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Optical Nanosensors for Detecting Proteins and Biomarkers in Individual Living Cells

Tuan Vo-Dinh

Summary

Recently, nanotechnology has been revolutionizing important areas in molecular biology and medicine, especially diagnostics and therapy at the molecular and cellular levels. The combination of nanotechnology, biology, advanced materials, and photonics opens up the possibility of detecting and manipulating atoms and molecules using nanodevices. This capability has the potential for a wide variety of medical uses at the cellular level. One of the most recent technological advances has been in the area of nanosensors. This chapter describes the principle of optical nanosensors, their development, and their applications for in vivo analysis of proteins and biomarkers in individual living cells. Nanosensors were fabricated with optical fibers pulled down to tips with distal ends in nanoscale dimensions. Nanosensors with immobilized bioreceptor probes (e.g., antibodies, enzyme substrate) that are selective to target analyte molecules are also referred to as nanobiosensors. Laser light is launched into the fiber, and the resulting evanescent field at the tip of the fiber is used to excite target molecules bound to the antibody molecules. A photometric detection system is used to detect the optical signal (e.g., fluorescence) originating from the analyte molecules or from the analyte-bioreceptor reaction.

Key Words: Nanosensor; nanoprobe; nanotechnology; biosensor; antibody; single cell; benzopyrene tetrol; cancer.

1. Introduction

Minimally invasive analysis of proteins and related cellular signaling pathways inside single intact cells is becoming increasingly important because cells in a population respond asynchronously to external stimuli. There is a need to further our understanding of basic cellular signaling processes in order to obtain new information that is not available from population-averaged cellular measurements. A further advantage of living-cell analysis is that it allows us to

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understand the exact pathways by which signaling pathways move through the architecture of the cell. Not only is there a need to resolve such measurements temporally; there is also a need to resolve them spatially. For these reasons, continued progress in cellular physiology requires new protein measurement strategies that can be applied to individual cells with great temporal and spatial resolution.

Advances in nanotechnology and photonics have recently led to a new generation of devices for probing the cellular machinery, elucidating intimate life processes occurring at the molecular level that were heretofore invisible to human inquiry ***(1)***. Recent advances in nanotechnology have led to the development of biosensor devices having nanoscale dimensions; such sizes make them suitable for probing biomolecules such as proteins inside individual living single cells. These nanotools could provide new information that could greatly improve our understanding of cellular function, thereby revolutionizing cell biology.

Fiberoptic sensors provide useful tools for remote *in situ* monitoring. Nanoscale fiberoptic sensors would be suitable for sensing intracellular/intercellular physiological and biological parameters in microenvironments. A wide variety of fiberoptic chemical sensors and biosensors have been developed in our laboratory for environmental and biochemical monitoring ***(2–9)***. Submicron fibers have been developed for use in near-field optics ***(10,11)***. Tapered fibers with submicron tip diameters between 20 and 500 nm have also been developed for near-field scanning optical microscopy (NSOM). NSOM was used to achieve subwavelength 100-nm spatial resolution in Raman detection ***(12,13)***. Tan et al. ***(14,15)*** have developed and used chemical nanosensors to perform measurements of calcium and nitric oxide, among other physicochemicals in single cells. Vo-Dinh et al. have developed nanosensors with antibody probes ***(16–23)*** and enzyme substrate–based probes ***(24)*** to detect biochemical targets and proteins inside living single cells. This chapter describes the operating principle, instrumentation, protocols, and applications of optical nanosensors.

2. Principle of Biosensors and Nanosensors

2.1. Near-Field Optics and Nanosensors

Nanofibers were originally developed for use in NSOM, which is a technique involving light sources or detectors that are smaller than the wavelength of light ***(10)***. The first method developed for performing these experiments was to place a pinhole in front of the detector, thus effectively reducing the detector's size. In a later variation of these pinholes, an excitation probe with dimensions smaller than the wavelength of the light was used for sample inter-

rogation. Betzig and Chichester ***(11)*** developed one such probe capable of obtaining measurements with a spatial resolution of approx 12 nm. The probe was constructed by using a micropipet puller to pull a single-mode optical fiber to a tip diameter of 20 nm and then coating the walls of the fiber with 100 nm of aluminum to confine the excitation radiation to the tip. With this nanoprobe, images of a pattern were reconstructed from a raster scan performed in the illumination mode, with the probe acting as a localized light source.

Near-field microscopy has received great interest resulting from its extremely high spatial resolution (subwavelength) ***(10)***. For example, a relatively new method known as near-field surface-enhanced Raman spectroscopy (NF-SERS) has been used for the measurement of single-dye and dye-labeled DNA molecules with a resolution of 100 nm ***(12,13)***. In this work, DNA strands labeled with the dye brilliant cresyl blue were spotted onto a SERS-active substrate that was prepared by evaporation of silver on a nanoparticle-coated substrate. The silver-coated nanostructured substrates are capable of inducing the SERS effect, which can enhance the Raman signal of the adsorbate molecules up to 10^8 times ***(25)***. NF-SERS spectra were collected by illuminating the sample using the nanoprobe and detecting the SERS signals using a spectrometer equipped with a charge-coupled device (CCD). Raster scanning the fiber probe over the sample and normalizing for surface topography using Rayleigh scattered light produced a two-dimensional (2D) SERS image of the DNA on the surface of the substrate with subwavelength spatial resolution. Near-field optical microscopy promises to be an area of growing research that could provide an imaging tool for monitoring individual cells and even biological molecules. Single-molecule detection and imaging schemes using nanofibers could open new possibilities in the investigation of the complex biochemical reactions and pathways in biological and cellular systems.

