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Nanobiosensors: Probing the Sanctuary of Individual Living Cells

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Abstract Recently, nanotechnology has been revolutionizing important areas in molecular biology, especially diagnostics and therapy at the molecular and cellular level. The combination of nanotechnology, biology, and photonics opens the possibility of detecting and manipulating atoms and molecules using nanodevices, which have the potential for a wide variety of medical uses at the cellular level. The nanoprobes were fabricated with optical fibers pulled down to tips with distal ends having sizes of approximately 30–50 nm. 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 nanobiosensors, it has become possible to probe individual chemical species in specific locations throughout a cell. This article provides an overview of the principle, development, and applications of optical nanosensor systems for in vivo bioanalysis at the single-cell level. The fiberoptics nanoprobes were covalently bound with antibodies that are selective to target analyte molecules. Excitation 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. The fluorescence emission from the analyte molecules is then collected via a microscope. The usefulness and potential of this nanotechnology-based biosensor systems in biological research and applications in single-cell analysis are discussed. J. Cell. Biochem. Suppl. 39: 154–161, 2002. © 2002 Wiley-Liss, Inc.

Key words: nanosensor; nanotechnology; biosensor; antibody; benzo[a]pyrene; cancer

Advances in nanotechnology have recently led to the development of fiberoptics-based nanosensor systems having nanoscale dimensions suitable for intracellular measurements. The possibilities to monitor in vivo processes within living cells could dramatically improve our understanding of cellular function, thereby revolutionizing cell biology. Fiberoptic sensors provide significant advantages for in situ monitoring applications due to the optical nature of the excitation and detection modalities. Fiberoptics sensors are not affected by electromagnetic interferences from static electricity, strong magnetic fields, or surface potentials. Another advantage of fiberoptic sensors is the small size of optical fibers, which allow sensing intracellular/intercellular physiological and biological parameters in microenvironments. Biosensors, which use biological probes coupled to a transducer, have been developed during the last two decades for environmental, industrial, and biomedical diagnostics. Extensive research and development activities in our laboratory have been devoted to the development of a variety of fiberoptic chemical sensors, biosensors [Vo-Dinh et al., 1987, 1991, 1993]. The application of a submicron fiberoptic chemical sensor has been reported [Betzig et al., 1991; Betzig and Chichester, 1993]. Submicron tapered optical fibers with distal diameters between 20 and 500 nm have been employed to study the submicron spatial resolution achievable using near-field scanning optical microscopy (NSOM). The combination of NSOM and surface-enhanced Raman scattering (SERS) has been demonstrated to detect chemicals on solid substrates

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with sub-wavelength 100-nm spatial resolution [Deckert et al., 1998; Zeisel et al., 1998]. Submicron optical fiber probes have been developed for chemical analyses [Tan et al., 1992a,b]. Nanosensors with bioreceptors such as antibodies have been developed and used to detect biochemical targets inside single cells [Cullum and Vo-Dinh, 2000; Cullum et al., 2000; Vo-Dinh and Cullum, 2000; Vo-Dinh et al., 2000a,b]. In this article, we provide an overview of the principle, development, and applications of fiberoptics nanosensor systems using antibody-based probes. The article provides background information on biosensors, a description of the fabrication methods for fiberoptics nanosensors and detection systems, and applications in single-cell analysis.

DEVELOPMENT OF NANOBIOSENSORS

A biosensor is generally defined as a measurement system that consists of a probe with biological recognition element, often called a bioreceptor, and 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, for example, an electrical signal. Bioreceptors are used because they are important elements to specificity for biosensor technologies. They allow binding the specific analyte of interest to the sensor for the measurement with minimum interference from other components in complex mixtures. 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 utilizes a biochemical mechanism for recognition. The sampling component of a biosensor contains a bio-sensitive layer that can either contain bioreceptors or be made of bioreceptors covalently attached to the transducer. Various techniques (optical, electrochemical, and mass-sensitive) can be used for detection in biosensors. In the 1980s, advances in spectrochemical instrumentation, laser miniaturization, biotechnology, and fiberoptics research have provided opportunities for novel approaches to the development of sensors for the detection of chemicals and biological materials of environmental and biomedical interest. Since the first development of a remote fiber-optics immunosensor for in situ detection of the chemical carcinogen benzo[a]pyrene (BaP) [Vo-Dinh et al., 1987], antibodies have become common bioreceptors used in biosensors today.

