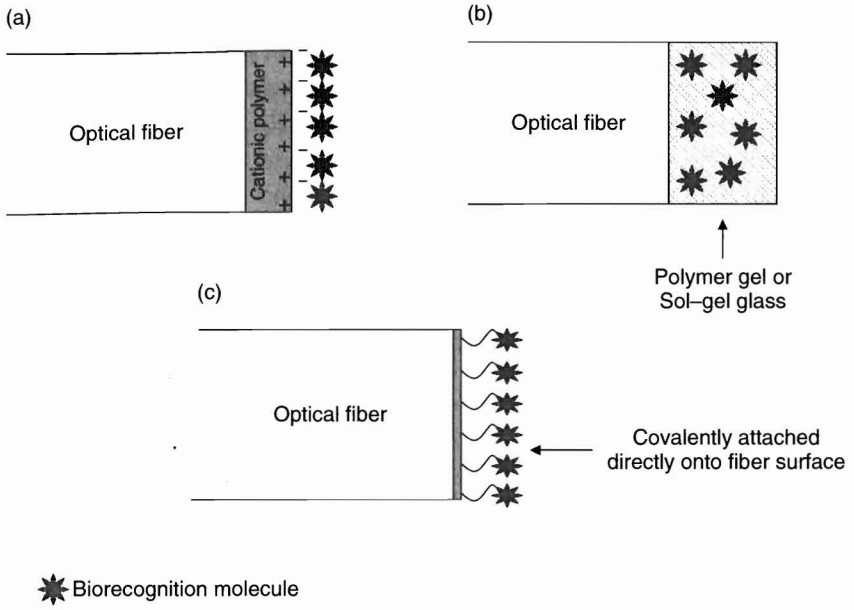


Figure 1.12 Schematic diagram of three different immobilization techniques employed in bio-optrodes. (a) Absorption/electrostatic. (b) Entrapment. (c) Covalent immobilization.

and highly reproducible but may be affected by changes in the medium pH or by changes in other ion concentrations.

Entrapment immobilization involves physical entrapment of sensing biomolecules within a porous matrix (Figure 1.12b). The biomolecules are suspended in a monomer solution, which is then polymerized to a gel causing the molecules to be entrapped. Such polymers can be either thermally or photochemically initiated and attached to the fiber surface by dip-coating procedures (Healey *et al.*, 1995). The immobilized molecules usually do not leach out of the matrix and can retain their biochemical activity. Polyacrylamide gels are most commonly used for entrapment immobilization, although agarose and calcium alginate gels have also been used (Polyak *et al.*, 2001). Fine *et al.* (2006) recently described a novel entrapment method using PVA-based hydrogels; the

mild entrapment conditions allowed immobilization of living luminescent yeast cells for biodetection of estrogenic endocrine-disrupting chemicals. One important limitation of this approach is the slow diffusion rates of the analytes and products through the immobilization matrix, which increases the bio-optrode response time.

Optically transparent sol-gel glasses are also used for biological sensing molecule entrapment (Jordan *et al.*, 1996; Dunn *et al.*, 1998, 2001; Kishen *et al.*, 2003; Kwok *et al.*, 2005). Sol-gel glasses are produced by hydrolysis and polycondensation of organometallic compounds, such as tetraethyl orthosilicate ($Si(OCH_3)_4$). The sensing biomolecules are added to the reaction mixture during the formation of the sol or gel. Sol-gel glasses prepared by this method contain interconnected pores formed by a three-dimensional SiO_2 network. As a result, the biomolecules and dyes are trapped but small analytes can readily diffuse in and out of the pores. The main advantages of the sol-gel glass immobilization method are the chemical, photochemical, and mechanical stability of the immobilized layer. Disadvantages of sol-gel glass immobilization are the slow response times in aqueous media and the fragility of thin sol-gel glass films compared with polymer films.

Functional groups in the sensing biomolecules can be covalently bound to reactive groups on the surface of optical fibers, allowing robust immobilization and hence giving the bio-optrodes long lifetimes in storage of anywhere from 4 to 14 months (Eggins, 1996) (Figure 1.12c). The fiber surface can be chemically modified using silanization reactions (Weetall, 1993). For example, the fiber surface can be aminosilanized to form amine functional groups on the fiber surface followed by reaction with –COOH groups on the enzyme or antibody. Amine-modified surfaces can also covalently bind to the biomolecule's amine groups using bifunctional cross-linkers such as glutaraldehyde. Covalent immobilization methods are usually more complicated and time-consuming compared with the other immobilization techniques, but are very reliable since the biomolecules and dyes are not likely to leach out. It should be noted that covalent immobilization might change the biomolecule activity. If the binding occurs at crucial sites (e.g., an enzyme-active site or an antibody-binding site), activity can be lost completely. To avoid such

inactivation, substrate, inhibitors, and other effectors are often included in the immobilization medium to protect the active or binding site of the biomolecules. In recent years, new techniques such as using Protein A for optimized antibody orientation (Anderson *et al.*, 1997) have been developed, which controls the immobilized molecule's orientation on the sensing surface and may result in an increase in the immobilization efficiency (Sackmann, 1996).

A more generalized and widely used binding method involves the use of avidin–biotin chemistry. The fiber surface can be modified with biotin groups and bind avidin-modified biomolecular conjugates or vice versa. This method is very attractive since many biotin- or avidin-labeled enzymes, antibodies, and nucleic acids are commercially available. Luo and Walt (1989) first reported using avidin–biotin coupling to fabricate enzyme-based bio-optrodes for penicillin, ethyl butyrate, and urea. This method has hence been widely used by other research groups (Wilchek and Bayer, 1990; Konry *et al.*, 2003; Viveros *et al.*, 2006).

A novel photon-initiated covalent-binding method was recently reported by Konry *et al.* (2005). An electropolymerizable pyrrole–benzophenone film was deposited on optical fiber tips, and a biological receptor was then covalently bound to the film through photoreaction. The photoreaction process included triplet-state excitation, hydrogen abstraction, and radical recombination, resulting in covalent immobilization of nucleic acids or proteins bearing amino acids with sterically accessible C–H bonds. This immobilization technique is easily applicable to a wide variety of biomolecules and combines the advantages of photolithography with electrochemical addressing of polymer films.

1.2. History

Optical fiber-based biosensors evolved from chemical optrodes. The first optical fiber-based chemical sensor was developed by Lubbers and Opitz (1975). Their device was designed to measure CO_2 and O_2 and was used in biological fluids. A few years later, biological molecules were coupled to the optical fiber-based chemical sensors and bio-optrodes

were formed. One of the first bio-optrodes included a glucose biosensor (Arnold, 1985) fabricated by coupling the enzyme glucose oxidase to an O_2 optrode. A penicillin biosensor was also fabricated by immobilizing the enzyme penicillinase and a pH sensitive fluorescent dye on a polymer membrane covalently attached to the tip of a glass optical fiber (Kulp *et al.*, 1987). In the following years, many bio-optrodes with different recognition molecules have been developed and are described in several books (Wolfbeis, 1991; Blum *et al.*, 1994; Lopez, 2002) and reviews (Rabbany *et al.*, 1994; Fraser, 1995; Aboul-Enein *et al.*, 2000; Mehrvar *et al.*, 2000; Wolfbeis, 2000a, 2000b, 2000c, 2000d, 2002, 2004, 2006; Epstein and Walt, 2003; Monk and Walt, 2004a; Brogan and Walt, 2005). Although the bio-optrode basic configuration has not changed much from the one proposed by Lubbers and Opitz (1975), new types of optical fibers, optical instruments, biorecognition molecules, and indicators have been integrated into bio-optrodes. These materials, combined with new immobilization techniques and advanced optical approaches, have led to the development of more sophisticated, selective, and sensitive bio-optrodes. Advances in two fields influenced bio-optrode development in the last decade. First was the development of new fiber optic technologies developed for telecommunication applications. Second, advances in molecular biology techniques have allowed specific biorecognition molecules to be designed. Integration of technologies from these two fields has led to the development of advanced bio-optrode technologies such as multianalyte bio-optrodes, reagentless bio-optrodes, and nano bio-optrodes.

1.3. State of the art for bio-optrodes

In this section, a few examples of new bio-optrode technologies and applications will be described. Although many novel and interesting papers related to bio-optrode developments have been published in recent years, we focus here on several examples that emphasize the diversity of existing bio-optrode technologies. In addition, a few examples of bio-optrode applications in the industrial, environmental, and clinical fields will be described.

1.3.1. Nano bio-optrodes

One of the most exciting advances in bio-optrode development is the miniaturization of sensors to submicron dimensions. Nanotechnology facilitates research in this field and leads to the development of new nano bio-optrodes (Cullum and Vo-Dinh, 2000; Kasili *et al.*, 2004; Kasili and Vo-Dinh, 2005; Vo-Dinh and Kasili, 2005). The main importance of such biosensors is their ability to monitor physiological and biological processes inside a single living cell and thereby expand our knowledge about complex intracellular process.

In order to prepare nano bio-optrodes, optical fibers a few nanometers in diameter are fabricated. The fabrication process involves pulling optical fibers with an initial diameter of a few microns, using a modified micropipette puller optimized for optical fiber pulling. After pulling, tapered fibers are formed with typical distal end (tip) diameters of 20–80 nm.

This technique was used by Kopelman and coworkers to make a nanofiber optic chemical sensor for monitoring intracellular pH inside living cells (Tan *et al.*, 1992). Changes in pH were measured by immobilizing a pH-sensitive dye to the fiber tip. The same design was used to prepare an enzyme-based nano bio-optrode for nitric oxide detection (Barker *et al.*, 1998). Fluorescently labeled cytochrome *c′*, which undergoes conformational changes in the presence of NO, was immobilized to the fiber tip. Changes in NO concentrations were correlated to changes in the energy transfer between cytochrome *c′* and the fluorescent dye.

An antibody-based nano bio-optrode for the fluorescent analyte benzo[a]pyrene tetrol (BPT) was also fabricated for detection inside a single living cell (Vo-Dinh *et al.*, 2000). The nano bio-optrode was prepared by first fabricating nanofiber tips by pulling them from a larger diameter (600 μm) silica optical fiber using a special fiber-pulling device (Sutter Instruments P-2000). This method was based on local heating of a glass fiber using a CO_2 laser while pulling the fiber apart and then coating the tapered fiber's outside walls with a thin silver, gold, or aluminum layer (100–200 nm) using a vacuum evaporator as shown in Figure 1.13a. In this system, the fiber was held at an angle relative to the

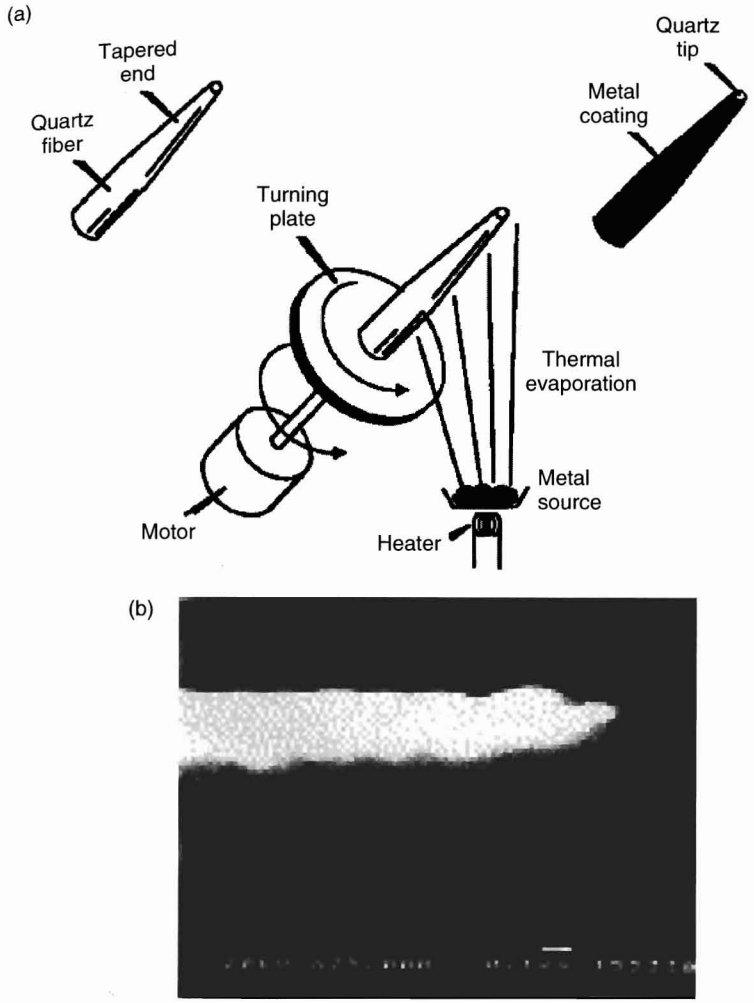


Figure 1.13 Nano bio-optrodes. (a) Fabricating a nanofiber optic tip. An optical fiber is heated and pulled and a tapered end with submicron diameter is formed. The tapered fiber side walls are then coated with a thin metal layer, using thermal evaporation, in order to prevent excitation light leakage. Biorecognition molecules can be immobilized on the fiber tip (Vo-Dinh *et al.*, 2000). Reprinted with permission from *Nat. Biotechnol.* (b) Scanning force micrograph (SFM) of nanofiber (Vo-Dinh *et al.*, 2001). Reprinted with permission from Elsevier Science.

metal vapor, resulting in a coating on the side of the fiber, leaving the tip uncoated. This coating prevented light leakage from the fiber's walls and enabled propagation of the excitation energy down the tapered sides of the nanofiber, bringing maximum light intensity to the fiber tip. The fiber's uncoated tip surface was then silanized in order to covalently attach anti-BPT antibodies. The final nano bio-optrode tip diameter was 200–300 nm. Bio-optrodes of this size have several advantages over larger bio-optrodes including fast response time and higher sensitivity. Using BPT nano bio-optrodes, BPT concentrations as low as ~300 zeptomoles were detected (Vo-Dinh *et al.*, 2001).