2.2. Biosensor Components

Over the years, new techniques in biosensing have set the stage for great advances in the field of biological research. The two fundamental operating principles of a biosensor are biological recognition and sensing. Therefore, a biosensor can be generally defined as a device that consists of two basic components connected in series: (1) a biological recognition element, often called a bioreceptor; and (2) a transducer ***(4,18)***. The biosensor detects molecules and transforms this recognition into another type of signal using a transducer. The interaction of the analyte with the bioreceptor is designed to produce an effect measured by the transducer, which converts the information into a measurable effect, such as an electrical signal. A bioreceptor is a biological molecular species (e.g., an antibody, an enzyme, a protein, or a nucleic acid) or a living biological system (e.g., cells, tissue, or whole organisms) that uses

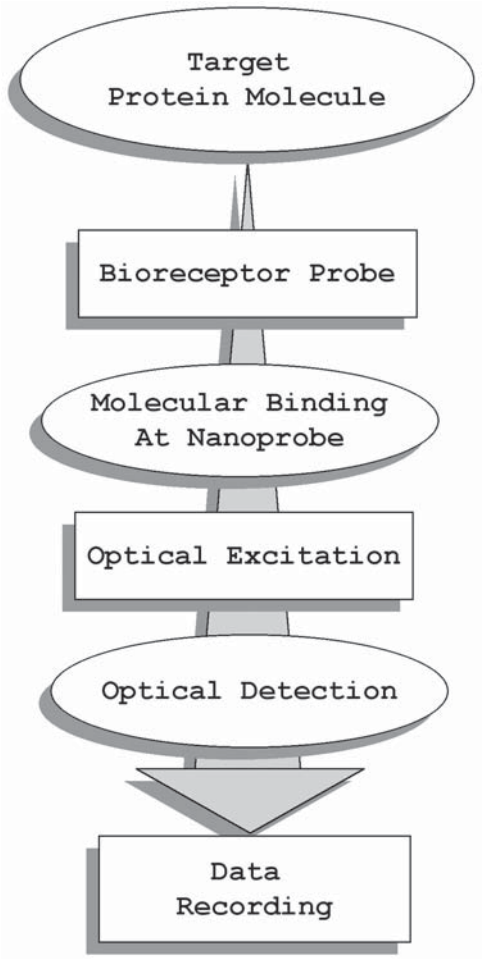

Fig. 1. Principle of biosensing system.

biochemical mechanisms for recognition. The main purpose of the recognition system is to provide the sensor with a high degree of selectivity for the analyte to be measured.

Several transduction methods are used in biosensors. The three main methods are based on optical detection, electrochemical detection, and mass-based detection. Other detection methods include voltaic and magnetic. New types of biosensor transducers are continually being developed. Each of the three main classes contains many different subclasses, creating a large number of possible transduction methods or combinations of methods. This chapter focuses on optical transduction methods. **Figure 1** illustrates the conceptual principle of

the biosensing process using a bioreceptor probe and an optical detection method.

Recent advances in nanotechnology leading to the development of submicron optical fibers have opened new horizons for intracellular measurements. Typically, the tip diameter of the optical fiber used in these sensors ranges between 20 and 100 nm. These sensors are based on the same basic principles as more conventional optical biosensors, except for the excitation process. Because the diameter of the optical fiber's tip is significantly less than the wavelength of light used for excitation of the analyte, photons cannot escape from the tip of the fiber to be absorbed by the species of interest, as is the case in larger fiberoptic sensors. Instead, in a fiberoptic nanosensor, after the photons have traveled as far down the fiber as possible, excitons or evanescent fields continue to travel through the remainder of the tip, providing excitation for the fluorescent species of interest present in the biosensing layer. An additional feature of evanescent excitation is that only species that are in extremely close proximity to the fiber's nanoprobe can be excited, thereby precluding the excitation of interfering fluorescent species elsewhere on the sample. Thus, any signal collected is from molecular species extremely close to the fiber (within the 100-nm near-field environment). This feature allows for an unprecedented level of localization.

2.3. Bioreceptors

The key to specificity with biosensor technologies lies in the bioreceptor molecules used. Bioreceptors are responsible for binding the analyte of interest to the sensor for measurement. Bioreceptors can take many forms and are as numerous as the different analytes that have been monitored using biosensors. However, bioreceptors can generally be classified into five major categories: antibodies/antigens, enzymes, nucleic acids/DNA, cellular structures/cells, and biomimetics. This chapter deals with biosensor systems using antibody probes, often called immunosensors, with optical detection. Another type of probe that involves an enzyme substrate is discussed in **Subheading 4.**

Immunological reactions involving the antigen-antibody binding reaction provide the basis for the specificity of immunoassays. Antibodies are complex biomolecules, made up of hundreds of individual amino acids arranged in a highly ordered sequence. Antibodies are produced by immune system cells when such cells are exposed to substances or molecules called antigens. The antibodies called forth following antigen exposure have recognition or binding sites for specific molecular structures (or substructures) of the antigen. The way in which an antigen and an antigen-specific antibody interact is analogous to a lock-and-key fit, in which specific configurations of a unique key enable it to open a lock. In the same way, an antigen-specific antibody fits its unique

antigen in a highly specific manner, so that the three-dimensional (3D) structures of antigen and antibody molecules are complementary. Owing to this 3D shape fitting, and the diversity inherent in individual antibody makeup, it is possible to find an antibody that can recognize and bind to any one of a large variety of molecular shapes.