A nanobiosensor (also called nanosensor) is a biosensor that has dimensions on the nanometer-size scale. Typical tip diameters of the fiberoptics used in these sensors range 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, the photons cannot escape from the tip of the fiber to be absorbed by the species of interest, as is the case in larger fiber optic sensors. Instead, in a fiber-optic 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 tip (i.e., antigens bound to the antibody probes) can be excited, thereby precluding the excitation of interfering fluorescent species within other locations of the sample.

The fabrication of near-field submicron optical probes is a crucial prerequisite for the development of nanosensors. There are two methods for preparing the nanofiber tips. The most frequently used technique is the so-called "heat and pull" method. It is based on local heating of a glass fiber using a laser or a filament and subsequently pulling the fiber apart. The resulting tip shapes depend strongly on the temperature and the timing of the procedure. The second method is based on chemical etching of glass fibers [Hoffmann et al., 1995]. Recently, a variation of the standard etching scheme was proposed, where the taper is formed inside the polymer cladding of the glass fibers [Lambelet et al., 1998].

Figure 1 illustrates the experimental steps involved in the fabrication of nanosensors using the heat and pull method [Vo-Dinh et al., 2000a]. Fabrication of nanosensors involved techniques capable of making optical fibers with submicron-size diameter core. Since these nanoprobes are not commercially available, they have to be fabricated in the laboratory. One procedure consists of pulling from a larger silica optical fiber using a special fiber-pulling device (Sutter Instruments P-2000). This