The optical measurement system used with the nano bio-optrode is shown in Figure 1.14a. Laser light was transmitted through the fiber and used to excite the captured BPT molecules. Changes in fluorescence

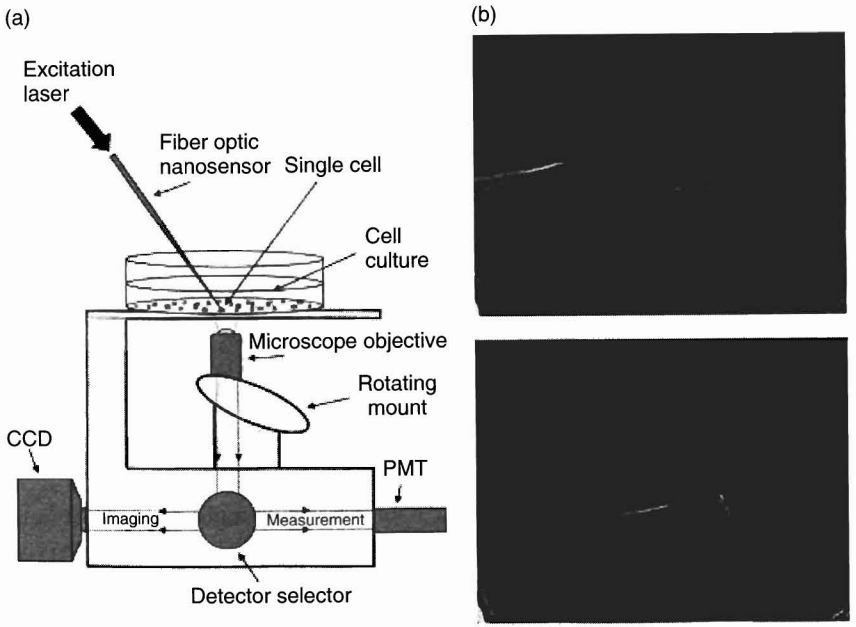


Figure 1.14 Measurements inside a single live cell using a nano bio-optrode. (a) The optical measurement system. (b) A nano bio-optrode inside a single cell (Vo-Dinh *et al.*, 2001). Reprinted with permission from Elsevier Science.

signals due to the presence of bound BPT molecules were transmitted through the microscope objective and measured using a photomultiplier tube (PMT). Using this experimental setup, BPT molecules inside single living cells were measured. The fiber's tip was inserted into the cell (Figure 1.14b) and incubated for 5 min inside the cells to allow the antibodies to bind the antigen (BPT). The fiber was then removed from the cell and the fluorescence signal obtained from the bound BPT was immediately measured. Concentrations as low as 9.6×10^{-11} M were measured inside the cells. The ability to measure concentrations of specific analytes inside single living cells with nano bio-optrodes can lead to a better understanding of many cellular processes such as transport mechanisms through cellular membranes, signal transduction pathways, complex enzymatic reactions, and even gene expression.

1.3.2. Multianalyte sensing

One of the main challenges of any sensor device is to detect several analytes simultaneously. Multianalyte sensing is important for clinical, environmental, and industrial analysis. For example, measuring the presence of proteins, antibodies, DNA sequences, antibiotics, viruses, and bacteria in single blood samples can provide physicians with rapid and comprehensive information about a patient's medical condition. Several approaches have been described for multianalyte bio-optrode fabrication (Li and Walt, 1995; Healey *et al.*, 1997a; Michael *et al.*, 1998; Anderson *et al.*, 2000; Walt, 2000; Chovin *et al.* 2004).

The conventional approach to preparing multianalyte sensors is to simply bundle multiple individual optical sensors. In this approach to multianalyte sensing, several optical fibers are assembled, each containing a different immobilized biorecognition molecule on a single fiber bundle. This approach was used to fabricate multianalyte biosensors for detecting different DNA target sequences simultaneously (Ferguson *et al.*, 1996). Eight optical fibers, each with a different immobilized DNA probe, were bundled together as shown in Figure 1.15a. The bundled fiber's distal end was inserted into the sample solution containing a fluorescein isothiocyanate–labeled oligonucleotide with a sequence complementary to one of the probe sequences. The fluorescence signals

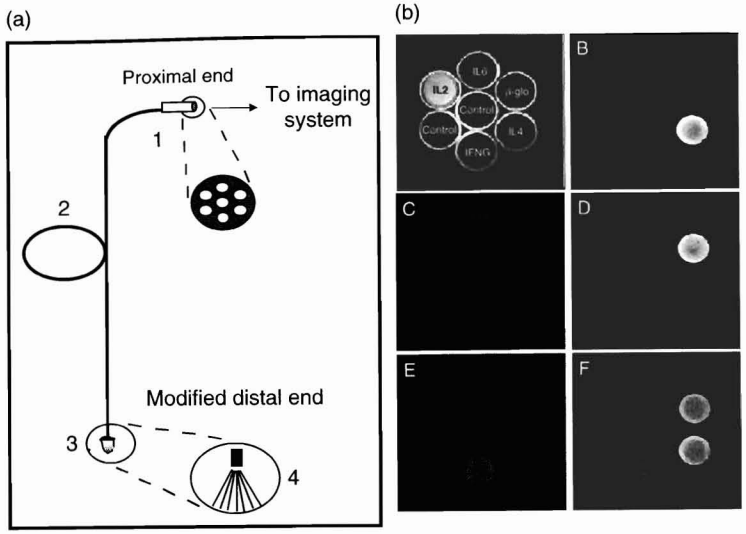


Figure 1.15 Multianalyte bio-optrode for oligonucleotide detection. (a) Schematic diagram of bio-optrode setup. Individual optical fibers, each with a specific immobilized oligonucleotide probe sequence are bundled together. The fiber's distal end is incubated with the sample and the signals obtained at the proximal end are measured using a CCD detector. (b) Fluorescence images acquired after incubating the multianalyte bio-optrode in solutions containing different target sequences. Image F show the bio-optrode response to the presence of three different targets in the sample (Ferguson *et al.*, 1996). Reprinted with permission from *Nature Biotechnol.*

were measured from the fiber's proximal end. Figure 1.15b shows that, when only one target sequence is present, a signal is obtained only from the fiber (bright signal) that contains the complementary probe sequence; the rest of the fibers in the bundle do not respond. When several target sequences were present, signals from several fibers carrying the complementary probes were observed (Figure 1.15b).

In different work, the specificity of this approach was demonstrated (Healey *et al.*, 1997b). Two probes were prepared, one that was complementary to the H-Ras oncogene sequence and a second probe containing a similar sequence but with a single base pair mismatch. When

the hybridization reaction was performed at low temperature, both sequences hybridized to the probe, but at high temperature only the wild-type sequence hybridized. This experiment shows that these sensors can be used for point mutation detection.

The same sensor configuration can be applied for different sensing elements such as antibodies, enzymes, or whole cells. This approach, theoretically, is not limited in the number of individual fibers (each with a different sensing chemistry) that can be used simultaneously; however, the array size grows as more sensing elements are added.

An alternative approach involves fabrication of discrete sensing regions, each containing different biosensing elements, at precise spatial locations on an imaging fiber's distal end (Figure 1.16a). The sensing regions can be formed using photopolymerization techniques (Pantano and Walt, 1995). The imaging fiber's proximal end is first prefunctionalized with a polymerizable silane. The fiber is then dipped into a solution containing monomer, cross-linker, indicators, photoinitiator, and the sensing biomolecules. Using a pinhole, light is focused onto a small area ($\sim$30 μm in diameter) on the imaging fiber's proximal end. Light travels through the imaging fiber, from the illuminated area at the proximal end to the distal end. At the distal end, the light activates a photoinitiator and the polymer layer is formed only on the illuminated area (Figure 1.16b). For the formation of the next sensing polymer, light is focused on a different area at the proximal end and the fiber's distal end is dipped into a polymerization solution containing different sensing biomolecules.

Initially, this approach was used to fabricate a multianalyte sensor for pH, CO_2, and O_2 by forming sensing regions with different fluorescent dyes on a single optical imaging fiber face (Ferguson *et al.*, 1997). Based on this initial work, multianalyte biosensors for detecting penicillin and pH were developed (Healey and Walt, 1995; Healey *et al.*, 1997a). This sensor incorporated two sensing regions: in one region the enzyme penicillinase was immobilized together with a pH indicator and in the second region only the pH indicator was immobilized. In the presence of penicillin, the penicillinase activity resulted in the formation of H^+,

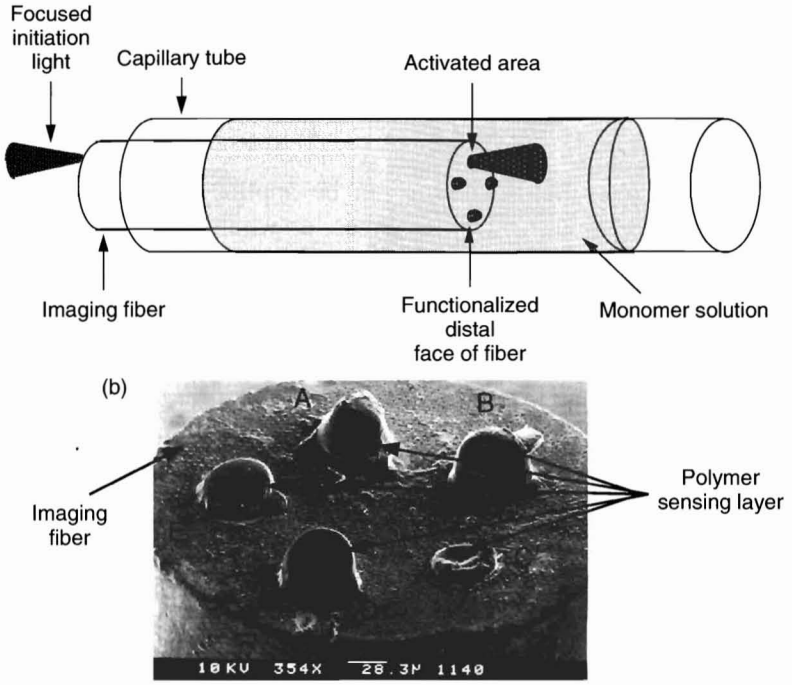


Figure 1.16 Multianalyte bio-optrode with different biosensing elements immobilized in polymers attached to an imaging fiber. (a) Setup of photopolymerization procedure used to fabricate the bio-optrode. Reprinted from Pantano and Walt (1995) with permission from the American Chemical Society. (b) Scanning electron micrograph of immobilized sensing polymer on an imaging fiber (Ferguson *et al.*, 1997). Reprinted with permission from Elsevier Science.

causing a decrease in the local pH in the polymer's microenvironment. By simultaneously monitoring pH changes in both the sensing regions (with and without the enzyme), the changes related to the enzymatic activity could be discriminated from pH changes in the bulk solution. Thus, this dual sensor was able to detect penicillin and could account for changes in the solution pH (Figure 1.17). A similar approach was used to fabricate glucose and O_2 biosensors. The enzyme glucose oxidase was used and the depletion of O_2 in the presence of glucose was monitored (Li and Walt, 1995). A separate sensor for O_2 was also

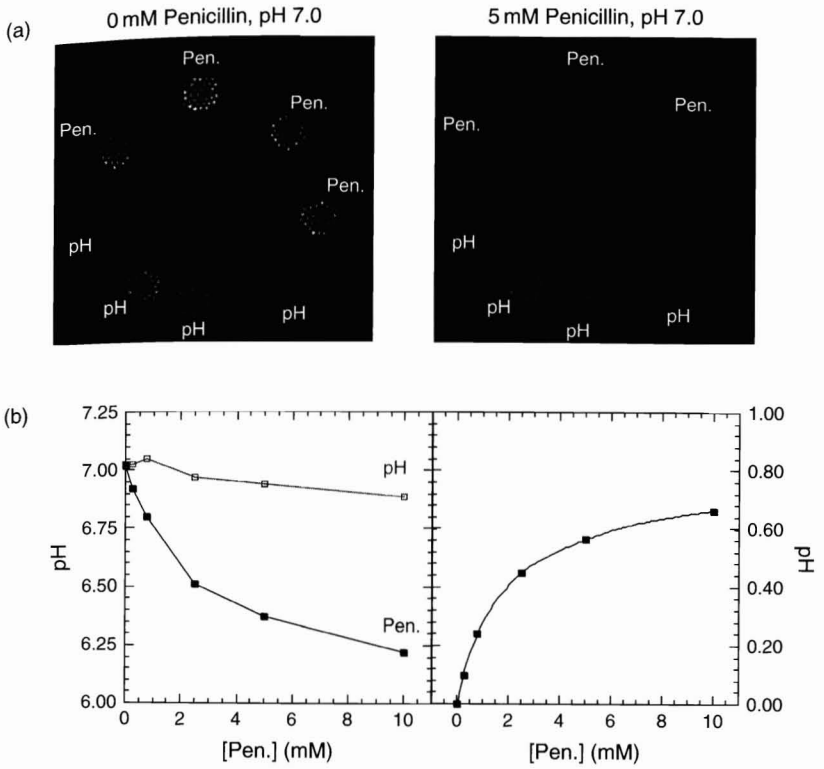


Figure 1.17 Imaging fiber-based penicillin and pH bio-optrode. (a) Response of bio-optrode, similar to the one described in Figure 1.15, with penicillin-sensitive polymer regions (containing the enzyme penicillinase) and pH-sensitive polymer regions. When the penicillin concentration is increased, only the fluorescence intensity from the penicillin-sensitive polymer regions increases. (b) Bio-optrode response to penicillin (solid squares) and pH (empty squares) are shown in the left plot. The difference between the buffer pH and the microenvironmental pH at the penicillin-sensitive polymer is shown in the right plot (Healey and Walt, 1995). Reprinted with permission from the American Chemical Society.

prepared on the same imaging fiber. When the glucose sensor signals were compared with the signals obtained from the sensing region that contained only the O_2 indicator, the concentration of glucose could be quantitatively determined. Both biosensors can be used to determine the

analyte concentrations in different environments. In addition, they can provide information about both the biochemical analytes and the pH or O_2 concentrations using a single imaging optical fiber. A possible future application for such biosensors may be for *in vivo* multianalyte sensing, where early changes in drug levels, glucose, O_2, and pH are important.

In both of these approaches (sensor bundling or photopolymerization), when more than 20 optical fibers or polymer regions are required, the bundle of fibers may become too big or the photopolymerization protocol may become complicated. A new approach to overcome this limitation based on the unique characteristics of optical imaging fibers (see Section 1.1.1) has been proposed (Michael *et al.*, 1998; Walt, 2000) and explored extensively (Ferguson *et al.*, 2000; Steemers *et al.*, 2000, 2005; Biran and Walt, 2002; Epstein *et al.*, 2002, 2003a, 2003b, 2003c; Biran *et al.*, 2003; Szunerits *et al.*, 2003; Kuang *et al.*, 2004a, 2004b; Ahn and Walt, 2005; Bowden *et al.*, 2005; DiCesare *et al.*, 2005; Gunderson *et al.*, 2005, 2006; Kuang and Walt, 2005; Song *et al.*, 2005, 2006; Ahn *et al.*, 2006; Blicharz and Walt, 2006; Fan *et al.*, 2006; Rissin and Walt, 2006a, 2006b; Dragoy Whitaker and Walt, 2007). Imaging fibers consist of thousands of optical fibers coherently bundled together, with each individual fiber maintaining its ability to carry its own light signal from one end of the fiber to the other. Thus, by attaching a sensing material to each individual fiber's distal end, an array of thousands of sensing elements can be constructed on the tip of a single imaging fiber array. In practice, microwells are fabricated on the end of each individual fiber by selectively etching the fiber cores. This process results in the formation of a high-density microwell array on the imaging fiber tip as shown in Figure 1.18a. The sensing elements are prepared by immobilizing fluorescent indicators and/or biorecognition molecules to the microsphere surfaces. The microspheres and microwells are matched in size such that the microspheres can be distributed into the microwells to form an array of sensing elements (Figure 1.18b). When different biorecognition molecules are immobilized on different microspheres, the array can be used to detect multiple analytes. A CCD detector is used to monitor and spatially resolve the fluorescence signals obtained from each microsphere (Figure 1.18c and d). Imaging and data analysis software are used to calculate the analyte concentrations.