This unique property of antibodies is the key to their usefulness in immunosensors; their ability to recognize molecular structures allows one to develop antibodies that bind specifically to chemicals, biomolecules, microorganism components, and so on. One can then use such antibodies as specific probes to recognize and bind to an analyte of interest that is present, even in extremely small amounts, within a large number of other chemical substances. Since the first development of a remote fiberoptics immunosensor for *in situ* detection of the chemical carcinogen benzo[*a*]pyrene (BaP) ***(2)***, antibodies have become commonly used as bioreceptors in biosensors.

3. Materials and Methods

3.1. Fabrication of Fiberoptics Nanoprobes

This section describes the protocols and instruments used in the fabrication of fiberoptics nanoprobes. Since fiberoptic nanoprobes are not commercially available, investigators must fabricate them in their own laboratories. Two methods are generally used for preparing the nanofiber tips. The so-called heat-and-pull method is the most commonly used. This method consists of local heating of a glass fiber using a laser or a filament and subsequently pulling the fiber apart. The shape of the nanofiber tips obtained depends on controllable experimental parameters such as the temperature and the timing of the procedure. The second method, often referred to as Turner's method, involves chemical etching of glass fibers. In a variation of the standard etching scheme, the taper is formed inside the polymer cladding of the glass fibers.

The experimental procedures for the fabrication of nanofibers using the heat-and-pull procedure, used by our laboratory, is schematically shown in **Fig. 2** ***(21)***. The heat-and-pull procedure consists of pulling a larger silica optical fiber to produce the tapered nanotip fiber using a special fiber-pulling device (Sutter Instruments P-2000). This method yields fibers with submicron diameters. One end of a 600-μm silica/silica fiber is polished to a 0.3-μm finish with an Ultratec fiber polisher. The other end of the optical fiber is then pulled to a submicron length using a fiber puller. A scanning electron microscopy photograph of one of the fiber probes fabricated for studies is shown in **Fig. 3**. The distal end of the nanofiber is approx 30 nm.

To prevent leakage of the excitation light on the tapered side of the fiber, the sidewall of the tapered end is then coated with a thin layer (100 nm thick) of

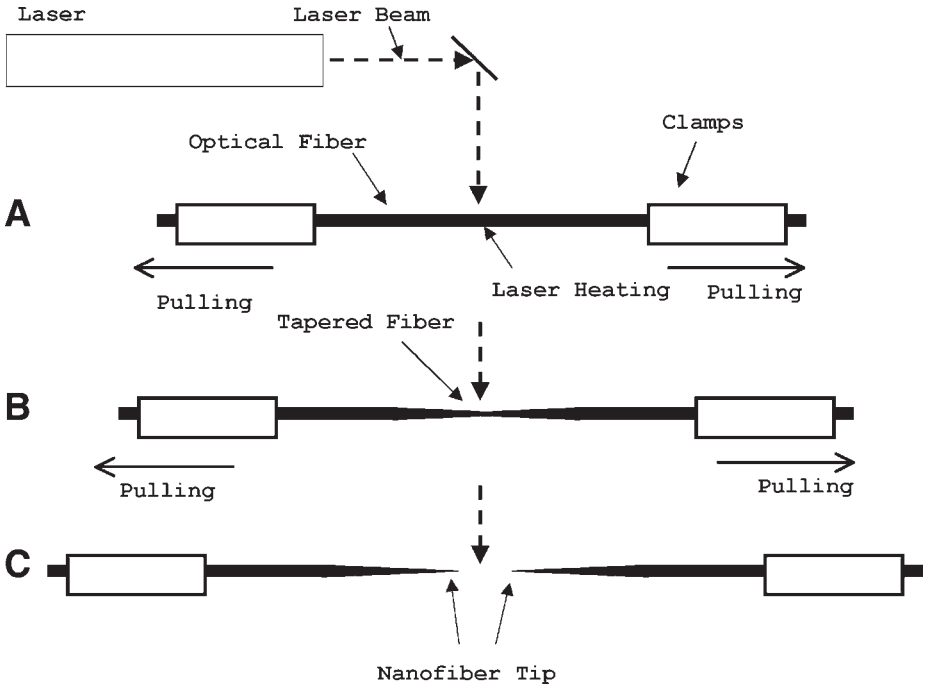

Fig. 2. Fabrication of nanofibers using heat-and-pull technique.