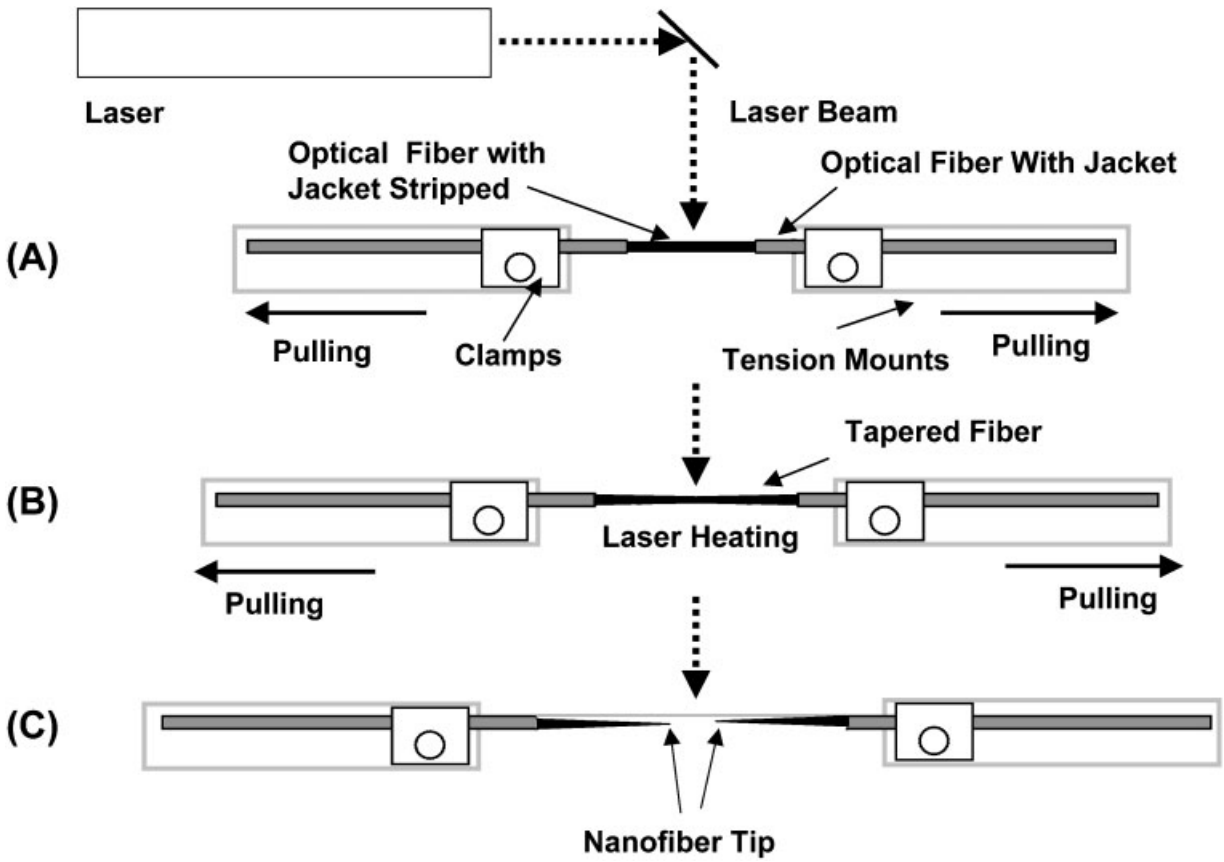

Fig. 1. Fabrication of nanofibers. Using the ''heat and pull'' method.

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. Figure 2 shows a scanning election microscopy photograph of one of the fiber probes fabricated for our preliminary studies. The scale on the photograph of this sample indicates that the distal end of the fiber is approximately 40 nm.

The side wall of the tapered end is then coated with a thin layer of silver, aluminum, or gold (100–300 nm) to prevent light leakage of the excitation light on the tapered side of the fiber. The coating procedure is designed to leave the distal end of the fiber free for subsequent binding with bioreceptors. The fiber probe is attached on a rotating plate inside a thermal evaporation chamber. The fiber axis and the evaporation direction formed an angle of approximately 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. Since the fiber tip is pointed away from the metal source, it remains free from any metal coating. The tapered end is coated with 300–400 nm of silver in a Cooke Vacuum Evaporator system using a thermal source at 10^{-6} Torr. With the metal coating, the size of the probe tip is approximately 250–300 nm.

The next step in the preparation of the biosensor probes involves covalent immobilization of receptors onto the fiber tip. Antibodies can be immobilized onto the fiberoptics probes by using a method previously described. Briefly, the fiber is derivatized in 10% glycidoxypropyltrimethylsilane (GOPS) in H_2O (v/v) at 90°C for 3 h. The pH of the mixture is maintained below 3 with concentrated HCl (1 M). After derivatization, the fiber was 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 1,1′ carbonyldiimidazole (CDI) in acetonitrile for 20 min followed by rinsing with acetonitrile and then phosphate buffered saline (PBS). The fiber tip is then incubated in a