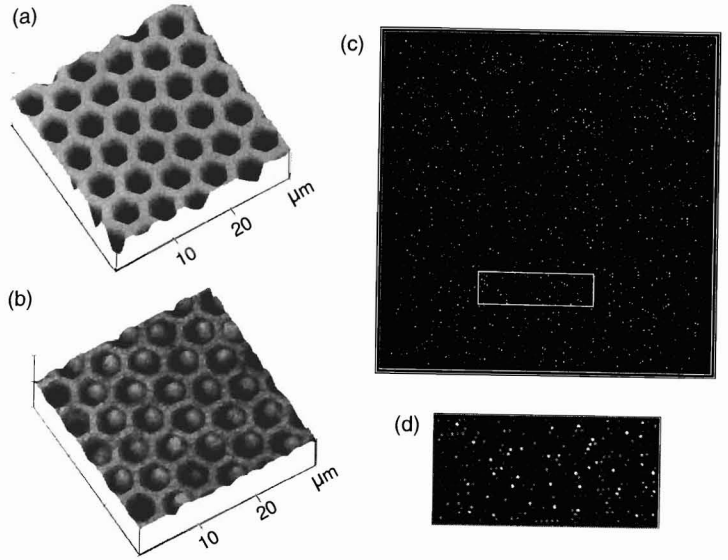


Figure 1.18 High-density multianalyte bio-optrode composed of microsphere array on an imaging fiber. (a) SFM of microwell array fabricated by selectively etching the cores of the individual fibers composing the imaging fiber. (b) The sensing microspheres are distributed in the microwell. (c) Fluorescence image of a DNA sensor array with ~13 000 DNA probe microspheres. (d) Small region of the array showing the different fluorescence responses obtained from the different sensing microspheres (Walt, 2000). Reprinted with permission from the American Association for the Advancement of Science. (see Plate 2)

These sensor arrays are prepared by randomly distributing the microspheres into the wells. In order to allow multianalyte sensing, the location of each sensing microsphere must be determined. The microsphere registration process involves using one of several encoding/decoding schemes. When the microspheres are prepared, each type of microsphere is modified such that it carries a unique optical marker in addition to the biorecognition element. This marker can be a fluorescent dye or a combination of several different fluorescent dyes. Different markers are used for the different microsphere types, allowing each of the microspheres carrying a certain type of biomolecule to be encoded with a unique optical signature. For example, as shown in Figure 1.19, three

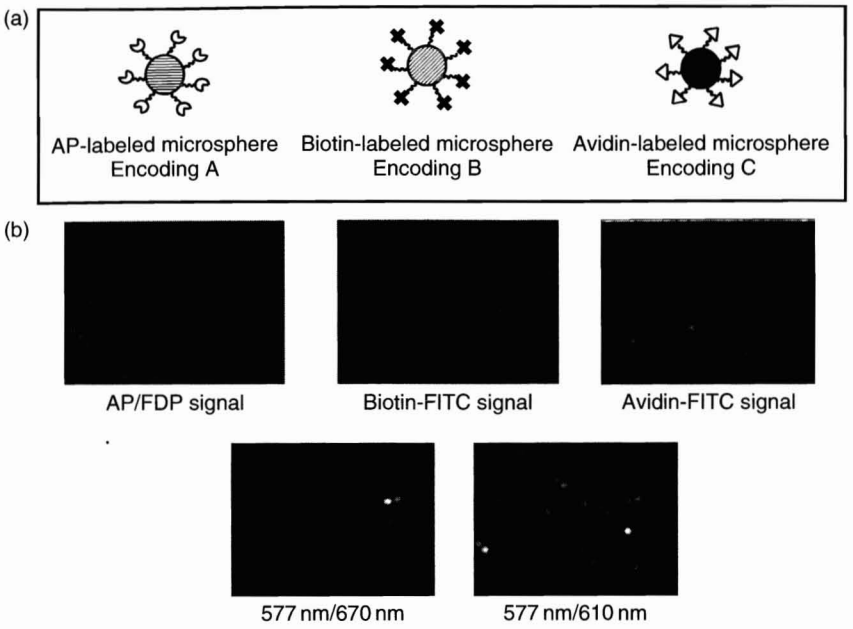


Figure 1.19 Randomly ordered array bio-optrode. (a) Schematic representation of the biorecognition elements immobilized on different sets of encoded microspheres (AP). The microspheres were encoded using three different ratios of two fluorescence dyes: indodicarbocyanine (DiIC) and Texas red cadaverine (TRC), both dyes are excited at 577 nm and emit at 670 and 610 nm, respectively. (b) The three microspheres types are mixed and randomly distributed into the microwell array. Fluorescence responses in the presence of the AP fluorogenic substrate, avidin–FITC, and biotin–FITC are shown on the top images. The identity of each microsphere was determined by calculating the emission ratio 670 nm/610 nm obtained using 577 nm excitation light (bottom images) (Michael *et al.*, 1998). Reprinted with permission from the American Chemical Society.

types of microspheres were prepared by immobilizing the enzyme AP to one group of microspheres, avidin to the second group, and biotin to the third group (Figure 1.19a). Each type was encoded with different concentrations of two fluorescent dyes. When the three microsphere types were mixed and randomly distributed into the microwell array, their location could be determined by applying the appropriate excitation

and emission wavelengths to establish the different fluorescent markers on each bead. This biosensor was used for multianalyte detection of fluorescein diphosphate, biotin–FITC, and avidin–FITC, as shown in Figure 1.19b. For each analyte, several different microspheres produced fluorescence emission signals, indicated by the bright spots on the array images.

These images demonstrate two main advantages of this technology. First, the presence of replicates of each microsphere type provides statistically significant results and reduces the probability of both false negatives and false positives. Second, averaging signals from many identical individual sensing elements results in higher signal-to-noise ratios. This multianalyte biosensor design was also used to develop a DNA biosensor with the ability to detect 25 different fluorescently labeled DNA sequences simultaneously (Ferguson *et al.*, 2000). Using a similar approach, Epstein *et al.* developed a microsensor array for simultaneous detection of multiple DNA sequences with a detection limit of 10^{-21} mol (approximately 600 DNA molecules). Recently, this methodology was applied to the detection of pathogenic bacteria (Ahn and Walt, 2005), biological warfare agents (Song *et al.*, 2006), and fabrication of an advanced platform for genomic analysis (Epstein *et al.*, 2003). Another biosensor, comprising microspheres with different immobilized molecular beacons, was used to detect three different unlabeled DNA sequences (Steemers *et al.*, 2000). This microsphere array platform has been commercialized by Illumina Inc., and many novel applications, such as high-throughput genotyping, have been successfully carried out using this system (Gunderson *et al.*, 2005, 2006; Steemers *et al.*, 2005; Fan *et al.*, 2006).

Microspheres with immobilized antibodies were used for simultaneous detection of the clinically important drugs digoxin and theophylline (Szurdoki *et al.*, 2001). Most recently, microsphere immunoassay arrays were used for simultaneous detection of five inflammatory cytokines (Blicharz and Walt, 2006). This multiplexed array is presently being evaluated for its potential for using saliva as a noninvasive diagnostic fluid for pulmonary inflammatory diseases such as asthma.

Alternatively, living cells could be directly immobilized on the surface of the microwells. By utilizing the genetic responses and signal transduction mechanisms in living cells, Walt and co-workers (Kuang *et al.*, 2004a, 2004b, 2005) developed cell assays for genotoxin monitoring and simultaneous monitoring of "promiscuous" drug effects using single cells of multiple cell types.

Multianalyte bio-optrodes are still in the early stages of development. Due to their importance for many analytical applications, it is expected that research efforts will continue to advance the capabilities of such sensors.

1.3.3. Single-molecule detection

Single-molecule detection represents the ultimate goal of ultrasensitive chemical analysis. Several approaches for fabricating bio-optrodes for single-molecule detection have been described (Fang and Tan, 1999; Loescher *et al.*, 1999; Rissin and Walt, 2006a, 2006b). Capture and detection of single molecules of β-galactosidase on a femtoliter fiber optic array was recently reported by Rissin and Walt (2006b). Twenty-four thousand reaction vessels, each 46 fl in size, were generated at the distal face of a polished 1 mm fiber optic array using an acid etch. Each well was then functionalized with biotin and was capable of capturing the target, streptavidin β-galactosidase (SβG) (Figure 1.20a).

After array modification, the biotinylated fiber arrays were incubated in PBS buffer containing varying amounts of SβG. The concentration was adjusted so that statistically either one molecule or no molecules bind to each well during incubation. The wells were then incubated with a fluorogenic substrate of β-galactosidase and sealed using a silicone gasket sandwiched between a microscope slide and the fiber array (Figure 1.20b). Single enzyme molecules were detected in the individual reaction vessels after generation of a sufficient number of fluorescent product molecules (Figure 1.20c). A linear relationship was observed between the percentage of reaction vessels that captured an enzyme molecule and the amount of enzyme present in the interrogated sample. This result suggests the viability of using this method for detecting

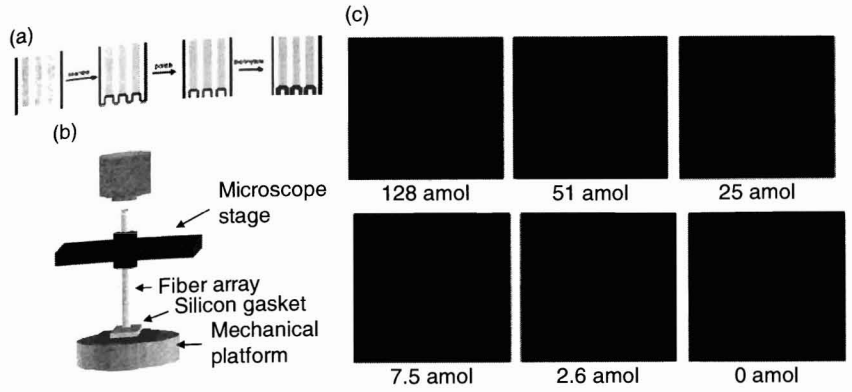


Figure 1.20 (a) A side-view cross-section schematic representing the etched bundle modifications. From left to right: An etched fiber, modification with an amino-functionalized silane, removal of the amine modification from the cladding material via polishing, and functionalization of the fiber bundle with biotin succinimidyl ester (Rissin and Walt, 2006a). (b) A simple schematic of the femtoliter array and microscope platform. The fiber array is locked into the microscope stage, a drop of substrate solution is placed on the silicone gasket, and the mechanical platform applies pressure to the distal end of the fiber, thereby sealing the solution in each femtoliter chamber (Rissin and Walt, 2006b). (c) A portion of the fiber arrays, each incubated with different concentrations of the analyte, SβG. Individual SβG molecules were captured in the biotin-functionalized femtoliter wells at the end of fiber (pseudocolor added using IPlab Software). Reprinted with permission from the American Chemical Society.

ultra-low amounts of target such as DNA or antigens. Such quantitative single-molecule detection techniques could circumvent additional time-consuming and complex steps such as immuno-PCR amplification and will push the limits of ultra-low detection.

1.3.4. Reagentless bio-optrodes for homogeneous assay

One limitation common to many bio-optrode technologies is the requirement for external reagents to be added to the analytical assay. For example, when antibodies are used as recognition molecules in a sandwich assay, there is a need to add a secondary labeled antibody in

order to measure the analyte concentration (Figure 1.10c). The same requirement applies to a competition assay where a labeled antigen is used (Figure 1.10b). Most nucleic acid bio-optrodes are based on pre-labeling the target sequence with fluorescent dye. The necessity to add reagents complicates the assay procedure and limits the acceptance of bio-optrodes as standard and simple analytical tools. Therefore, many research efforts have concentrated on developing bio-optrodes for "mix and measure" assays where no reagents are added. In this section, several approaches for reagentless (also called homogeneous) bio-optrode fabrication will be described.

One approach for reagentless bio-optrode fabrication is based on monitoring conformational changes in the biorecognition molecule following analyte binding. The conformational changes are usually detected using FRET as the transduction mechanism. In one example, molecular beacons were used to detect unlabeled DNA sequences (Steemers *et al.*, 2000). Molecular beacon structures consist of single-stranded DNA in a hairpin configuration with a fluorophore and quencher attached to opposite termini (Tyagi and Kramer, 1996). The molecule's 3′ and 5′ ends are complementary to one another and form the hairpin structure. The probe sequence, which is complementary to the target sequence, is located in the center (Figure 1.21a). In the absence of target, the fluorophore and quencher are within the requisite energy transfer distance, resulting in fluorescence quenching (Figure 1.21a). Upon target binding, a conformational change occurs, the hairpin separates (denatures), and the fluorescence signal increases (Figure 1.21b). Using an imaging fiber-based, molecular-beacon bio-optrode, three different sequences from mutant genes related to cystic fibrosis have been simultaneously detected (Steemers *et al.*, 2000). The multianalyte, imaging fiber-based bio-optrode was prepared as previously described in Section 1.3.2. Each type of molecular beacon probe was immobilized to beads encoded with unique optical signatures. The resulting three types of beads were randomly distributed into a microwell array and used for the analysis of three different target sequences simultaneously.

In a similar manner, fluorescence donor and acceptor pairs can be incorporated into proteins and used as reporters for substrate-binding

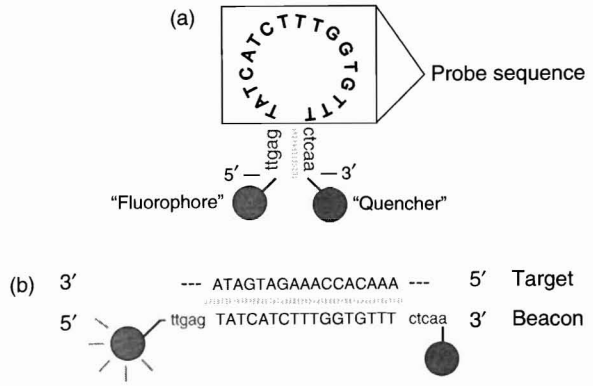


Figure 1.21 Molecular beacon structure. (a) The hairpin structure is formed due to the complementary sequences near the 3′ and 5′ ends. The single strand "loop" contains the probe sequence. In this configuration, the fluorophore and quencher are in proximity and therefore no fluorescence signal is produced. (b) When a target sequence binds, the molecular beacon structure changes causing separation of the fluorophore and quencher resulting in a fluorescence signal change.

events. In one approach, the enzyme carbonic anhydrase, which binds metal ions with high affinity and selectivity, was used to fabricate Zn^{2+}, Co^{2+}, and Cu^{2+} bio-optrodes (Thompson and Jones, 1993; Thompson *et al.*, 1996). Donor molecules, such as Cyt-5 or Cyt-3 dyes, were bound to primary amines in the protein, using *N*-hydroxysuccinimide esters of the dyes as modification reagents. The acceptor molecules in this case were the Co^{2+} and Cu^{2+} analytes themselves, which exhibit weak d–d absorbance bands at long wavelengths. Thus, upon analyte binding, a decrease in the donor fluorescence was observed. The decrease was measured by monitoring the time-dependent phase angle change at a fixed frequency upon binding of the metal ion. Results for two different concentrations of Co^{2+} are shown in Figure 1.22. The fiber configuration included an entrapped enzyme in a polyacrylamide layer immobilized to the tip of an optical fiber.