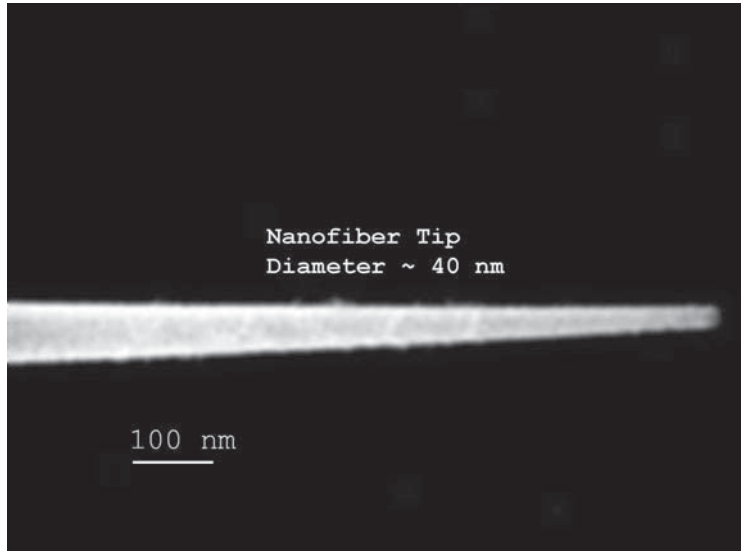

Fig. 3. Scanning electron micrograph of uncoated nanoprobe. The size of the fiber tip diameter is approx 40 nm.

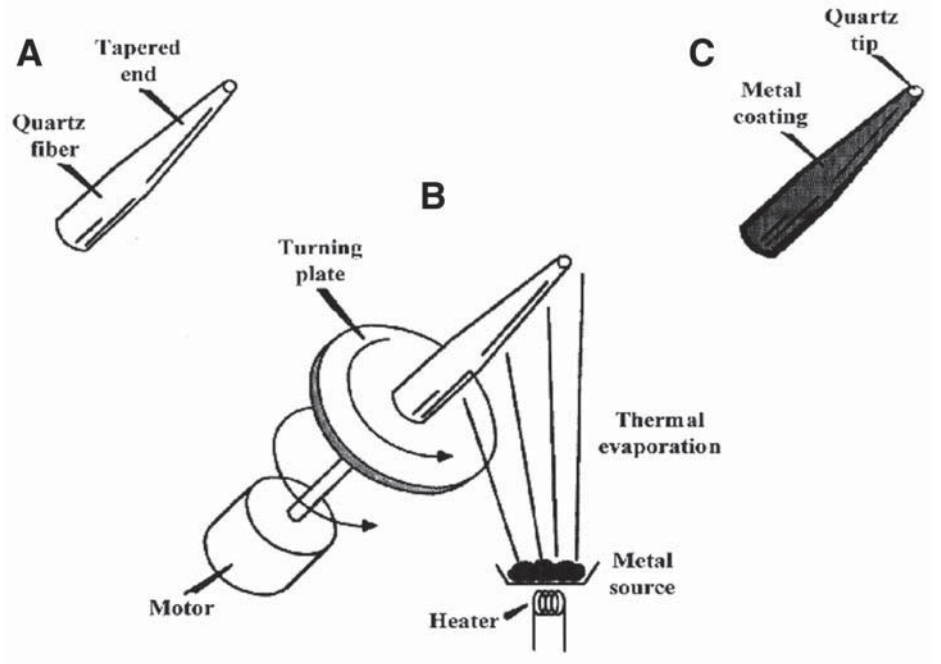

Fig. 4. Protocol used for coating nanofiber tip with silver: **(A)** uncoated fiber tip; **(B)** fiber coating using metal evaporation over rotating tip; **(C)** coated fiber. (From **ref. *21***.)

metal, such as silver, aluminum, or gold, using a thermal evaporation metal-coating device. The coating procedure is schematically illustrated in **Fig. 4** ***(21)***. The metal coating is only for the sidewall and leaves the distal end of the fiber free for subsequent binding with bioreceptors. The fiber probe is attached to a rotating plate inside a thermal evaporation chamber ***(4,19,21)***. The fiber axis and the evaporation direction form an angle of approx 45°. While the probe is rotated, the metal is allowed to evaporate onto the tapered side of the fiber tip to form a thin coating. The tapered end is coated with 300 to 400 nm of silver in a Cooke Vacuum Evaporator system using a thermal source at 10^{-6} torr. Since the fiber tip is pointed away from the metal source, it remains free from any metal coating. With the metal coating, the size of the probe tip is approx 250 to 300 nm (**Fig. 5**).

Another method for fabricating optical nanofibers involves chemical etching using HF. There are two variations of the HF etching method: one method involves the use of a mixture of HF acid and organic solvent, known as Turner etching ***(26)***; and the other uses only HF, known as tube etching ***(27–29)***. In the Turner method, a fiber is placed in the meniscus between the HF and the