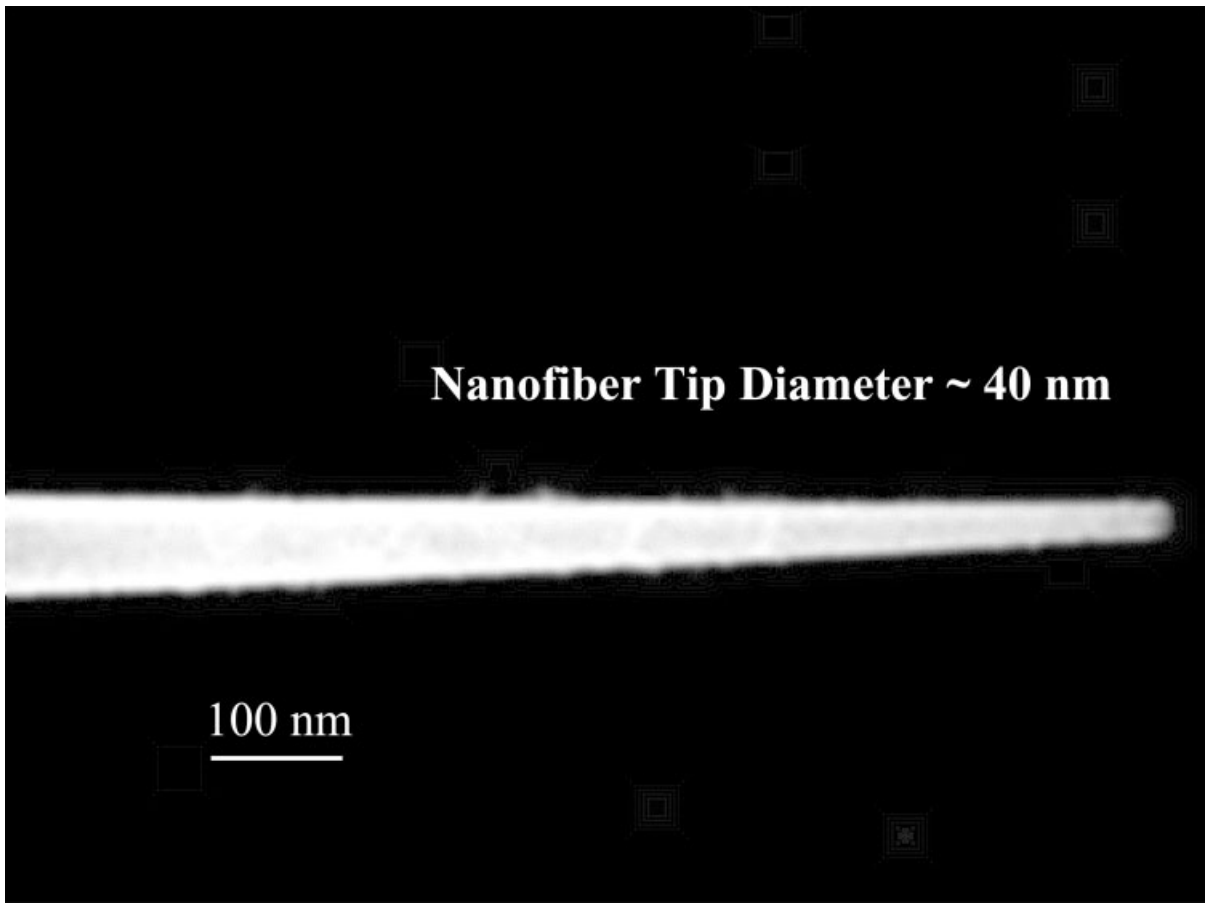

Fig. 2. Scanning electron photograph of an optical nanofiber. The size of the fiber tip diameter is approximately 40 nm.

1.2 mg/ml anti-benzopyrene tetrol (BPT) solution (PBS solvent) for 4 days at 4°C and then stored overnight in PBS to hydrolyze any unreacted sites. The fibers are then stored at 4°C with the antibody immobilized tips stored in PBS. This procedure has been shown to maintain over 95% antibody activity for benzopyrene tetrol (BPT) [Vo-Dinh et al., 2000a].

MONITORING SUBCOMPARTMENTS OF LIVING CELLS

A unique application of nanosensor involves monitoring BPT in single cells in vivo [Cullum and Vo-Dinh, 2000; Cullum et al., 2000; Vo-Dinh and Cullum, 2000; Vo-Dinh et al., 2000a,b]. The antibody probe was targeted against BPT, an important biological compound, which was used as a biomarker of human exposure to the carcinogen BaP, a polycyclic aromatic hydrocarbon (PAH) 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 [Vo-Dinh, 1989]. 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/BRL, Grand Island, NY) supplemented with 10% fetal bovine serum and an additional 1 mM glutamine (Gibco). In preparation for an experiment, 1×10^5 cells in 5 ml of medium were seeded into 60-mm diameter dishes (Corning Costar Corp., Corning, NY). The growth of the cells was monitored daily by microscopic observation, and when the cells reached a state of confluence of 50–60%, BPT was added and left in contact with the cells for 18 h (i.e., overnight). 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. Benzopyrene tetrol was prepared as a 1-mM stock solution in reagent grade methanol and further diluted in reagent grade ethanol (95%) prior to addition to the cells. The final concentration of BPT in the culture medium of the dish was 1×10^{-7} M and the final alcohol concentration (combination of methanol and ethanol) was 0.1%. Following chemical treatment, the medium containing BPT was aspirated and replaced with standard growth medium, prior to the nanoprobe procedure.

Monitoring BPT in single cells using the nanosensor was carried out in the following way. A culture dish of cells was placed on the pre-warmed microscope stage, and the nanoprobe, mounted on the micropipette holder, was moved into position (i.e., in the same plane of the cells), using bright field microscopic illumination, so that the tip was outside the cell to be probed. The total magnification was usually 400 ×. All room light and microscope illumination light were extinguished, the laser shutter opened, and laser light allowed to illuminate the optical fiber and excitation light transmitted into 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 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 a short way into the cytoplasm, but care was taken not to penetrate the nuclear envelope. The laser was again opened, and readings were then taken and recorded as a function of time during which the nanoprobe was inside the cell.