A related approach for fabricating reagentless enzyme-based biosensors is based on transducing conformational changes occurring upon substrate

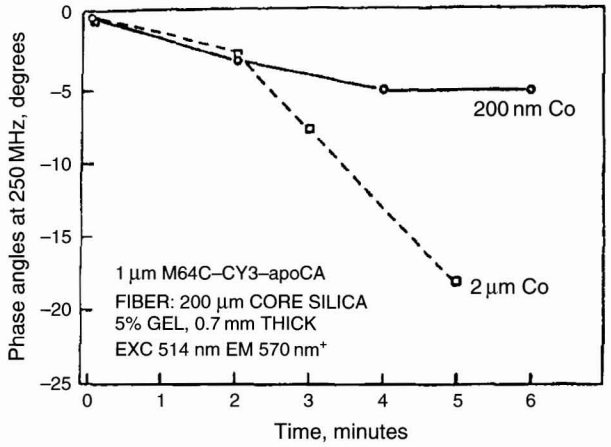
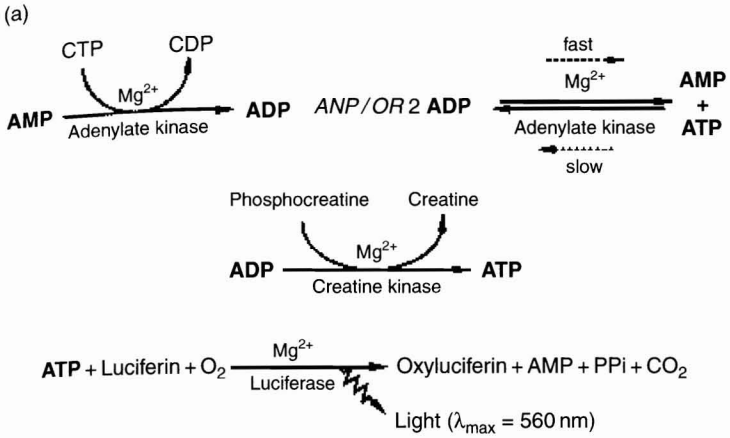


Figure 1.22 Reagentless Co^{2+} bio-optrode. The enzyme carbonic anhydrase was labeled with a fluorescent donor and immobilized to the fiber optic distal end. When Co^{2+} binds to the enzyme, the donor's fluorescence is quenched and the signal decreases (Thompson *et al.*, 1996). Reprinted with permission from Elsevier Science.

binding into FRET signals. Proteins such as calmodulin, maltose-binding protein, and phosphate-binding protein undergo conformational changes upon substrate binding and have been used to prepare such biosensors (Hellinga and Marvin, 1998). Using genetic engineering, two FRET fluorescent groups (acceptor and donor) were incorporated on two different cysteine residues; upon a conformational change, the spatial arrangement of these cysteines was altered, resulting in a FRET signal change.

Another example of a reagentless enzyme-based bio-optrode was recently described (Michel *et al.*, 1998a). The sensor used a controlled-release polymer similar to that used in previous work (Luo and Walt, 1989; Barnard and Walt, 1991) for controlled delivery of substrate in the enzymatic microenvironment. It was designed to detect the three-adenylate nucleotides (ATP, ADP, AMP) using a three-enzyme reaction sequence. Three enzymes were used: adenylate kinase, creatine kinase, and luciferase. The enzymes were compartmentalized in such a way that the product of the first reaction would be accessible to serve as the substrate for the subsequent reactions shown in Figure 1.23a. The final



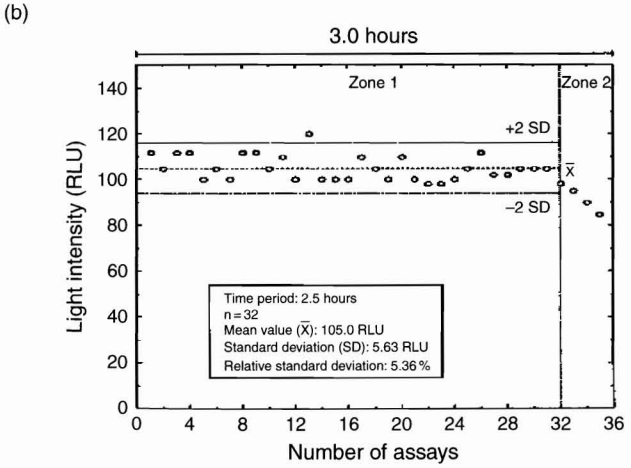


Figure 1.23 Reagentless bio-optrode for AMP, ADP and ATP detection. (a) Schematic of the enzymatic reactions employed for the measurements. (b) Repetitive measurements of ATP using controlled released of luciferin from acrylic microspheres. The light intensity was measured after each injection of 25 pmol ATP. The self-contained bio-optrode reproducibility over three hours (32 repetitive injections) is shown (Michel *et al.*, 1998a). Reprinted with permission from Elsevier Science.

indicator reaction for all three analytes involved the luciferase reaction. In previous designs, the co-substrate for this reaction, luciferin, was externally added to the flow cell. In the new design, luciferin was incorporated into acrylic microspheres. When the microspheres were immobilized together with the enzymes on the fiber surface, they slowly released the luciferin, allowing continuous detection for 3 h (Figure 1.23b). This generic approach is appropriate for the controlled release of cosubstrates or cofactors, which can be used in different enzyme-based bio-optrodes.

In recent years, fiber optic SPR sensors have emerged as a new approach for reagentless biodetection (Battaglia *et al.*, 2005; Chau *et al.*, 2006). In the study by Battaglia *et al.* (2005), the fiber probe tip was coated with 50 nm of Au and then modified with a 16-mercaptohexadecanoic acid to form a self-assembled monolayer. Various anti-cytokine antibodies were immobilized on the self-assembled monolayer via an amine coupling reaction. The shift in the minimum SPR wavelength was proportional to the amount of analyte captured by the immobilized antibodies and was measured using a white-light fiber optic SPR refractometer (Obando and Booksh, 1999). Detection limits at or below 1 ng/ml in both buffered saline solution and spiked cell culture medium were achieved for this label-free assay. The ability to detect multiple markers in complex media with a label-free method offers a promising approach for future clinical applications.

1.3.5. Environmental applications

Many bio-optrodes have been proposed for use in environmental applications (Rogers and Poziomek, 1996; Marty *et al.*, 1998; Rogers and Gerlach, 1999; Schobel *et al.*, 2000; Holst and Mizaikoff, 2002; Wolfbeis, 2002; Vannela and Adriaens, 2006). For remote monitoring, only the fiber tip containing the biorecognition element has to be located at the measurement site (e.g., lakes, rivers, sewage streams), while the optical detection instrumentation can be located in a protected location away from the site. Optical fibers are small in diameter and flexible, and therefore can be located in places inaccessible to other sensing devices. In addition, the optical fiber's durable structure protects it from

harsh environmental conditions. At present, although some prototype environmental monitoring fiber optic biosensors are commercially available, such as the Analyte 2000™ and RAPTOR™ developed by Research International and the Naval Research Laboratory, environmental bio-optrodes are still largely in the research and development stage with most of the research focused on detection scheme development and optimization.

Bio-optrodes have been described for detecting pesticides such as terbutryn (Bier *et al.*, 1992), parathion (Eldefrawi *et al.*, 1995), metsulfuron-methyl (Xing *et al.*, 2000), and imazethapyr (Wong *et al.*, 1993). One such example is a bio-optrode for the detection of 2,4-dichlorophenoxyacetic acid (2,4-D) in water (Wittmann *et al.*, 1996). In this system, an optical fiber with an immobilized analyte, 2,4-D, was placed into a flow cell. The assay procedure involved several steps:

(1) The fiber was incubated with fluorescently labeled monoclonal antibody for 2,4-D and the initial (maximum) fluorescence signal was measured. The fiber was then washed with buffer.
(2) The sample was incubated with fluorescently labeled monoclonal antibody for 2,4-D.
(3) The fiber was incubated with a mixture of sample and labeled antibody.
(4) The fiber was washed and the signal was measured.

When a high concentration of analyte (>1000 μg/l) was present in the sample, a low signal was obtained because most of the antibodies were occupied with the sample analyte and could not bind to the 2,4-D immobilized on the fiber. This bio-optrode was used to measure concentrations ranging between 0.2 and 100 μg/l. This concentration range is suitable for environmental applications where the permitted level of 2,4-D in drinking water cannot exceed 0.1 μg/l. The sensing layer could be regenerated by washing with proteinase K. This procedure enabled the bio-optrode to be used for more than eight weeks and in more than 500 successive measurements. Such bio-optrodes have the potential to be useful for online analysis of drinking water and to serve as warning devices for hazardous pesticide contamination.

Fiber optic-based biosensors have also been used to detect toxins and bacteria, such as staphylococcal enterotoxin B, ricin, *Bacillus anthracis*, and *Francisella tularensis* (Anderson and Rowe-Taitt, 2001).

Enzyme-based bio-optrodes for environmental applications have also been developed. The most common approach employs enzyme inhibition as the sensing mechanism. The inhibition of acetylcholinesterase by an organophosphate pesticide has been used in several sensors (Eldefrawi *et al.*, 1995; Xavier *et al.*, 2000; Choi *et al.*, 2001; Doong and Tsai, 2001), and is described in Section 1.1.4.1.

A different enzyme-based bio-optrode that uses a chemiluminescence reaction for detection of phenolic compounds was recently described (Ramos *et al.*, 2001). This bio-optrode is based on the enhancement of the luminol–H_2O_2–horseradish peroxidase chemiluminescence reaction by phenolic compounds. Using this bio-optrode, *p*-iodophenol, *p*-coumaric acid, and 2-naphthol were detected in concentrations as low as 0.83 μM, 15 nm, and 48 nM, respectively. The bio-optrode was fabricated by entrapping the enzyme in a sol-gel layer; the gel was prepared directly on the fiber tip. The assay was performed by inserting the fiber with the immobilized enzyme into a test tube containing the analyte, luminol, and H_2O_2. The chemiluminescence intensity maximum at 5 min was the output signal.

Whole cells have also been used for environmental bio-optrode construction. Recombinant *E. coli* cells overexpressing the enzyme OPH were immobilized to an optical fiber and used to detect organophosphate nerve agents, as described earlier (Section 1.1.4.1) (Mulchandani *et al.*, 1998). The bio-optrode detection limits were 3 μM for paraoxon and parathion and 5 μM for coumaphos. The sensor was stable over a 1-month period and used for over 75 repeated measurements. Recently, a fiber optic biosensor for the measurement of 1,2-dichloroethane in aqueous solution was developed by immobilizing whole cells of *Xanthobacter autotrophicus* GJ10 in calcium alginate on the tip of a fiber optic fluoresceinamine-based pH optrode (Campbell *et al.*, 2006). An enzyme in the *X. autotrophicus* cells, haloalkane dehalogenase DhlA, hydrolytically cleaves a chlorine atom from dichloroethane, generating hydrochloric acid. This enzyme-generated pH change was detected by the fiber optic pH sensor. Dichloroethane as low as 11 mg/l could be reproducibly measured in 8–10 min with a linear response up to at least 65 mg/l.

Using a different approach, in which the cell's genetic response was used as the sensing mechanism, a whole cell bio-optrode was used for detection of naphthalene and salicylate (Heitzer *et al.*, 1994; Ripp *et al.*, 2001). The sensing was performed by *Pseudomonas fluorescens* HK44 cells carrying a plasmid containing a fusion of the *nahG* gene, which is induced by naphthalene and salicylate, and the *luxCDABE* reporter gene, coding for the enzyme luciferase. The cells were immobilized onto the surface of a liquid light guide or an optical fiber by using strontium alginate. The bio-optrode tip was placed in a measurement flow cell that simultaneously received a waste stream solution and a maintenance medium. A rapid increase in bioluminescence was obtained when either naphthalene and salicylate were present in the waste stream. Real environmental samples of pollutant mixtures containing naphthalene were also tested using this system. High bioluminescence was obtained when aqueous solutions saturated with JP-4 jet fuel or aqueous leachates from contaminated soil were tested.

Using a similar approach, Hakkila *et al.* (2004) used recombinant *E. coli* cells carrying genes responsive to the presence of bioavailable heavy metal ions (e.g., Hg^{2+}, Cu^{2+}, Cd^{2+}, and Pb^{2+}) fused to firefly luciferase reporter gene. The cells were immobilized onto the tip of an optical fiber and the device was used to accurately and reproducibly detect bioavailable heavy metals in 17 synthetic and 3 environmental blind samples. In the same manner, Biran *et al.* (2003) fabricated an Hg^{2+} biosensor by immobilizing *E. coli* cells, possessing the *lacZ* reporter gene fused to the heavy metal-responsive gene promoter *zntA*, on the face of an optical imaging fiber containing a high-density array of microwells. A plasmid carrying the gene encoding enhanced cyan fluorescent protein was also introduced into this sensing strain to identify the cell locations in the array. Single cell *lacZ* expression was measured when the array was exposed to mercury and a response to 100 nm Hg^{2+} could be detected

after a 1-h incubation time. Another bio-optrode was also reported in which genetically modified *E. coli* cells produced bioluminescence in response to the presence of genotoxic agents (Polyak *et al.*, 2001). This bio-optrode was able to detect mitomycin C as low as 25 μg/l in less than 2 h.

The main importance of genetic response–based bio-optrodes for environmental analysis is the information they provide about the bioavailability of the analytes. This parameter is very important and helps to decide whether and how to treat a polluted site and which remediation strategies to employ.

1.3.6. Clinical applications

The development of bio-optrodes for clinical applications is another promising field and is focused on two types of applications: (a) *in vivo* detection inside a patient, (b) *ex vivo* detection when clinical samples are analyzed at the patient's bedside. The *in vivo* bio-optrodes would enable continuous monitoring of important analyte concentrations and would dramatically improve clinical procedures such as heart bypass surgery and critical care procedures in patients with compromised respiratory conditions. The optical fiber's small diameter, flexibility, nontoxic nature, durability, and lack of direct electrical connections make them highly suitable for *in vivo* applications. Moreover, optical fibers have already proven to be valuable for *in vivo* clinical applications such as endoscopic procedures and laser power transmission for surgical procedures. For example, endoscopes are routinely used in endoscopic surgery for gall bladder removal and for chest and knee surgery. In principle, bio-optrodes can be coupled to such devices and used to provide analytical information during endoscopic surgeries. At present, such bio-optrodes have not been implemented because of blood compatibility problems in which a thrombus (clot) forms around the sensor tip and affects the measurement accuracy. Recently, other *in vivo* chemical optrodes, such as the ones used by Baldini (2003) for *in vivo* monitoring of bile, carbon dioxide, and pH, have been reported.