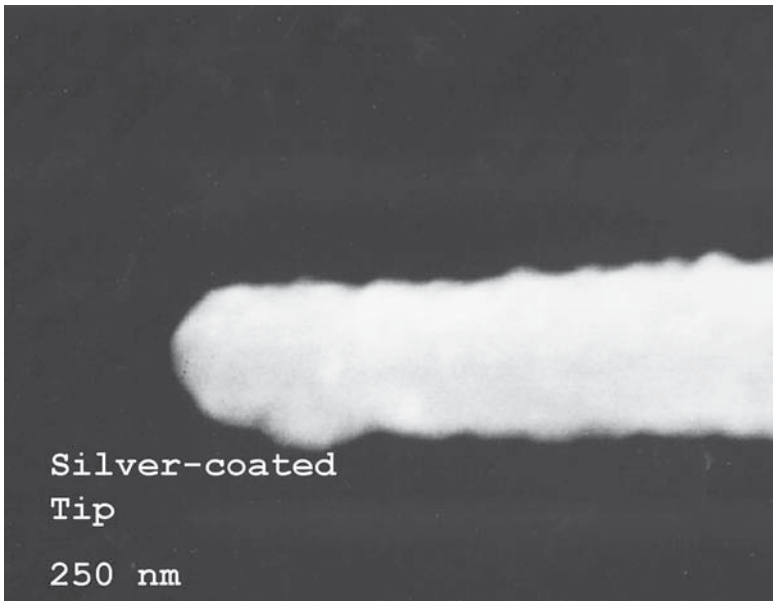

Fig. 5. Scanning electron micrograph of fiberoptics nanosensor with silver-coated sidewall.

organic overlayer; over time, a small tip with a smooth, large, angled taper is formed. This large taper angle provides much more light at the tip of the fiber, and the additional light, in turn, greatly increases the sensitivity of the nanosensors.

The reproducibility of the Turner method is strongly affected by environmental parameters such as temperature and vibration because of the dual chemical nature of the etching process. To avoid this problem, researchers developed a variation of the etching method involving tube etching. In this procedure, an optical fiber with a silica core and an organic cladding material is placed in an HF solution. The HF slowly dissolves the silica core, producing a fiber with a large taper angle and a nanometer-size tip. The HF begins first to dissolve the fiber's silica core, while not affecting the organic cladding material. This unaffected cladding creates localized convective currents in the HF solution, which cause a tip to be formed. After a period of time, more of the silica core is dissolved until the dissolved silica emerges above the surface of the HF solution. At this juncture, the HF is drawn up the cladding walls via capillary action and runs down the silica core to produce a nanometer-size tip. By varying the time of HF exposure and the depth to which the fiber is submerged in the HF solution, one can control the size of the fiber tip and the angle of the taper.

Once the tip has been formed, the protruding cladding can be removed either with a suitable organic solvent or by simply burning it off. Nanotips fabricated using etching procedures, which can be designed to have sharp tips, have been used in NSOM studies to detect SERS-labeled DNA molecules on solid substrates at subwavelength spatial resolution ***(12,13)***.

3.2. Immobilization of Bioreceptors Onto a Nanoprobe

The preparation of nanosensor probes involves covalent immobilization of receptors onto the nanofiber tip. For antibody binding, several strategies can be used to retain the antibody at the sensing probe. Whatever procedure is involved, one requirement is that the antibody retain its antigen-binding activity as much as possible. Perhaps the easiest procedure involves enclosure of the antibody in solution within a semipermeable membrane cap that fits over the end of the sensor ***(2)***. However, this design is more complicated and would increase the size of the tip of the nanosensor.

Antibodies can be immobilized onto nanofiber probes by using a chemical immobilization method. The fiber is derivatized in 10% GOPS in H_2O (v/v) at 90°C for 3 h. The pH of the mixture is maintained below 3.0 with concentrated HCl (1 *M*). After derivatization, the fiber is washed in ethanol and dried overnight in a vacuum oven at 105°C. The fiber is then coated with silver as described previously. The derivatized fiber is activated in a solution of 100 mg/mL of 1,1' carbonyldiimidazole in acetonitrile for 20 min, followed by rinsing with acetonitrile and then phosphate-buffered saline (PBS). The fiber tip is then incubated in a 1.2 mg/mL antibody solution (PBS solvent) for 4 d at 4°C and stored overnight in PBS to hydrolyze any unreacted sites. The fibers are stored at 4°C with the antibody-immobilized tips stored in PBS. This procedure has been shown to maintain >95% antibody activity ***(21)***.

3.3. Experimental Protocol

This section provides a description of the protocols used for growing cell cultures for analysis using the nanosensors. Cell cultures were grown in a water-jacketed cell culture incubator at 37°C in an atmosphere of 5% CO_2 in air. Clone 9 cells, a rat liver epithelial cell line, were grown in Ham's F-12 medium (Gibco) supplemented with 10% fetal bovine serum and an additional 1 m*M* glutamine (Gibco). In preparation for an experiment, 1×10^5 cells in 5 mL of medium were seeded into standard dishes (Corning Costar). Growth of the cells was monitored daily by microscopic observation. When the cells reached a state of confluence of 50 to 60%, the analyte solution was added and left in contact with the cells for 18 h (i.e., overnight). This procedure is designed to incubate the cells with the analyte molecules for subsequent monitoring using the nanosensors.

The growth conditions were chosen so that the cells would be in log phase growth during the chemical treatment but would not be so close to confluence that a confluent monolayer would form by the termination of the chemical exposure. The analyte solution was prepared as a 1 m*M* stock solution in reagent-grade methanol and further diluted in reagent-grade ethanol (95%) prior to addition to the cells. Following chemical treatment, the medium containing the analyte was aspirated and replaced with standard growth medium prior to the nanoprobe procedure.