Figure 3 shows a schematic diagram of the optical measurement system used for monitoring single cells using the nanosensors. The 325-nm line of a 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 terminated with a SMA connector. The antibody immobilized tapered fiber was coupled to the delivery

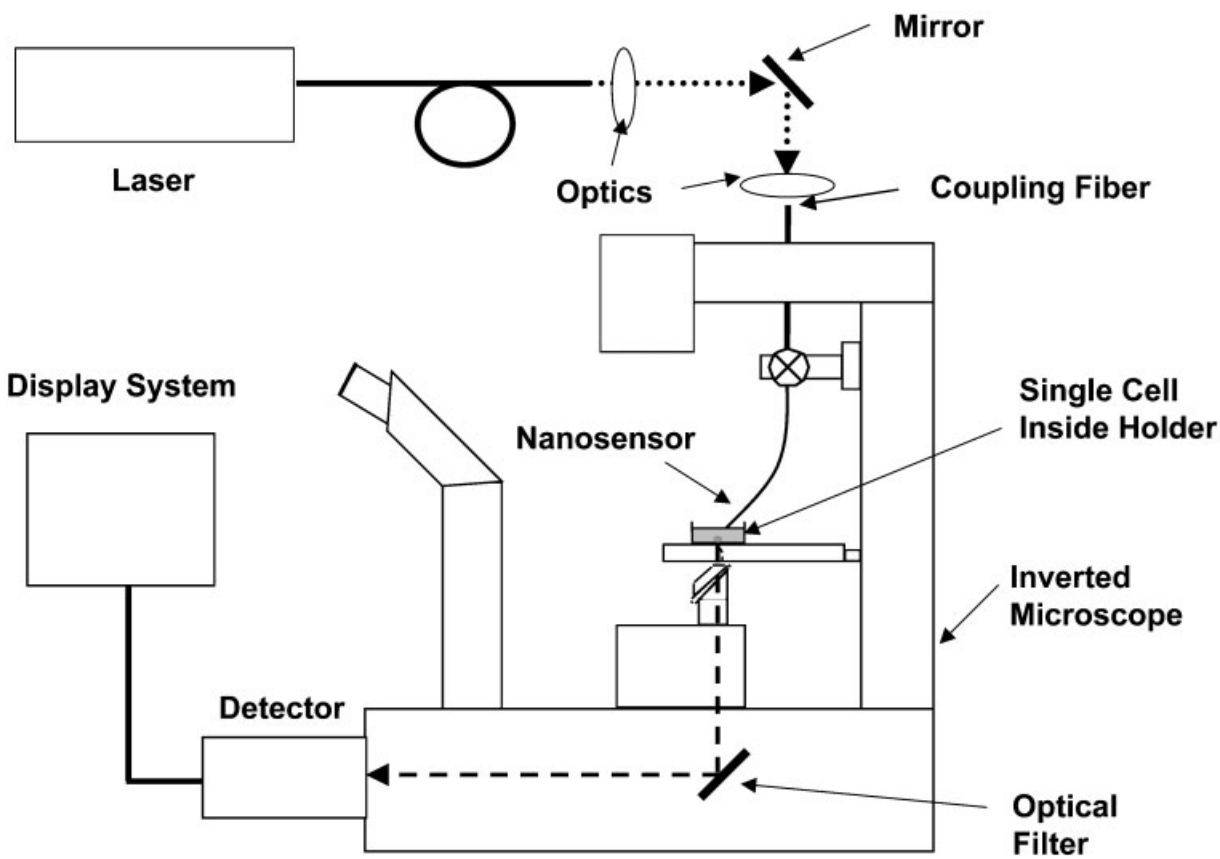

Fig. 3. Instrumental system for fluorescence measurements of single cells using nanosensors.