The second clinical application for bio-optrodes is *ex vivo* diagnostics, mainly in critical care situations. Most diagnostic tests are presently performed in a centralized laboratory. Samples must be collected with the attendant transport, storage, and chain-of-custody issues. The remote location of the laboratory delays the medical diagnosis. In order to provide rapid diagnostic tests, analytical devices, such as bio-optrodes, can be used to bring the laboratory closer to the patient. These point-of-care devices should be sensitive, selective, self-contained (no need to add reagents), and simple to operate. They should also be small in size in order to be conveniently located near the patient. In addition, it is preferable that the sampling unit in contact with the sample (e.g., blood, urine) be disposable. Bio-optrode devices of this type are still not commercially available, but there are similar chemical-based fiber optic sensor devices used routinely in clinics. In these devices, fluorescent dyes are used as indicators for monitoring blood gases (PO_2, PCO_2) and pH. In one device, the immobilized dyes are incorporated into a disposable apparatus that is inserted into an extracorporeal blood circuit on one side and connected to a fiber bundle on the other (Owen, 1996). These sensors are mainly used to monitor blood gases during open-heart surgery. Another device is used for a paracorporeal measurement at the patient's bedside (Martin *et al.*, 1994). The sensors are placed into an external tube connected to an arterial blood line. Blood samples are periodically and automatically pumped into the tube, analyzed by the sensors, and then returned to the blood line. In this way, the blood can be monitored semi-continuously without requiring blood samples to be taken from the patient. It should be possible to incorporate bio-optrodes into such devices and use them to monitor other clinically important analytes.

For both *in vivo* and *ex vivo* applications, the first step in bio-optrode development is to establish sensitive sensing mechanisms that can be used to recognize specific analytes in a complex sample such as blood, urine, or other human fluids. Many examples of bio-optrodes directed for clinical applications have been proposed (Meadows, 1996; Vidal *et al.*, 1996; Vo-Dinh and Cullum, 2000; Knory *et al.*, 2004; Watterson *et al.*, 2004). Several glucose bio-optrodes, based on the enzyme-catalyzed reaction of glucose with glucose oxidase, have been prepared

or proposed (Moreno-Bondi *et al.*, 1990; Li and Walt, 1995; Marquette *et al.*, 2000; Zhu *et al.*, 2002). A submicrometer glucose bio-optrode has been prepared (Rosenzweig and Kopelman, 1996a, 1996b). In this bio-optrode, the consumption of molecular oxygen is measured by the fluorescence quenching of ruthenium complexes and serves as a reporter for glucose concentration. The enzyme and the indicator are immobilized in an acrylic polymer support on the fiber tip. The bio-optrode response is very fast (2 s) and concentrations as low as 1×10^{-15} mol were detected. Pasic *et al.* (2006) recently described a microdialysis-based bio-optrode for continuous online glucose monitoring. The bio-optrode was used *in vitro* to monitor glucose-spiked plasma continuously for 3 days. It was further evaluated by online monitoring the glucose level of a healthy volunteer for 24 h. A commercial microdialysis system was used to maintain constant air saturation of the measuring fluid in the cell. A reference oxygen optrode was used to compensate for response changes caused by events such as bacterial growth and temperature fluctuations. The authors suggested that this sensor could potentially be used for continuous glucose monitoring of patients in intensive care units.

Several bio-optrodes were developed for the sensitive detection of important cardiac markers such as myoglobin, cardiac troponin I(cTnI), and brain natriuretic peptide (Hanbury *et al.*, 1997; Masson *et al.*, 2004; Hong *et al.*, 2006). These markers are critical for rapid and accurate heart attack diagnosis and prognosis. The self-contained antibody-based bio-optrode developed by Hanbury *et al.* (1997) is potentially clinically important since it can serve as a method for monitoring the extent of myocardial infarction. Myoglobin was detected by immobilizing a fluorescently labeled monoclonal antibody in polyacrylamide gel on the tip of an optical fiber (Figure 1.24a). Cascade Blue was used both as the fluorescent labeling agent and as a FRET donor molecule. When myoglobin was captured by the antibody, fluorescence energy transfer occurred between Cascade Blue and the myoglobin heme group (acceptor). Fluorescence quenching of Cascade Blue was then correlated to the myoglobin concentration (Figure 1.24b). The polyacrylamide gel was optimized to serve as a size selective filter allowing only low molecular mass molecules to penetrate and interact with the antibodies. Using this approach, myoglobin (16 500 Da) could be discriminated

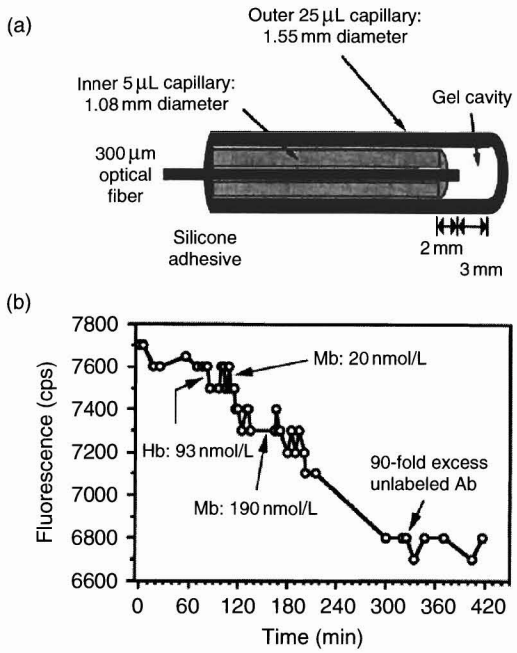


Figure 1.24 Reagentless bio-optrode for myoglobin detection. (a) Bio-optrode setup. (b) Myoglobin bio-optrode responses. The bio-optrode responses in PBS buffer (0–80 min) and after incubations with hemoglobin (Hb), myoglobin (Mb), and unlabeled myoglobin antibody (Ab) are shown (Hanbury *et al.*, 1997). Reprinted with permission from *Clin. Chem.*

from hemoglobin (bigger than 70 kDa). The size selection was necessary since antibodies for myoglobin can also bind hemoglobin. As shown in Figure 1.24b, a significant response was obtained when the bio-optrode was incubated with myoglobin but no response was obtained with hemoglobin (93 nmol/l). The detection limit of this bio-optrode was 5 nmol/l (83 µg/l), which is near the clinical decision limit for myocardial infarction diagnostics. The limitation of using the gel layer was the increased response time due to the low diffusion rate through the gel layer. In addition, when the gel layer was used, the bio-optrode response was irreversible even when the bio-optrode was incubated in a solution containing a high concentration of myoglobin antibodies (Figure 1.22b).

Irreversible sensor responses could limit the use of this bio-optrode for continuous monitoring applications.

Another example of a bio-optrode for heart-related disease diagnostics is the D-dimer antigen bio-optrode (Grant and Glass, 1999). D-Dimer antigen is formed when vascular occlusions are treated with a thrombolytic agent to lyse the clot. This treatment involves inserting a microcatheter at the occlusion site and injecting thrombolytic agents. Although thrombolytic therapy can help in preventing strokes, it suffers from several limitations that bio-optrodes can help to overcome. One limitation of thrombolytic therapy is the difficulty in determining whether the occlusion occurred from an atherosclerotic plaque or from a thrombus. Using the bio-optrode, initiation of D-dimer antigen formation following the injection of a small amount of thrombolytic agent would indicate the presence of a thrombus clot. If D-dimer antigens are not detected after the thrombolytic agent injection, it would be an indication that the occlusion is caused by an atherosclerotic plaque and alternative treatment would be required. In addition, in the case of a thrombus clot, the bio-optrode can be used for online monitoring of the thrombolytic agent dosage needed to dissolve the clot by monitoring the D-dimer antigen formation during the procedure. The principle of operation for this sensor is similar to that of the myoglobin bio-optrode described above. A fluorescently labeled antibody was immobilized in a sol-gel on an optical fiber tip and fluorescence quenching was observed when D-dimer antigen bound. D-Dimer antigen was detected in human plasma and in whole blood at a concentration of 0.56–6 μg/ml, which is within the clinically relevant range. The limitations of this bio-optrode were its poor reversibility and the short storage lifetime of the immobilized antibodies (4 weeks).

In addition to bio-optrodes directed to *in vivo* applications, bio-optrodes for point-of-care medical diagnostics have also been developed. These bio-optrodes offer a miniature design and a fast response time for analytes such as hepatitis C virus (Konry, 2005), bilirubin (Li and Rosenzweig, 1997), cholesterol (Marazuela *et al.*, 1997), and D-amino acids (Zhang *et al.*, 1995).

1.3.7. Industrial applications including bioprocess monitoring

Cell culture-based bioprocesses are very complex to control since they are sensitive to minor changes in the chemical composition of the fermentation medium. Therefore, tools for *online* monitoring of different analyte concentrations during the bioprocess are highly desirable. Bio-optrodes offer several advantages in such applications. The ability to use optical fibers directly inside fermentors (*in situ*) eliminates the need to periodically remove samples for analysis in a remote analytical laboratory. Once inside the fermentor, bio-optrodes can be used for sensitive and selective online monitoring of different analytes. Fermentation substrates and products such as proteins, antibodies, and antibiotics can be monitored. Other parameters related to the biological status of the cells, such as cell viability and activity, can also be measured. The ability to perform this measurement from a remote location (e.g., central control room) without using wires offers another important advantage. The main obstacle, which prevents wide use of *in situ* bio-optrodes (or any type of biosensor), is the need to sterilize the probe, which may damage the sensing biomolecules. For this reason, most bio-optrodes for bioprocess monitoring use a flow system in which a sample of the medium is taken from the fermentor and is delivered to the sensor (Dremel *et al.*, 1992; Mulchandani and Bassi, 1995; Scheper *et al.*, 1996; Marose *et al.*, 1999).

In one example, an FIA-based enzyme bio-optrode system was used for simultaneous detection of five different analytes (glucose, lactate, glutamate, glutamine, and ammonia) in samples removed during animal cell cultivation (Spohn *et al.*, 1995). The system was based on chemiluminescence detection and consisted of five optical fibers, each with a different immobilized enzyme. Each fiber was inserted into a different flow cell and, when the sample was injected, each fiber's response was measured. The results were combined and the concentrations of the different analytes were determined. Figure 1.25 shows results from a 350-h monitoring of an animal cell culture medium. In another example, an optical penicillin/pH biosensor was developed (Healey and Walt, 1995) to simultaneously monitor the pH and the concentration of penicillin produced during *Penicillium chrysogenum* fermentation process. In a

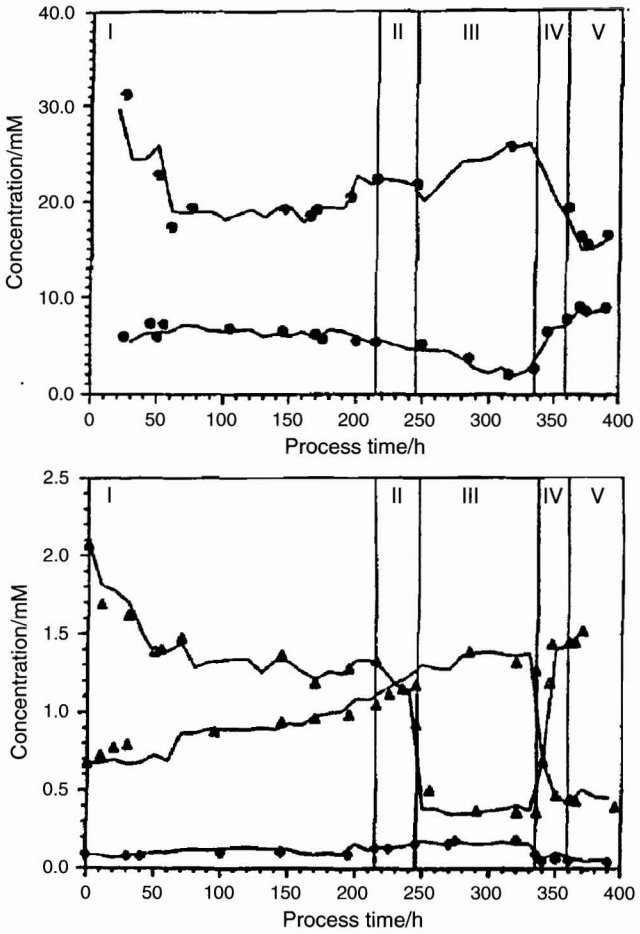


Figure 1.25 Five-channel enzyme-based bio-optrode for continuous monitoring of lactate, glucose, glutamine, ammonia, and glutamate (from top to bottom) during animal cell cultivation. Chemiluminescence measurements were employed to determine the analyte concentrations using five optical fibers, each with a different immobilized enzyme (Spohn *et al.*, 1995). Reprinted with permission from Elsevier Science.

similar way, antibody-based or nucleic acid-based bio-optrodes can be used to monitor different bioprocesses.

1.4. Advantages and limitations of bio-optrode technology

Bio-optrodes offer several advantages over other biosensing technologies based on the unique characteristics of optical fibers. The optical fiber's small dimensions, flexibility, and ability to transmit optical signals for long distances allow them to be used for remote sensing in places where other biosensors cannot be used. In addition, their ability to function without any direct electrical connection to the sample makes them safer than electrochemical biosensors. Optical sensors are also free from electromagnetic interference. Bio-optrodes are intrinsically simpler than electrode-based biosensors since no reference electrodes are needed; if desired, however, reference fibers can be interrogated in parallel to those used to measure analyte. Moreover, the development of new biorecognition molecules, such as those containing FRET-based dyes, enables the fabrication of self-contained bio-optrodes where no additional reagents are needed. Bio-optrodes based on imaging fibers offer additional advantages since they allow multiplexing with multianalyte sensing capabilities.

It is expected that as new optical technologies are developed for telecommunication applications, they will be adopted for bio-optrodes. These technologies include miniaturization of light sources, detectors, and optical fiber components (Kostov and Rao, 2000; Lechuga, 2005). Furthermore, new developments in the area of nanotechnology should eventually enable development of new bio-optrodes at the nanometer scale (Kasili and Vo-Dinh, 2005).

Nevertheless, bio-optrode technologies suffer from several drawbacks. Some of these drawbacks are common to all biosensor devices; the most restrictive of these drawbacks is the poor stability of the biological recognition molecules. Such molecules tend to be sensitive to pH or temperature changes and therefore generally have short lifetimes. Another important limitation is the high cost of some of the purified

biological sensing materials. Regeneration of sensing biomolecules is usually problematic and, in most cases, fresh biorecognition molecules are required for each assay. In addition, there are several problems related to the immobilization process including loss in activity, leaching of reagents, and the decreased response time due to slow diffusion of analytes through the immobilized layer.

Several other bio-optrode limitations are related to the nature of optical fibers. Since light signals are the measured parameter, bio-optrodes are sensitive to ambient light interference and precautions must be taken either to exclude light or to employ optical designs with lock-in detection capabilities. In most bio-optrodes, there is a need to use indicator dyes in order to transduce the biorecognition events. The dyes have to be immobilized together with the biomolecules and therefore complicate the bio-optrode fabrication. In addition, the dyes may leach from the immobilization matrix or may lose their characteristics because of photobleaching.