Monitoring target analyte molecules in single cells was then performed using antibody nanoprobes as follows: a culture dish of cells was placed on a prewarmed microscope stage, and the nanoprobe, mounted on a micropipet holder, was moved into position (i.e., in the same plane as the cells), using bright-field microscopic illumination, so that the tip was outside the cell to be probed. The total magnification was usually ×400. Under no room light and no microscopic illumination, the laser shutter was opened to illuminate the optical fiber for excitation of the analyte molecules bound on the antibodies at the fiber tip. Usually, if the silver coating on the nanoprobe was appropriate, no light leaked out of the sidewall of the tapered fiber. Only a faint glow of laser excitation at the tip could be observed on the nanoprobe. A reading was first taken with the nanoprobe outside the cell and the laser shutter closed. The nanoprobe was then moved into the cell, inside the cell membrane and extending into the cellular compartments of interest. The laser was again opened, and readings were taken and recorded as a function of the time during which the nanoprobe was inside the cell.

3.4. Optical Detection Instrument

The optical detection system used for monitoring single cells with the nanosensor is schematically illustrated in **Fig. 6** ***(18–21)***. Laser excitation light (either the 325-nm line of an HeCd laser [Omnichrome; 8-mW laser power] or the 488-nm line of an argon ion laser [Coherent; 10 mW]) was focused onto a 600-µm delivery fiber, which was connected to the nanofiber through an SMA connector. The nanofiber was secured to a micromanipulator on a microscope. The experimental setup used to probe single cells was adapted to this purpose from a standard micromanipulation/microinjection apparatus. A Nikon Diaphot 300 inverted microscope with a Diaphot 300/Diaphot 200 Incubator to maintain the cell cultures at approx 37°C on the microscope stage was used for these experiments. The micromanipulation equipment used consisted of MN-2 Narishige 3D manipulators for coarse adjustment, and Narishige MMW-23 3D hydraulic micromanipulators for final movements. The optical fiber nanoprobe was mounted on a micropipet holder (World Precision Instruments). The fluorescence emitted from the cells was collected by the microscope objective and

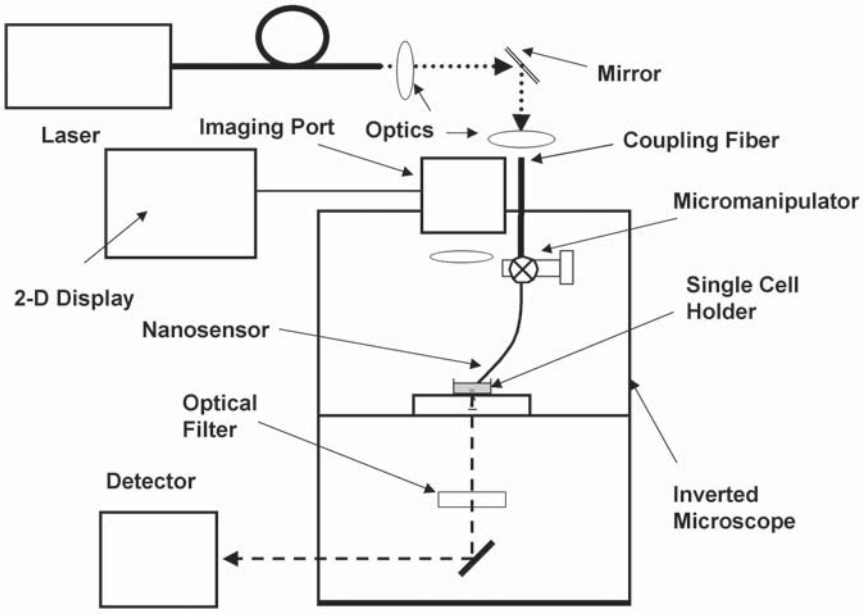

Fig. 6. Instrument using nanosensors for fluorescence measurements of single cells.

passed through an appropriate long-pass dichroic mirror to eliminate the laser excitation scatter light. The fluorescence beam was then focused onto a photomultiplier tube (PMT) for detection. The output from the PMT was passed through a picoammeter and recorded on a strip chart recorder or a personal computer for further data treatment. To record the fluorescence of analyte molecules binding to antibodies at the fiber tip, a Hamamatsu PMT detector assembly (HC125-2) was mounted in the front port of the Diaphot 300 microscope, and fluorescence was collected via this optical path (80% of available light at the focal plane can be collected through the front port). A CCD mounted onto another port of the microscope could be used to record images of the nanosensor monitoring single cells.

4. Applications

4.1. Monitoring Biomarkers in Single Living Cells

The nanoscale size of this new class of sensors allows for measurements in the smallest of environments. One such environment that has evoked a great deal of interest is that of individual cells. Using these nanosensors, it has become possible to probe individual chemical species in specific locations throughout a cell. Previously, such measurements could be performed only by

fluorescence microscopy, in which a fluorescent dye was inserted into a cell and allowed to diffuse throughout the cell. Depending on the fluorescent dye that was chosen, changes in the fluorescence properties of the dye could then be monitored, in an imaging modality, as the dye came in contact with the analyte of interest. Since this technique relies on imaging the fluorescent dye, it requires the homogeneous dispersion of the dye through the various locations in the cell, which is limited by intracellular conditions (e.g., pH) or often does not even occur owing to compartmentalization by the cell. Fiberoptic nanosensors, therefore, could offer significant improvements over such methods and eliminate the problems associated with cellular diffusion.