fiber through the SMA connector and was secured to the micromanipulators on the microscope. The fluorescence emitted from the cells was collected by the microscope objective and passed through a 400-nm longpass dichroic mirror and 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 (PC) for further data treatment. 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 (Nikon, Inc., Melville, NY) with Diaphot 300/Diaphot 200 Incubator, to maintain the cell cultures at approximately 37°C on the microscope stage, was used for these experiments. The micromanipulation equipment used consisted of MN-2 (Narishige Co., Ltd., Tokyo, Japan) Narishige three-dimensional manipulators for coarse adjustment, and Narishige MMW-23 three-dimensional hydraulic micromanipulators for final movements. The optical fiber nanoprobe was mounted on a micropipette holder (World Precision Instruments, Inc., Sarasota, FL). To record the fluorescence of BPT 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).

Since cells have very small sizes (1–10 μm) the success of intracellular investigations depends largely not only on the sensitivity of the measurement system, but also on the selectivity of the probe and also on the small size of the probes. The measurements were performed on rat liver epithelial cells (Clone 9) used as the model cell system. The cells had been previously incubated with BPT molecules prior to measurements. The results demonstrated the possibility of in situ measurements of BPT inside a single cell. Figure 4 shows a photograph of an antibody-based nanosensor inside a single cell. The small size of the probe allowed manipulation of the nanosensor at specific locations within the cells.

The nanoprobes in our studies were single-use bioprobes because they were used to obtain only one measurement at a specific time and could not be reused due to the strong association constant of the antibody–antigen binding process. Multiple (e.g., five) recordings of the fluorescence signals could be taken with each measurement using a specific nanoprobe. We have made a series of calibration measurements of solutions containing different BPT concentrations in order to obtain a quantitative estimation of the amount of BPT molecules detected. 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 versus the concentration of BPT, and fitting these data with an exponential function in order to simulate a saturated condition, a concentration of $(9.6 \pm 0.2) \times 10^{-11}$ M has been determined for BPT in the individual cell investigated [Cullum et al., 2000].

The introduction of nanoprobes into cells could raise questions about the effect of such treatment upon cell viability. The use of glass micropipets to introduce dyes or carry out gene transfer experiments has been shown to have minimal effects upon cell viability, provided that the attendant procedures do not produce too much trauma. Similarly, we have found that the introduction of a nanoprobe into the cell cytoplasm does not seem to affect cellular function/viability. This was empirically established in several experiments, where, after nanoprobe penetration and equilibration for 5 min, the probe was withdrawn, and the cells thus penetrated were found to subsequently undergo mitosis, indicating at least that one round of cell division could take place, following the probing event. The studies demonstrated that the nanosensors are minimally invasive tools appropriate for monitoring biomolecular processes inside sub-compartments of living cells.

CONCLUSION

Today, the interest for research in cellular biology at the single-cell level is rapidly growing because of the availability of 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, discover novel phenomena and processes, and provide science with a wide range of tools, materials,