1.5. Potential for improving performance or expanding current capabilities

As with any sensing or monitoring device, the ideal bio-optrode should be specific, sensitive, simple to fabricate and use, well adapted to the measurement environment (e.g., detect specific analytes in a complex sample), reliable, and self-contained. When used as a sensor, it should be operated in a continuous and reversible manner. When used as a probe, it should include a simple and disposable unit that contains the sensing molecules. In addition, for many applications, bio-optrodes should be small, able to detect multiple analytes simultaneously, and enable measurements in remote sites. At present, no bio-optrode device has achieved all these ideal performance capabilities. Nevertheless, based on new bio-optrode technologies currently under development, it is expected that the next generation of bio-optrodes will come closer to achieving these goals.

The development of new bio-optrode technologies and devices is highly dependent on advances in several different fields. Advances in biology, chemistry, materials science, optics, electrical engineering, mechanical engineering, and computer engineering are expected to inspire new bio-optrode technology development. In this section, a few new key technologies and their future impacts on the bio-optrode field are discussed.

1.5.1. New optical fibers and instrumentation

Optical fibers have attracted attention mainly due to their use in telecommunications. New technologies have been developed for fabricating optical fibers with very efficient light transmission capabilities. Fibers can transmit extremely high amounts of data when used in both single or bundle format. These characteristics will advance the development of real-time multianalyte bio-optrodes for various analytical applications.

Improved, smaller, and less-expensive light sources and detectors are driving consumer electronics. Integration of these components into bio-optrodes can lead to miniaturization and commercialization of bio-optrode devices (Kostov and Rao, 2000). Among the different possible light sources, light emitting diodes (LEDs) are very attractive to use in bio-optrodes. LEDs are very small, cover the entire visible spectrum, produce optical power in the range of 0.1–5 mW, and have a very long life (100 000 h) and low cost (~$2). Once LEDs at a particular wavelength have been demonstrated and commercialized, laser diodes are usually available within a few years. Laser diodes have higher power output and are nearly monochromatic, whereas LEDs have a relatively broad spectral emission output. Another interesting new light source is the scintillation light source that can be used as a high-stability light source for the UV and blue region (Potyrailo *et al.*, 1998). These sources are based on long-lived radioisotopes in scintillation crystals, which convert the radioactive emission (typically beta particles) into emitted light. These sources are extremely stable, can be used without external power sources, and have an expected life of 20 years.

In recent years, new generations of miniaturized and improved light detectors, such as photodiodes (PDs) and PMTs, have been developed (Kostov and Rao, 2000). The most sensitive detectors are avalanche

PDs. CCD chips are also rapidly developing; chips with higher signal/noise ratios, wider dynamic ranges, more pixels, and lower dark currents have been developed. In addition, CCD detectors have been miniaturized and integrated into small devices. Image intensifiers have been integrated into CCD cameras to increase light detection sensitivity. Although CCD chip prices have been dramatically reduced, scientific grade CCD cameras are still very expensive (~$7000–$20 000). A competing technology to CCD is the complementary metal oxide semiconductor (CMOS) technology. Recent developments in this technology have demonstrated light detection capabilities similar to CCD detectors. The advantages of this technology are lower cost, simpler fabrication process, and the ability to use it for very fast image acquisition (32 000 pictures per second) because frame transfers are not required as all the processing is done on chip. Both CCD and CMOS technologies are being rapidly integrated into future bio-optrode devices.

1.5.2. New biological recognition elements

The "heart" of any bio-optrode is the biological recognition element that initiates the detection process by its interaction with the analyte. Development of new biological recognition elements will increase the number and types of analytes that can be detected by bio-optrodes. New advances in molecular biology techniques allow the design or selection of new recognition molecules. Among new recognition molecules, perhaps the one group attracting the most attention is engineered antibodies, such as small monovalent recombinant antibody fragments (Fab, scFv) and engineered multivalent variants (diabodies, triabodies, tetrabodies, minibodies, and single-domain antibodies) (Holliger and Hudson, 2005; see Chapter 12). For example, using phage display technology it is possible to screen and identify a single-chain antibody (scFv) with specificity for almost any analyte (Hoogenboom *et al.*, 1998). The recombinant scFv molecule is a smaller version of an antibody molecule containing the antigen-binding site. Genetically modified bacteriophages, each presenting a unique scFv molecule on its surface, are used in the screening process. Phage presenting scFvs with higher affinities to the analyte are selected. The system is designed in such a way that the sequence coding for the scFv presented in the selected phage can be readily identified.

Once inserted in an expression vector and transformed into a host, large quantities of the selected scFv molecule can be produced. This process is very powerful since it allows antibodies to be identified and isolated in a very short time (Goldman *et al.*, 2000). Moreover, once the antibody is found, it takes only a few days to produce it in large quantities. In addition, an scFv molecule can be specifically designed to be used in biosensor devices by adding immobilization capabilities to the molecule at the genetic level. For example, scFvs were fused to the cellulose-binding domain) resulting in scFv molecules that can be easily immobilized to a cellulose membrane (Berdichevsky *et al.*, 1999). Engineered proteins (Hellinga and Marvin, 1998; De Lorimier *et al.*, 2002, 2006; Looger *et al.*, 2003) and antibodies (Kramer and Hock, 2004) have already been used widely on biosensing platforms, and they will likely be integrated with bio-optrodes in the near future.

Using molecular biology techniques, several other biorecognition molecules have been designed. Genetic fusion between antibody molecules and the GFP results in self-fluorescent antibodies, also called fluorobodies, and eliminates the need to label the antibody with a fluorescent dye. Biological recognition elements isolated using combinatorial approaches, such as aptamers, are described in detail in Chapter 13. Biomimetic polymer materials for bio-optrode applications are described in Chapter 14.

1.5.3. Imaging and biosensing

The coupling of chemical and biosensing capabilities to optical fiber-based imaging devices (e.g., endoscopes) is expected to attract much attention in the future. Optical imaging fibers are used in endoscopes since they can carry images from one end of the fiber to the other due to the coherent nature of the fibers. This imaging capability can be utilized to simultaneously image and measure local analyte concentrations with micron-scale resolution (Bronk *et al.*, 1995; Michael and Walt, 1999; Issberner *et al.*, 2002). The distal face of an imaging fiber is coated with an analyte-sensitive layer (typically a biorecognition molecule and fluorescent indicator), which produces a microsensor array capable of spatially resolving analyte concentrations. The concept is shown in

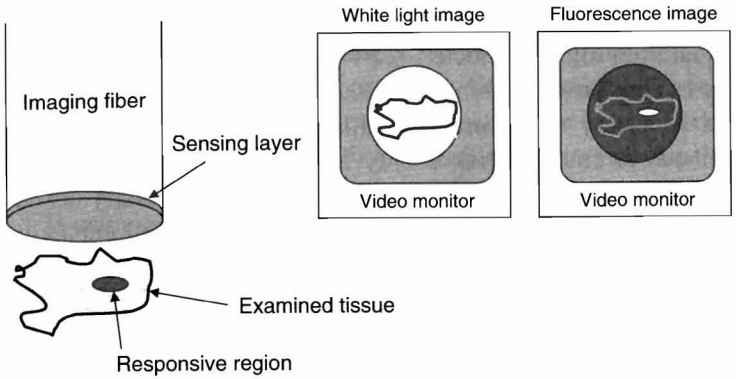


Figure 1.26 Combined imaging and biosensing concept. The technique provides the ability to both view tissue slices or individual cells and measure the release or consumption of different analytes using fluorescence techniques.

Figure 1.26. For example, an L-glutamate imaging fiber bio-optrode was fabricated by coating the imaging fibers with a layer of gel containing L-glutamate oxidase, poly(acrylamide-*co*-*N*-acryloxysuccinimide), and a pH-sensitive fluorescent dye, SNAFL (Issberner *et al.*, 2002). The fiber was used both to image and to chemically detect L-glutamate released from the foregut plexus of the Lepidopteran, *Manduca sexta* with spatial resolution of $3 \sim 4\,\mu m$ and a detection limit between 10 and $100\,\mu M$.

L-Glutamate release was measured *in vivo* following electrical stimulation of the esophageal nerve immediately posterior to the frontal ganglion. First, the fiber was placed over the specimen and a white-light image was taken through the fiber to visualize the specimen's morphology and to observe some of the medium-sized nerve branches ($5 \sim 10\,\mu m$ width) in order to locate and position the fiber tip in a specified area. The imaging fiber bio-optrode was then switched to the fluorescence mode to detect and localize *in vivo* the release of L-glutamate and its subsequent re-uptake or diffusion away from the release site. The ability to observe the location of neurotransmitter release, with microscale spatial resolution, provides a powerful tool for neuroscience researchers. Moreover, this report demonstrated the feasibility of using imaging fiber bio-optrodes to detect neurotransmitter release from nerve

endings *in vivo* with physiologically relevant resolution, suggesting the possibility of clinical applications with this technique. For example, a suspected cancer tumor could be examined based both on its morphology and on its response to specific antibodies immobilized on the imaging fiber tip.

1.5.4. Data analysis

New bio-optrode technologies are expected to provide a large amount of analytical data from each measurement. For example, multianalyte bio-optrodes can measure the concentration of many different analytes simultaneously. When performed in continuous fashion (e.g., multiple measurements every second), these measurements generate a high volume of data, especially as bio-optrode arrays are now being developed for genomic analysis. In order to acquire, analyze, and save such high data volumes, sophisticated software should be developed or adapted from other data-intensive applications (Figure 1.27).

The most significant computerized task is the data analysis because it may affect the specificity, sensitivity, and reproducibility of the bio-optrode. Analysis of biosensor measurements may be complicated due to the high variability in the activity of biorecognition molecules and because the measurements are usually performed in a complex sample matrix. It was previously shown that even in a simple FIA biosensor system for measurement of a single analyte in bioprocess samples, there is a need to use advanced computational analysis in order to improve the sensitivity, selectivity, and reproducibility of the measurement (Hitzmann *et al.*, 1998). For example, in typical bioprocess samples, the pH or ion concentrations change during cultivation; these changes may affect the biosensor's enzyme activity or antibody-binding properties. In addition, inhibitors, proteases, and nucleases can be produced during the bioprocess, which will affect protein- or nucleic acid-based biosensors. In order to overcome these problems, multivariate evaluation techniques such as neural networks have been used. Scheper and coworkers (Muller *et al.*, 1997) applied a neural network approach to improve the analysis of signals obtained from a bio-optrode for penicillin. A neural network approach was necessary due to the sensitivity of the measurement to

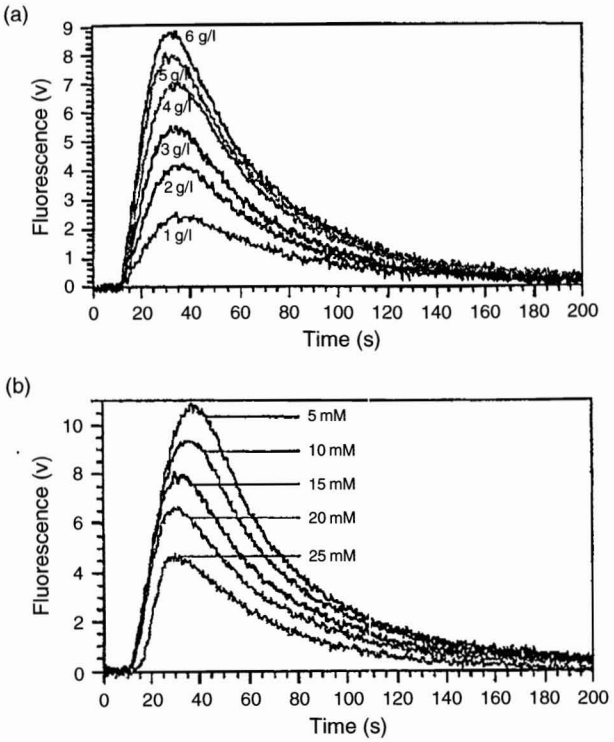


Figure 1.27 Penicillin detection using a FIA-based bio-optrode. (a) Fluorescence signals obtained in response to different penicillin concentrations. (b) Fluorescence signals obtained with different buffer ion concentrations and a fixed penicillin concentration (5 g/l). Neural network analysis was applied to analyze the relationships between these two responses (Muller *et al.*, 1997). Reprinted with permission from Elsevier Science.

changes in buffer ion concentration (Figure 1.25). The neural network was used to simultaneously evaluate the ion and penicillin concentrations from a single measurement based on characteristic signal shape variations. The shape characteristics were thought to be useful because in a preliminary experiment, multiple measurements of the same sample showed reproducible signal shapes. The results from this neural network showed errors of less than 11% for six different penicillin concentrations measured at five different ion concentrations.

To evaluate single-cell reporter-gene transcriptional activation and expression in yeast two-hybrid (Y2H) systems statistically, Walt and coworkers (Whitaker and Walt, 2007) have used a distribution fitting analysis tool, *t*-test, and one-way analysis of variance (ANOVA) model. This statistical analysis allowed elucidation of potential correlations between protein–protein interactions and reporter gene expression levels at varying stringency conditions. Plotting and normal fitting of these data provided a visual illustration of how different yeast strains expressed reporter genes under different conditions. These simple examples demonstrate the power of such computational techniques in the analysis of bio-optrode signals. It is clear that such techniques will be essential for analyzing signals from multianalyte bio-optrodes. Advanced computational methods have recently been used for the analysis of chemical sensor arrays (Jurs *et al.*, 2000), and it is expected that they will be adapted to the analysis of bio-optrode measurements. The generation of high amounts of information from future multianalyte bio-optrodes is expected to shift the emphasis from signal measurement to data analysis.

References

Aboul-Enein, H.Y., Stefan, R.I., van Staden, J.F. *et al.* (2000) *Crit. Rev. Anal. Chem.*, **30**, 271.

Ahn, S. and Walt, D.R. (2005) *Anal. Chem.*, **77**, 5041.

Ahn, S., Kulis, D.M., Erdner, D.L. *et al.* (2006) *App. Environmental Microbiol.*, **72**, 5742.

Anderson, G.P. and Rowe-Taitt, C.A. (2001) *Proc. SPIE – Int. Soc. Opt. Eng.*, **4206**, 58.

Anderson, G.P., Jacoby, M.A., Ligler, F.S., and King, K.D. (1997) *Biosens. Bioelectron.*, **12**, 329.

Anderson, G.P., King, K.D., Gaffney, K.L., and Johnson, L.H. (2000) *Biosens. Bioelectron.*, **14**, 771.

Arnold, M.A. (1985) *Anal. Chem.*, **57**, 565.

Balcer, H.I., Kwon, H.J., and Kang, K.A. (2002) *Ann. Biomed. Eng.*, **30**, 141.

Baldini, F. (2003) *Anal. Bioanal. Chem.*, **375**, 732.

Bard, A.J. (2004) Introduction. In *Electrogenerated Chemiluminescence* (A.J. Bard, ed.) New York: Marcel Dekker, p. 1.