Nanosensors with antibody-based probes to measure specific fluorescent targets inside a single cell have been demonstrated ***(18–23)***. Because cells are very small (1–10 μm), the success of intracellular investigations depends on several factors, including the sensitivity of the measurement system, the selectivity of the probe, and the small size of the nanofiber probes. Vo-Dinh et al. ***(21)*** reported the smallest cells to be nondestructively probed with a fiberoptic nanobiosensor. In their work, the antibody probe was targeted for benzo[*a*]-pyrene tetrol (BPT), an important biological compound, which was used as a biomarker of human exposure to the carcinogen BaP, a polycyclic aromatic hydrocarbon of great environmental and toxicological interest because of its mutagenic/carcinogenic properties and its ubiquitous presence in the environment. BaP has been identified as a chemical carcinogen in laboratory animal studies. The small size of the probe allowed manipulation of the nanosensor at specific locations within the cells. Before measurements, the cells were incubated with BPT using the experimental procedures described previously. Interrogation of single cells for the presence of BPT was then carried out using antibody nanoprobes for excitation and a photometric system for fluorescence signal detection.

Nanosensors for BPT were used to measure the intracellular concentrations of BPT in the cytoplasm of two different cell lines: human mammary carcinoma cells and rat liver epithelial cells (Clone 9). The rat liver epithelial cells were used as the model cell system after treatment of the culturing media with an excess of BPT. **Figure 7** shows a digital image of the nanosensor actually being inserted into a single human mammary carcinoma cell. The results demonstrated the possibility of *in situ* measurements of BPT inside a single cell.

Note that these nanosensors were equipped with single-use bioprobes because the probes were used to obtain only one measurement at a specific time and could not be reused owing to the strong association constant of the antibody-antigen binding process. Antibody probes can be regenerated, however, using ultrasound methods. Our laboratory has successfully developed a method using ultrasound (US) to noninvasively release antigen molecules from

Fig. 7. Monitoring of single human mammary carcinoma cell using fiberoptic nanosensor.

the antibodies and therefore regenerate antibody-based biosensors ***(30)***. The results of measurements with an antibody probe for the breast cancer antigen illustrate the effectiveness and the potential of the regenerable immunosensor. In this instance, US regeneration attained a 65% removal of the antigens bound to the monoclonal antibodies immobilized on the fiber surface. The US regeneration scheme is a nondestructive approach that has a great potential for application to nanosensors. The results demonstrate the effectiveness of this US-based approach in releasing the antigen from the antibody probe.

We have performed multiple calibration measurements of solutions containing different BPT concentrations to obtain quantitative estimates of the number of BPT molecules detected. Up to five recordings of the fluorescence signals could be taken with each measurement using a specific nanoprobe. For these calibration measurements, the fibers were placed in Petri dishes containing solutions of BPT with concentrations ranging from 1.56×10^{-10} to 1.56×10^{-8} *M*. By plotting the increase in fluorescence from one concentration to the next vs the concentration of BPT, and fitting these data with an exponential function in order to simulate a saturated condition, we determined a concentration of $(9.6 \pm 0.2) \times 10^{-11}$ *M* for BPT in the individual cell investigated ***(17–21)***.

Nanosensors were also developed for *in situ* measurements of the carcinogen BaP ***(30)***. Detection of BaP transport inside single cells is of great biomedical interest, because it can serve as a means for monitoring BaP exposure,

which can lead to DNA damage ***(28)***. To perform these measurements, it was necessary to use antibodies targeted to BaP. The fluorescent BaP molecules were bound by interaction with the immobilized antibody receptor, forming a receptor-ligand complex. Following laser excitation of this complex, a fluorescence response from BaP provided a basis for the quantification of BaP concentration in the cell being monitored. The fluorescence signal generated allows for a high sensitivity of detection. The intracellular measurements of BaP depend on the reaction times involved. The reaction time established in this study for antibody-BaP complexing was 5 min. This was used as a standard time to enable calibration from fiber to fiber. In addition, the nanosensors were calibrated through standard analytical procedures using measurements of known concentration of reference solutions.

4.2. Detection of Caspase Proteins Signaling Apoptosis in Single Living Cells

Over the last few years, there has been increasing interest in developing instruments and techniques for monitoring the onset of apoptosis in living cells. It has become increasingly apparent that the mitochondria play a major role in the apoptosis process. The key mitochondrial components regulating apoptosis exert their effects at two control points—the permeability transition pore complex (PTPC) situated at points where the inner and outer mitochondrial membranes come into close proximity, and the apoptosome located just outside the mitochondria. As shown in **Fig. 8**, anticancer drugs often induce apoptosis in cells, acting on the PTPC to induce the release of proapoptotic factors from the mitochondria. These factors stimulate the assembly of the apoptosome and the subsequent activation of the initiator caspase-9 and effector caspase-3, leading to apoptosis. The apoptosome is a major control point where caspase-9 and caspase-3 interact and activate one another. Caspase-9 processes caspase-3 at the apoptosome, thereby activating it. A feedback loop, in which processed caspase-3 cleaves and activates caspase-9, amplifies the production of activated caspases. A second positive feedback loop links the apoptosome and PTPC. Caspase-3 cleaves a number of components of the mitochondrial electron transport chain. These operations trigger the stepped-up production of reactive oxygen species (ROS) and efflux of cytochrome-c, which then acts at the apoptosome to amplify the amount of activated caspase-9 and caspase-3.