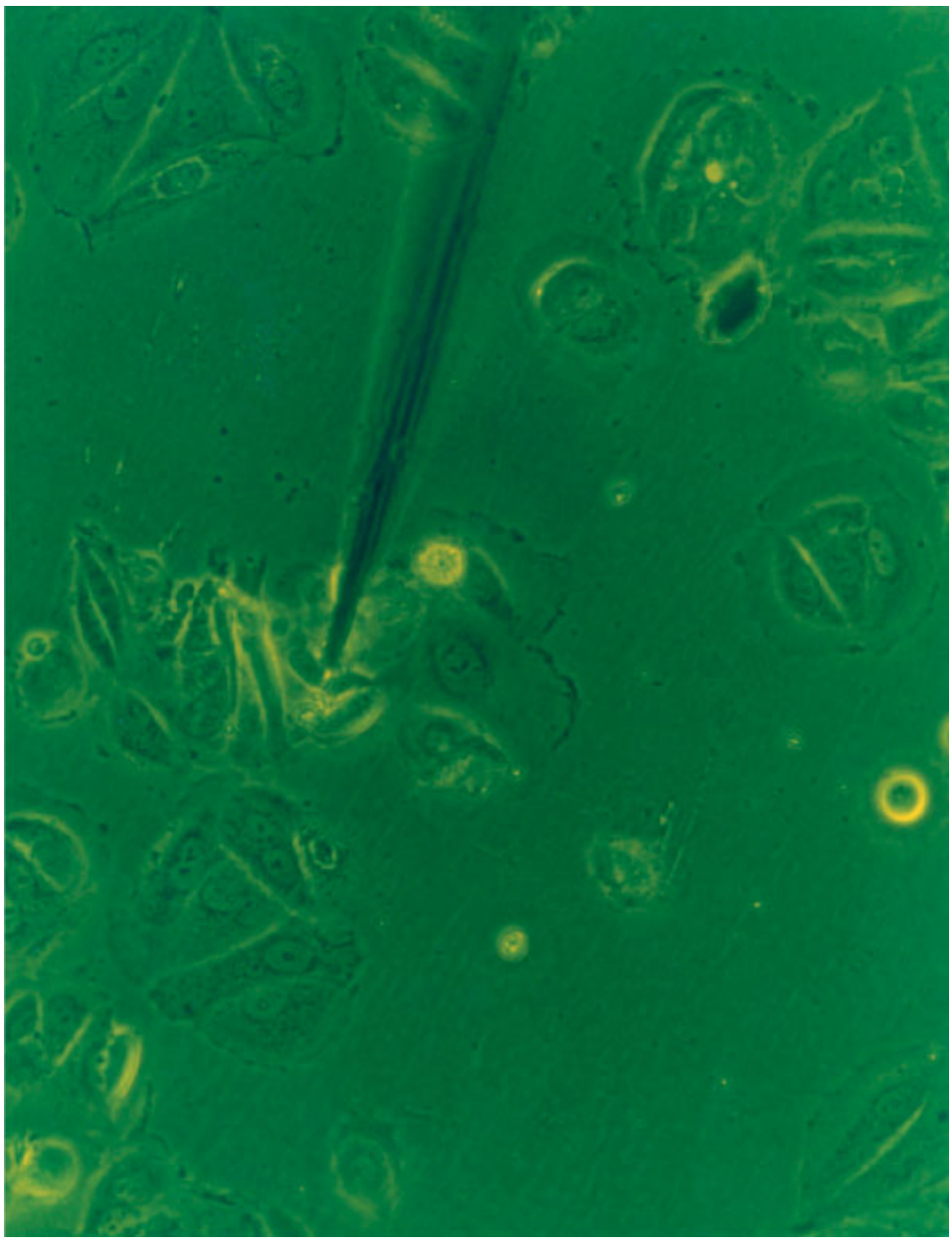

Fig. 4. Photograph of single-cell sensing using the nanosensor system [adapted from Vo-Dinh et al., 2000a].

devices, and systems with unique characteristics. Concrete knowledge of subcellular architecture and subcellular dynamic processes is important to fundamental biological understanding of cellular processes. Many traditional microscopy techniques involve incubation of cells with fluorescent dyes or with nanoparticles containing dye molecules and examining the interaction of these dyes with compounds of interest. However, when a dye is incubated into a cell, it is transported to certain intracellular sites that may or may not be where it is most likely to stay and not to areas where the investigator would like to monitor. The fluorescence signals which are supposed to reflect the interaction of the dyes with chemicals of interest, is generally directly related to the dye concentration as opposed to the analyte concentration. Only with optical nanosensors can excitation light be delivered to specific locations

inside cells. Combined with the molecular recognition of antibody probes, such optical sensors are capable of providing selectivity in location and analyte parameters.

Other approaches involve "fixing" of the sample before analysis; this fixing procedure often destroys cellular viability and may significantly alter intracellular architecture as compared to the living state. It is noteworthy that, after the measurements, the nanosensors are removed from the living cells. On the other hand, with other methods, the dyes molecules or nanoparticles introduced into the cells for sensing remain inside these cells. Nanosensors could provide the tools to investigate important biological processes at the cellular level in vivo. Not only can antibodies be developed against specific epitopes, but also an array of antibodies can be established, so as to investigate the overall structural architecture of a given protein. Finally, the most significant advantage of the nanosensors for cell monitoring is the minimal invasiveness of the technique. The integration of these advances in biotechnology and nanotechnology could lead to a new generation of nanosystems with unprecedented sensitivity and selectivity to probe sub-compartments of living cells at the molecular level.

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