Bard, A.J., Debad, J.D., Leland, J.K. *et al.* (2000) In *Encyclopedia of Analytical Chemistry* (R.A. Meyers, ed.) Chichester, UK: John Wiley & Sons, p. 9842.
Barker, S.L.R., Clark, H.A., Swallen, S.F. *et al.* (1999) *Anal. Chem.*, **71**, 1767.
Barker, S.L.R., Kopelman, R., Meyer, T.E., and Cusanovich, M.A. (1998) *Anal. Chem.*, **70**, 971.
Barnard, S.M. and Walt, D.R. (1991) *Science*, **251**, 927.
Battaglia, T.M., Masson, J.F., Sierks, M.R. *et al.* (2005) *Anal. Chem.*, **77**, 7016.
Berdichevsky, Y., Ben-Zeev, E., Lamed, R., and Benhar, I. (1999) *J. Immunol. Methods*, **228**, 151.
Berthold, J.W. and Lopushansky, R.L. (2004) *Proc. SPIE – Int. Soc. Opt. Eng.*, **5589**, 197.
Bier, F.F., Stocklein, W., Bocher, M. *et al.* (1992) *Sens. Actuators B Chem.*, **7**, 509.
Blicharz, T.M. and Walt, D.R. (2006) *Proc. SPIE – Int. Soc. Optical Eng.*, 6380 (Smart Medical and Biomedical Sensor Technology IV), 638010/1.
Blum, L.J., Gautier, S.M., and Coulet, P.R. (1993) *J. Biotechnol.*, **31**, 357.
Blum, L.J., Gautier, S.M., and Coulet, P.R. (1994) Fiber-optic biosensors based on luminometric detection. In *Food Biosensor Analysis* (G. Wagner and G.G. Guilbault, eds) New York: Marcel Dekker, p. 101.
Biran, I., Rissin, D.M., Ron, E.Z., and Walt, D.R. (2003) *Anal. Biochem.*, **315**, 106.
Biran, I. and Walt, D.R. (2002) *Anal. Chem.*, **74**, 3046.
Bowden, M., Song, L., and Walt, D.R. (2005) *Anal. Chem.*, **77**, 5583.
Brogan, K.L. and Walt, D.R. (2005) *Curr. Opin. Chem. Biol.*, **9**, 494.
Bronk, K.S., Michael, K.L., Pantano, P., and Walt, D.R. (1995) *Anal. Chem.*, **67**, 2750.
Bronstein, I., Martin, C.S., Fortin, J.J. *et al.* (1996) *Clin. Chem.*, 42, 1542.
Campbell, A.K. and Sala-Newby, G. (1993) Bioluminescent and chemluminescent indicators for molecular signaling and function in living cells. In *Fluorescence and Luminescence Probes for Biological Activity* (W.T. Mason, ed.) London: Academic Press, pp. 58–79.
Campbell, D.W., Mueller, C., and Reardon, K.F. (2006) *Biotech. Lett.*, **28**, 883.
Chalfie, M., Tu, Y., Euskirchen, G. *et al.* (1994) *Science*, **263**, 802.
Chandler, D.E., Majumdar, Z.K., Heiss, G.J., and Clegg, R.M. (2006) *J. Fluorescence*, **16**, 793.
Chang, A.C., Gillespie, J.B., and Tabacco, M.B. (2001) *Anal. Chem.*, **73**, 467.
Chau, L.-K., Lin, Y.-F., Cheng, S.-F., and Lin, T.-J. (2006) *Sens. Actuators B Chem.*, **B113**, 100.
Choi, J.-W., Kim, Y.-K., Lee, I.-H. *et al.* (2001) *Biosens. Bioelectron.*, **16**, 937.

Chovin, A., Garrigue, P., Vinatier, P., and Sojic, N. (2004) *Anal. Chem.* **76**, 357.
Cowan, J.J. and Arakawa, E.T. (1970) *Phys. Status Solid*, **1**, 695.
Cullum, B.M. and Vo-Dinh, T. (2000) *Trends Biotechnol.*, **18**, 388.
Cunningham, A.J. (1998) *Introduction to Bioanalytical Sensors*. New York: John Wiley & Sons, Inc., pp. 260–77.
Daunert, S., Barrett, G., Feliciano, J.S. *et al.* (2000) *Chem. Rev.*, **100**, 2705.
De Lorimier, R.M., Smith, J.J., Dwyer, M.A. *et al.* (2002) *Protein Sci.*, **11**, 2655.
De Lorimier, R.M., Tian, Y., and Hellinga, H.W. (2006) *Protein Sci.*, **15**, 1936.
Diaz, A.N., Peinado, M.C.R., and Minguez, M.C.T. (1998) *Anal. Chim. Acta*, **363**, 221.
DiCesare, C., Biran, I., and Walt, D.R. (2005) *Anal. Bioanal. Chem.*, **382**, 37.
Doong, R.A. and Tsai, H.C. (2001) *Anal. Chim. Acta*, **434**, 239.
Dremel, B.A.A., Li, S.Y., and Schmid, R.D. (1992) *Biosens. Bioelectron.*, **7**, 133.
Dunn, B., Cox, J., Lan, E., and Zink, J.I. (2001) *Abstr. Pap. Am. Chem. Soc.*, **221**, 473-COLL.
Dunn, B., Miller, J.M., Dave, B.C. *et al.* (1998) *Acta Mater.*, **46**, 737.
Durrieu, C. and Tran-Minh, C. (2002) *Ecotox. Environ. Safety*, **51**, 206.
Eggins, B.R. (1996) *Biosensors, an Introduction*. New York: John Wiley & Sons, Inc., pp. 1–117
Eldefrawi, M.E., Eldefrawi, A.T., Anis, N.A. *et al.* (1995) *Immunoanalysis of Agrochemicals*. Washington, DC: American Chemical Society, pp. 197–209.
Endo, H., Yonemori, Y., Musiya, K. *et al.* (2006) *Anal. Chim. Acta*, **573–574**, 117.
Epstein, J.R. and Walt, D.R. (2003) *Chem. Soc. Rev.*, **32**, 203.
Epstein, J.R., Ferguson, J.A., Lee, K.H., and Walt, D.R. (2003) *J. Am. Chem. Soc.*, **125**, 13753.
Epstein, J.R., Lee, M., and Walt, D.R. (2002) *Anal. Chem.*, **74**, 1836.
Epstein, J.R., Leung, A.P.K., Lee, K.H., and Walt, D.R. (2003) *Biosens. Bioelectron.*, **18**, 541.
Fan, J.B., Gunderson, K.L., Bibikova, M. *et al.* (2006) *Methods Enzymol.*, **410**, 57.
Fang, Q., Papaioannou, T., Jo, J.A. *et al.* (2004) *Rev. Sci. Instr.*, **75**, 151.
Fang, X. and Tan, W. (1999) *Anal. Chem.*, **71**, 3101.
Ferguson, J.A., Boles, T.C., Adams, C.P., and Walt, D.R. (1996) *Nat. Biotechnol.*, **14**, 1681.
Ferguson, J.A., Healey, B.G., Bronk, K.S. *et al.* (1997) *Anal. Chim. Acta*, **340**, 123.
Ferguson, J.A., Steemers, F.J., and Walt, D.R. (2000) *Anal. Chem.*, **72**, 5618.
Fine, T., Leskinen, P., Isobe, T. *et al.* (2006) *Biosens. Bioelectron.*, **21**, 2263.

Fraser, D. (1995) *Med. Device Technol.*, **6**, 28, 34.
Freeman, M.K. and Bachas, L.G. (1992), *Biosens. Bioelectron.*, **7**, 49.
Goldman, E.R., Pazirandeh, M.P., Mauro, J.M. *et al.* (2000) *J. Mol. Recognition*, **13**, 382.
Grant, S.A. and Glass, R.S. (1999) *IEEE Trans. Biomed. Eng.*, **46**, 1207.
Grant, S.A., Stringer, R.C., Studer, S. *et al.* (2005) *Biosen. Bioelectron.*, **21**, 438.
Gubitz, G., Schmid, M.G., Silviaeh, H., and Aboul-Enein, H.Y. (2001) *Crit. Rev. Anal. Chem.*, **31**, 167.
Gunderson, K.L., Steemers, F.J., Lee, G. *et al.* (2005) *Nat. Genet.* **37**, 549.
Gunderson, K.L., Steemers, F.J., Ren, H. *et al.* (2006) *Methods Enzymol.*, **410**, 359.
Hanbury, C.M., Miller, W.G., and Harris, R.B. (1997) *Clin. Chem.*, **43**, 2128.
Hakkila, K., Green, T., Leskinen, P. *et al.* (2004) *J. Appl. Toxicol.*, **24**, 333.
Harfenstellęr, M., Schilp, M., Eursch, A., and Zaeh, M.F. (2004) *Proc. SPIE – Int. Soc. Opt. Eng.*, **5590**, 57.
Healey, B.G. and Walt, D.R. (1995) *Anal. Chem.*, **67**, 4471.
Healey, B.G., Foran, S.E., and Walt, D.R. (1995) *Science*, **269**, 1078.
Healey, B.G., Li, L., and Walt, D.R. (1997a) *Biosens. Bioelectron.*, **12**, 521.
Healey, B.G., Matson, R.S., and Walt, D.R. (1997b) *Anal. Biochem.*, **251**, 270.
Heitzer, A., Malachowsky, K., Thonnard, J.E. *et al.* (1994) *Appl. Environ. Micro.*, **60**, 1487.
Hellinga, H.W. and Marvin, J.S. (1998) *Trends Biotechnol.*, **16**, 183.
Hitzmann, B., Ritzka, A., Ulber, R. *et al.* (1998) *J. Biotechnol.*, **65**, 15.
Hobel, W. and Polster, J. (1992) *Fresenius J. Anal. Chem.*, **343**, 101.
Holst, G. and Mizaikoff, B. (2002) Fiber optic sensors for environmental applications. In *Handbook of Optical Fibre Sensing Technology* (J.M. Lopez-Higuera, ed.) Chichester, UK: John Wiley & Sons, Inc, pp. 729–55.
Holliger, P. and Hudson, P.J. (2005) *Nat. Biotech.*, **23**, 1126.
Homola, J. and Yee, S.S. (1996) *Sens. Actuators B*, **37**, 145.
Hong, B. and Kang, K.A. (2006) *Biosens. Bioelectron.*, **21**, 1333.
Hoogenboom, H.R., de Bruine, A.P., Hufton, S.E. *et al.* (1998) *Immunotechnology*, **4**, 1.
Ikariyama, Y., Nishiguchi, S., Koyama, T. *et al.* (1997) *Anal. Chem.*, **69**, 2600.
Ince, R. and Narayanaswamy, R. (2006) *Anal. Chim. Acta*, **569**, 1.
Iqbal, S.S., Mayo, M.W., Bruno, J.G. *et al.* (2000) *Biosens. Bioelectron.*, **15**, 549.
Issberner, J.P., Schauer, C.L., Trimmer, B.A., and Walt, D.R. (2002) *J. Neuro. Met.*, **120**, 1.
Jin E.S., Norris, B.J., and Pantano, P. (2001) *Electroanalysis*, **13**, 1287.
Jordan, J.D., Dunbar, R.A., and Bright, F.V. (1996) *Anal. Chim. Acta*, **332**, 83.

Jorgenson, R.C. and Yee, S.S. (1993) *Sens. Actuators B*, **12**, 213.
Jurs, P.C., Bakken, G.A., and McClelland, H.E. (2000) *Chem. Rev.*, **100**, 2649.
Kasili, P.M., Song, J.M., and Vo-Dinh, T. (2004) *J. Am. Chem. Soc.*, **126**, 2799.
Kasili, P.M. and Vo-Dinh, T. (2005) *J. Nanosci. Nanotech.*, **5**, 2057.
Kaspar, B.H., Amstutz, P., and Plueckthun, A. (2005) *Nat. Bio.*, **23**, 1257.
Kishen, A., John, M.S., Lim, C.S., and Asundi, A. (2003) *Biosens. Bioelectron.*, **18**, 1371.
Knight, A.W. (1999) *Trends Anal. Chem.*, **18**, 47.
Kohler, S., Belkin, S., and Schmid, R.D. (2000) *Fresenius J. Anal. Chem.*, **366**, 769.
Komatsu, T., Kikuchi, K., Takakusa, H. *et al.* (2006) *J. Am. Chem. Soc.*, **128**, 15946.
Konry, T., Novoa, A., Avni, Y.S. *et al.* (2004) *Chem. Sensors*, **20**, 214.
Konry, T., Novoa, A., Cosnier, S., and Marks, R.S. (2003) *Anal. Chem.*, **75**, 2633.
Konry, T., Novoa, A., Shemer-Avni, Y. *et al.* (2005) *Anal. Chem.*, **77**, 1771.
Kostov, Y. and Rao, G. (2000) *Rev. Sci. Instruments*, **71**, 4361.
Kramer, K. and Hock, B. (2004) Antibodies for biosensors. In *Springer Series on Chemical Sensors and Biosensors*. Berlin: Springer GmbH, pp. 3–22.
Kuang, Y. and Walt, D.R. (2005) *Anal. Biochem.*, **345**, 320.
Kuang, Y., Biran, I., and Walt, D.R. (2004a) *Anal. Chem.*, **76**, 2902.
Kuang, Y., Biran, I., and Walt, D.R. (2004b) *Anal. Chem.*, **76**, 6282.
Kuboka, A.V., Bykh, A.T., and Svir, I.B. (2000) *Frsenius J. Anal. Chem.*, **368**, 439.
Kulp, T.J., Camins, I., Angel, S.M. *et al.* (1987) *Anal. Chem.*, **59**, 2849.
Kumagai, T. and Kajioka, H. (2002) Fibre optic gyroscope for industrial applications. In *Handbook of Optical Fibre Sensing Technology* (J.M. Lopez-Higuera, ed.) Chichester, UK: John Wiley & Sons, pp. 619–29.
Kumar, J., Jha, S.K., and D'Souza, S.F. (2006) *Biosens. Bioelectron.*, **21**, 2100.
Kuswandi, B., Andres, R., and Narayanaswamy, R. (2001) *Analyst*, **126**, 1469.
Kwok, N.-Y., Dong, S.J., Lo, W.H., and Wong, K.-Y. (2005) *Sens. Actuators B: Chem.*, **B110**, 289.
LaRossa, R.A. (1998) *Bioluminescence Methods and Protocols*, Vol. 102. Totowa, NJ: Humana Press, pp. 85–299.
Lambeck, P.V. (1991) *Proc. SPIE*, **115**, 100.
Lechuga, L.M. (2005) *Comprehensive Anal. Chem.*, **44**, 209.
Lee, M. and Walt, D.R. (2000) *Anal. Biochem.*, **282**, 142.
Lee, W.-B., Wu, J., Lee, Y.-I., and Sneddon, J. (2004) *Appl. Spectrosc. Rev.*, **39**, 27.