We have performed measurements to investigate the application and utility of nanosensors for monitoring the onset of the mitochondrial pathway of apoptosis in a single living cell by detecting enzymatic activities of caspase-9 ***(24)***. Minimally invasive analysis of single live MCF-7 cells for caspase-9 activity was demonstrated using the optical nanosensor, which employed a modification of an immunochemical assay format for the immobilization of

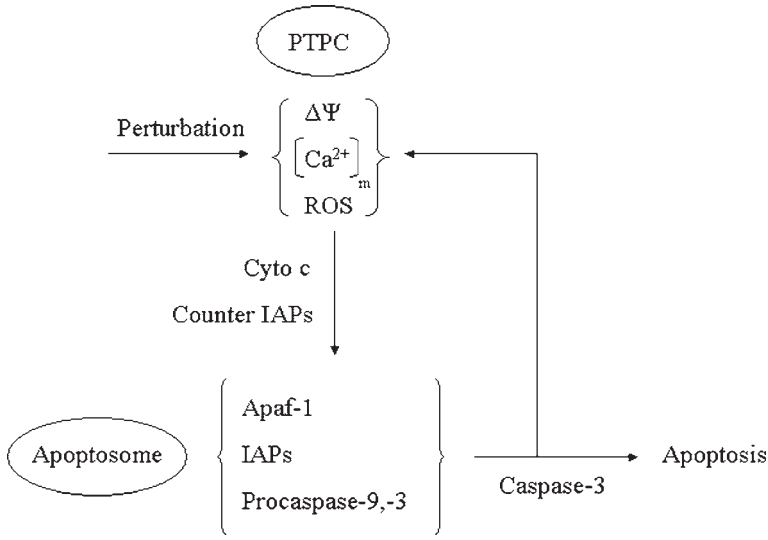

Fig. 8. Schematic diagram of mitochondrial apoptosis control unit.

the nonfluorescent enzyme substrate leucine-glutamic acid-histidine-aspartic acid-7-amino-4-methyl coumarin (LEHD-AMC). LEHD-AMC covalently attached to the tip of an optical nanosensor was cleaved during apoptosis by caspase-9, generating free AMC. An evanescent field was used to excite cleaved AMC, and the resulting fluorescence signal was detected. By quantitatively monitoring the changes in fluorescence signals, we detected caspase-9 activity within a single living MCF-7 cell. Photodynamic therapy protocols employing δ-aminolevulinic acid (ALA) were used to induce apoptosis ***(32)*** in MCF-7 cells. The substrate LEHD-AMC was cleaved by caspase-9, and the released AMC molecules were excited and emitted a fluorescence signal. By comparing the fluorescence from an apoptotic cell and an uninduced control, we detected and identified caspase-9 activity.

The results show that for the treated control group of cells, the fluorescence signal relative to the experimental group was insignificant. The fluorescence signals obtained from the cells that were both incubated with ALA and photoactivated were much higher than the signal obtained from both control groups. The presence and detection of cleaved AMC in single live MCF-7 cells as a result of these experiments is representative of caspase-9 activity and a hallmark of apoptosis. These results indicate that AMC, and hence apoptosis, can be monitored and measured using optical nanosensors within single living cells. These studies show the possibility of studying cells without having to disrupt the physiological makeup and, in the process, interfere with cellular biochemistry.

5. Conclusion

The nanosensor technologies described in this chapter belong to a new generation of nanotools that could dramatically change our fundamental understanding of the life process itself. Dynamic information on signaling processes inside living cells is important to a fundamental understanding of cellular processes. Many traditional microscopy techniques involve incubating cells with fluorescent dyes or nanoparticles and examining the interaction of these dyes with compounds of interest. However, when a dye or nanoparticle is incubated into a cell, it is transported to intracellular sites that may or may not be where it is most likely to stay and not to areas that the investigator would like to monitor. The fluorescence signals, which are supposed to reflect the interaction of the dyes with chemicals of interest, are generally directly related to dye concentration as opposed to analyte concentration. Only with optical nanosensors can excitation light be delivered to specific locations inside cells. To date, the nanosensor is the only technology that can be used to measure biotargets in a living cell without affecting cell viability. Combined with the exquisite molecular recognition of antibody probes, nanosensors have great potential to serve as powerful tools for exploring biomolecular processes in the subcompartments of living cells.

Nanosensors could ultimately lead to the development of new modalities for early diagnostics and medical treatment and prevention beyond the cellular level to that of individual organelles and even DNA, the building block of life. Today, research in biomedical science and engineering at the molecular level is growing exponentially because of the availability of these new investigative nanotools. These new analytical tools are capable of probing the nanometer world and will make it possible to characterize the chemical and mechanical properties of cells, probe the working of molecular protein machines, and discover novel phenomena and processes and also will provide science with a wide range of tools, materials, devices, and systems with unique capabilities. The marriage of genomics, electronics, biomaterials, and photonics is expected to revolutionize many areas of medicine in the twenty-first century. This ultimate technology convergence will open new horizons for the discovery of new tools and biomarkers for detection, diagnosis, and prevention studies, and new targets for therapeutic development.

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