Leland, J.K. and Powell, M.J. (1991) *J. Electroanal. Chem.*, **318**, 91.
Leshem, B., Sarfati, G., Novoa, A. *et al.* (2004) *Luminescence*, **19**, 69.
Leth, S., Maltoni, S., Simkus, R. *et al.* (2002) *Electroanalysis*, **14**, 35.
Li, L. and Walt, D.R. (1995) *Anal. Chem.*, **67**, 3746.
Li, X.P. and Rosenzweig, Z. (1997) *Anal. Chim. Acta*, **353**, 263.
Liu, X.J., Farmerie, W., Schuster, S., and Tan, W.H. (2000) *Anal. Biochem.*, **283**, 56.
Liu, X., Song, D., Zhang, Q. *et al.* (2005) *Trends. Anal. Chem.*, **24**, 887.
Looger, L.L., Dwyer, M.A., Smith, J.J., and Hellinga, H.W. (2003) *Nature*, **423**, 185.
Lopez-Higuera, J.M. (ed.) (2002) *Handbook of Optical Fibre Sensing Technology*. Chichester, UK: John Wiley & Sons.
Loescher, F., Ruckstuhl, T., and Seeger, S. (1998) *Advanced Materials*, **10**, 1005.
Lubbers, D.W. and Opitz, N. (1975) *Pflugers Archiv-Eur. J. Physiol.*, **359**, R145.
Luo, S. and Walt, D.R. (1989) *Anal. Chem.*, **61**, 1069.
Magrisso, M., Etzion, O., Pilch, G. *et al.* (2006) *Biosens. Bioelectron.*, **21**, 1210.
Maragos, C.M. and Thompson, V.S. (1999) *Nat. Tox.*, **7**, 371.
Marazuela, M.D., Cuesta, B., MorenoBondi, M.C., and Quejido, A. (1997) *Biosens. Bioelectron.*, **12**, 233.
Marazuela, M.D. and Moreno-Bondi, M.C. (2002) *Anal. Bioanal. Chem.*, **372**, 664.
Marose, S., Lindemann, C., Ulber, R., and Scheper, T. (1999) *Trends Biotechnol.*, **17**, 30.
Marquette, C.A. and Blum, L.J. (2006) *Anal. Bioanal. Chem.*, **385**, 546.
Marquette, C.A., Degiuli, A., and Blum, L.J. (2000) *Appl. Biochem. Biotechnol.*, **89**, 107.
Marquette, C.A., Leca, B.D., and Blum, L.J. (2001) *Luminescence*, **16**, 159.
Martin, R.C., Malin, S.F., Bartnik, D.J. *et al.* (1994) *Proc. SPIE*, **2131**, 426.
Marty, J.L., Leca, B., and Noguer, T. (1998) *Analysis*, **26**, M144.
Massey, M., Piunno, P.A.E., and Krull, U.J. (2005) In *Springer Series on Chemical Sensors and Biosensors*, 3 (Frontiers in Chemical Sensors) Berlin: Springer GmbH, pp. 227–60.
Masson, J.F., Barnhart, M., Battaglia, T.M. *et al.* (2004) *Analyst*, **129**, 855.
Masson, J.-F., Kim, Y.-C., Obando, L.A. *et al.* (2006) *App. Spec.*, **60**, 1241.
Massona, J.F., Obando, L., Beaudoin, S., and Booksh, K. (2004) *Talanta*, **62**, 865.
Meadows, D. (1996) *Adv. Drug Deliv. Rev.*, **21**, 179.

Mehrvar, M., Bis, C., Scharer, J.M. *et al.* (2000) *Anal. Sci.*, **16**, 677.
Michael, K.L. and Walt, D.R. (1999) *Anal. Biochem.*, **273**, 168.
Michael, K.L., Taylor, L.C., Schultz, S.L., and Walt, D.R. (1998) *Anal. Chem.*, **70**, 1242.
Michel, P.E., Gautier-Sauvigne, S.M., and Blum, L.J. (1998a) *Talanta*, **47**, 169.
Michel, P.E., Gautier-Sauvigne, S.M., and Blum, L.J. (1998b) *Anal. Chim. Acta*, **360**, 89.
Monk, D.J. and Walt, D.R. (2004a) *Anal. Bioanal. Chem.*, **379**, 931.
Monk, D.J. and Walt, D.R. (2004b) *J. Am. Chem. Soc.*, **126**, 11416.
Moreno-Bondi, M.C., Wolfbeis, O.S., Leiner, M.J., and Schaffar, B.P. (1990) *Anal. Chem.*, **62**, 2377.
Mulchandani, A. and Bassi, A.S. (1995) *Crit. Rev. Biotechnol.*, **15**, 105.
Mulchandani, A., Kaneva, I., and Chen, W. (1998) *Anal. Chem.*, **70**, 5042.
Muller, C., Hitzmann, B., Schubert, F., and Scheper, T. (1997) *Sens. Actuators B Chem.*, **40**, 71.
Narayanaswamy, R., and Wolfbeis, O.S. (eds) (2003) *Optical Sensors for Industrial, Environmental and Clinical Applications*. Berlin: Springer-Verlag.
Naylor, L.H. (1999) *Biochem. Pharmacol.*, **58**, 749.
Obando, L.A. and Booksh, K.S. (1999) *Anal. Chem.*, **71**, 5116.
Otto, A. (1968) *J. Phys.*, **216**, 398.
Owen, V.M. (1996) *Biosens. Bioelectron.*, **11**, R5.
Pantano, P. and Walt, D.R. (1995) *Anal. Chem.*, **67**, A481.
Pasic, A., Koehle, H., Schaupp, L. *et al.* (2006) *Anal. Bioanal. Chem.*, **386**, 1293.
Pilevar, S., Davis, C.C., and Portugal, F. (1998) *Anal. Chem.*, **70**, 2031.
Polyak, B., Bassis, E., Novodvorets, A. *et al.* (2001) *Sens. Actuators B Chem.*, **74**, 18.
Potyrailo, R.A., Hobbs, S.E., and Hieftje, G.M. (1998) *Anal. Chim. Acta*, **367**, 153.
Preininger, C., Klimant, I., and Wolfbeis, O.S. (1994) *Anal. Chem.*, **66**, 1841.
Quinn, J.G., Neill, S.O., Doyle, A. *et al.* (2000) *Anal. Biochem.*, **281**, 135.
Rabbany, S.Y., Donner, B.L., and Ligler, F.S. (1994) *Crit. Rev. Biomed. Eng.*, **22**, 307.
Ramos, M.C., Torijas, M.C., and Diaz, A.N. (2001) *Sens. Actuators B Chem.*, **73**, 71.
Richter M.M. (2004) *Chem. Rev.*, **104**, 3003.
Ripp S., Nivens, D.E., Ahn, Y. *et al.* (2001) *Environ. Sci. Technol.*, **34**, 846.
Rissin, D.M. and Walt, D.R. (2006a) *Nano Lett.*, **6**, 520.
Rissin, D.M. and Walt, D.R. (2006b) *J. Am. Chem. Soc.*, **128**, 6286.
Rogers, K.R. and Gerlach, C.L. (1999) *Environ. Sci. Technol.*, **33**, 500A.

Rogers, K.R. and Mascini, M. (1998) *Field Anal. Chem. Technol.*, **2**, 317.
Rogers, K.R. and Poziomek, E.J. (1996) *Chemosphere*, **33**, 1151.
Rosenzweig, Z. and Kopelman, R. (1996a) *Anal. Chem.*, **68**, 1408.
Rosenzweig, Z. and Kopelman, R. (1996b) *Sens. Actuators B Chem.*, **36**, 475.
Sackmann, E. (1996) *Science*, **271**, 43.
Scheper, T.H., Hilmer, J.M., Lammers, F. *et al.* (1996) *J. Chromatogr. A*, **725**, 3.
Schobel, U., Barzen, C., and Gauglitz, G. (2000) *Fresenius J. Anal. Chem.*, **366**, 646.
Serganova, I. and Blasberg, R. (2005) *Nuclear Med. Bio.*, **32**, 763.
Song, L., Ahn, S., and Walt, D.R. (2005) *Emerging Infectious Diseases*, **11**, 1629.
Song, L., Ahn, S., and Walt, D.R. (2006) *Anal. Chem.* **78**, 1023.
Spiridonova, V.A. and Kopylov, A.M. (2002) *Biochem. (Moscow)*, **67**, 706.
Spohn, U., Preuschoff, F., Blankenstein, G. *et al.* (1995) *Anal. Chim. Acta*, **303**, 109.
Steemers, F.J. and Walt, D.R. (1999) *Mikrochim. Acta*, **131**, 99.
Steemers, F.J., Chang, W., Lee, G. *et al.* (2006) *Nat. Methods*, **3**, 31.
Steemers, F.J., Ferguson, J.A., and Walt, D.R. (2000) *Nat. Biotechnol.*, **18**, 91.
Szunerits, S. and Walt, D.R. (2003) *Chemphyschem.*, **4**, 186.
Szunerits, S., Tam, J.M., Thouin, L. *et al.* (2003) *Anal. Chem.*, **75**, 4382.
Szurdoki, F., Michael, K.L., and Walt, D.R. (2001) *Anal. Biochem.*, **291**, 219.
Tan, W.H., Shi, Z.Y., Smith, S. *et al.* (1992) *Science*, **258**, 778.
Tang, L., Kwon, H.J., and Kang, K.A. (2004) *Biotech. Bioeng.*, **88**, 869.
Tang, L., Ren, Y., Hong, B., and Kang, K.A. (2006) *J. Biomed. Optics*, **11**, 021011/1.
Thompson, R.B. and Jones, E.R. (1993) *Anal. Chem.*, **65**, 730.
Thompson, R.B., Ge, Z.F., Patchan, M. *et al.* (1996) *Biosens. Bioelectron.*, **11**, 557.
Tyagi, S. and Kramer, F.R. (1996) *Nat. Biotechnol.*, **14**, 303.
Tyagi, S., Marras, S.A.E., and Kramer, F.R. (2000) *Nat. Biotechnol.*, **18**, 1191.
Vannela, R. and Adriaens, P. (2006) *Crit. Rev. Environ. Sci. Tech.*, **36**, 375.
Vidal, M.M., Delgadillo, I., Gil, M.H., and Alonso-Chamarro, J. (1996) *Biosens. Bioelectron.*, **11**, 347.
Viveros, L., Paliwal, S., McCrae, D. *et al.* (2006) *Sens. Actuators B Chem.*, **B115**, 150.
Vo-Dinh, T. and Cullum, B. (2000) *Fresenius J. Anal. Chem.*, **366**, 540.
Vo-Dinh, T. and Kasili, P. (2005) *Anal. Bioanal. Chem.*, **382**, 918.

Vo-Dinh, T., Alarie, J.P., Cullum, B.M., and Griffin, G.D. (2000) *Nat. Biotechnol.*, **18**, 764.
Vo-Dinh, T., Cullum, B.M., and Stokes, D.L. (2001) *Sens. Actuators B Chem.*, **74**, 2.
Wabuyele, M.B., Farquar, H., Stryjewski, W. *et al.* (2003) *J. Am. Chem. Soc.*, **125**, 6937.
Watterson, J.H., Raha, S., Kotoris, C.C. *et al.* (2004) *Nucleic Acids Res.*, **32**, e18.
Wygladacz, K. and Bakker, E. (2005) *Anal. Chim. Acta*, **532**, 61.
Walt, D.R. (1998) *Accounts Chem. Res.*, **31**, 267.
Walt, D.R. (2000) *Science*, **287**, 451.
Walt, D.R. (2002) *Curr. Opin. Chem. Bio.*, **6**, 689.
Walt, D.R. (2006) *BioTech.*, **41**, 529.
Wang, X. and Krull, U.J. (2005a) *J. Mat. Chem.*, **15**, 2801.
Wang, X. and Krull, U.J. (2005b) *Bioorg. Med. Chem. Lett.*, **15**, 1725.
Watterson, J.H., Piunno, P.A.E., Wust, C.C., and Krull, U.J. (2001) *Sens. Actuators B Chem.*, **B74**, 27.
Watterson, J.H., Raha, S., Kotoris, C.C. *et al.* (2004) *Nucleic Acids Res.*, **32**, e18/1.
Weetall, H.H. (1993) *Appl. Biochem. Biotechnol.*, 41, 157.
Whitaker, D.R. and Walt, D.R. (2007) *Anal. Biochem.*, **360**, 63.
Wilchek, M. and Bayer, E.A. (1990) *Avidin–Biotin Technology*. San Diego, CA: Academic Press.
Wittmann, C., Bier, F.F., Eremin, S.A., and Schmid, R.D. (1996) *J. Agric. Food Chem.*, **44**, 343.
Wu, M., Lin, Z., Duerkop, A., and Wolfbeis, O.S. (2004) *Anal. Bioanal. Chem.*, **380**, 619.
Wolfbeis, O.S. (1991) *Fiber Optic Chemical Sensors And Biosensors*, Vol. 2. Boca Raton, FL: CRC Press, pp. 193–257.
Wolfbeis, O.S. (1997) Chemical sensing using indicator dyes. In *Optical Fiber Sensors – Applications, Analysis, and Future Trends*, Vol. 4 (B. Culshaw and J. Dakin, eds) Norwood: Artech House, pp. 53–107.
Wolfbeis, O.S. (2000a) *Anal. Chem.* **72**, 81R.
Wolfbeis, O.S. (2000b) *Anal. Chem.* **74**, 2663.
Wolfbeis, O.S. (2000c) *Anal. Chem.* **76**, 3269.
Wolfbeis, O.S. (2000d) *Anal. Chem.* **78**, 3859.
Wolfbeis, O.S. (2004) *Springer Series on Chemical Sensors and Biosensors 1 (Optical Sensors)* Berlin: Springer-Verlag, pp. 1–34.
Wong, R.B., Anis, N., and Eldefrawi, M.E. (1993) *Anal. Chim. Acta*, **279**, 141.

Wortberg, M., Orban, M., Renneberg, R., and Cammann, K. (1997) Flurometric immunosensors. In *Handbook of Biosensors and Electronic Noses: Medicine, Food, and the Environment*, (E. Kress-Rogers, ed.) Boca Raton, FL: CRC Press, p. 369.
Xavier, M.P., Vallejo, B., Marazuela, M.D. *et al.* (2000) *Biosens. Bioelectron.*, **14**, 895.
Xing, W.-L., Ma, L.-R., Jiang, Z.-H. *et al.* (2000) *Talanta*, **52**, 879.
Zhang, W., Chang, H., and Rechnitz, G.A. (1997) *Anal. Chim. Acta*, **350**, 59.
Zhang, Z., Zhang, S., and Zhang, X. (2005) *Anal. Chim. Acta*, **541**, 37.
Zhang, Z.J., Gong, Z.L., and Ma, W.B. (1995) *Microchem. J.*, **52**, 131.
Zhao, C.Q., Anis, N.A., Rogers, K.R. *et al.* (1995) *J. Agric. Food Chem.*, **43**, 2308.
Zhu, L., Li, Y., and Zhu, G. (2002) *Sens. Actuators B Chem.*, **B86**, 209.

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Chapter 2

EVANESCENT WAVE FIBER OPTIC BIOSENSORS

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Evanescent wave fiber optic biosensors are a subset of fiber optic biosensors that perform the sensing function along the fiber's cylindrical length. In all optical fibers, light propagates by means of total internal reflection, wherein the propagating light is launched into waveguide at angles such that upon reaching the cladding–core interface, the energy is reflected and remains in the core of the fiber. Remarkably, however, for light reflecting at angles near the critical angle, a significant portion of the power extends into the cladding or medium which surrounds the core. This phenomenon, known as the evanescent wave, extends only to a short distance from the interface, with power dropping exponentially with distance. The evanescent wave has been exploited to allow for real-time interrogation of surface-specific recognition events.

2.1. Technical concept

Fiber optic biosensors all utilize the concept of total internal reflection (TIR), wherein the light, whether it be source or signal, transits the optical fiber by repeatedly reflecting off the cladding–core interface in