

# Nanosensors: Design and Application to Site-Specific Cellular Analyses

Brian M. Cullum, Guy D. Griffin and Tuan Vo-Dinh\*

Advanced Biomedical Science and Technology Group  
Oak Ridge National Laboratory; P.O. Box 2008; Oak Ridge, TN 378314101

## ABSTRACT

We report the development and application of an antibody-based nanoprobe for *in situ* measurements within a single cell. The nanoprobe has an antibody-based probe targeted to benzopyrene tetrol (BPT), a metabolite of the carcinogen benzo[a]pyrene (BaP) and the BaP-DNA adduct. Detection of BPT is of biomedical interest since this species can potentially serve as a biomarker for the monitoring of DNA damage due to BaP exposure and for possible pre-cancer diagnoses. The measurements were performed on the rat liver epithelial Clone 9 cell line, which was used as the model cell system. Nanoprobes were inserted into individual cells, incubated five minutes to allow antigen-antibody binding, and then removed for fluorescence detection. Prior to measurements, the cells had been treated with BPT. A concentration of  $9.6 \pm 0.2 \times 10^{-11}$  M has been determined for BPT in the individual cells investigated. The results demonstrate the possibility of *in situ* measurements inside a single cell using an antibody-based nanoprobe.

**Keywords:** nanoprobe, nanosensor, nanotechnology, bioprobe, antibody, benzo[a]pyrene, benzopyrene tetrol, carcinogen, cancer, intracellular measurement

## 1. INTRODUCTION

Fiberoptic chemical sensors and biosensors offer important advantages for *in situ* monitoring applications due to the optical nature of the detection signal. Our laboratory has been involved in the development of a variety of fiberoptic chemical sensors and biosensors.<sup>1-6</sup> Recent advances in nanotechnology leading to the development of optical fibers with submicron-sized dimensions have opened new horizons for intracellular measurements. The application of submicron fiberoptic chemical probes was pioneered by Kopelman and coworkers, who have developed probes for monitoring pH<sup>7,8</sup> and nitric oxide.<sup>9</sup> The use of submicron tapered optical fibers has also been demonstrated and used to investigate the possible spatial resolution achievable using near-field scanning optical microscopy.”

We have recently reported the development of nanosensors and *in situ* intracellular measurements of single cells using antibody-based nanoprobe.<sup>2,4-6</sup> Biosensor/bioprobe technology has been at the forefront of analytical instrumentation research. The nanoprobe described in this work has antibody-based receptors targeted against benzopyrene tetrol (BPT). We have selected BPT as the analyte model system because this species has been used as a biomarker of human exposure to benzo[a] pyrene (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.

## 2. NANOFIBER FABRICATION

A challenging aspect of this project involved the fabrication of reproducible nanoprobe for *in vivo* studies. Since optical fibers with submicron-size diameter cores are not commercially available, we have fabricated them by pulling larger silica optical fibers using a laser-based micropipette pulling device (Sutter Industries) that has been optimized for pulling silica fibers. Optimization of this fiber pulling device involved varying the following user input parameters: heat, velocity, delay and pull strength. Using this pulling method, fibers with submicron diameter tips were produced. Figure 1A shows a scanning electron micrograph of an example of a fiber probe fabricated using our fiber pulling procedure. The

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scale on the photograph indicates that the distal end of the fiber shown is approximately 50 nm. Because of the small diameter of this fiber tip, the light used to excite the sample is trapped inside the fiber, allowing for near-field excitation processes to be used. These near-field processes in turn cause the excitation volume of the sample to be minimized to the nanometer scale. However, by tapering these fibers to such small diameters in a heating pulling process, such as the micropipette puller technique just described, the cladding around the core of the optical fiber is stretched so thin that total internal reflection no longer occurs within the fiber. The resulting loss of the excitation light causes two primary detrimental effects, excitation of background autofluorescent proteins in the cell and a reduction in the intensity of the evanescent field at the tip of the fiber. To prevent this light leakage, the outside wall of the fiber,

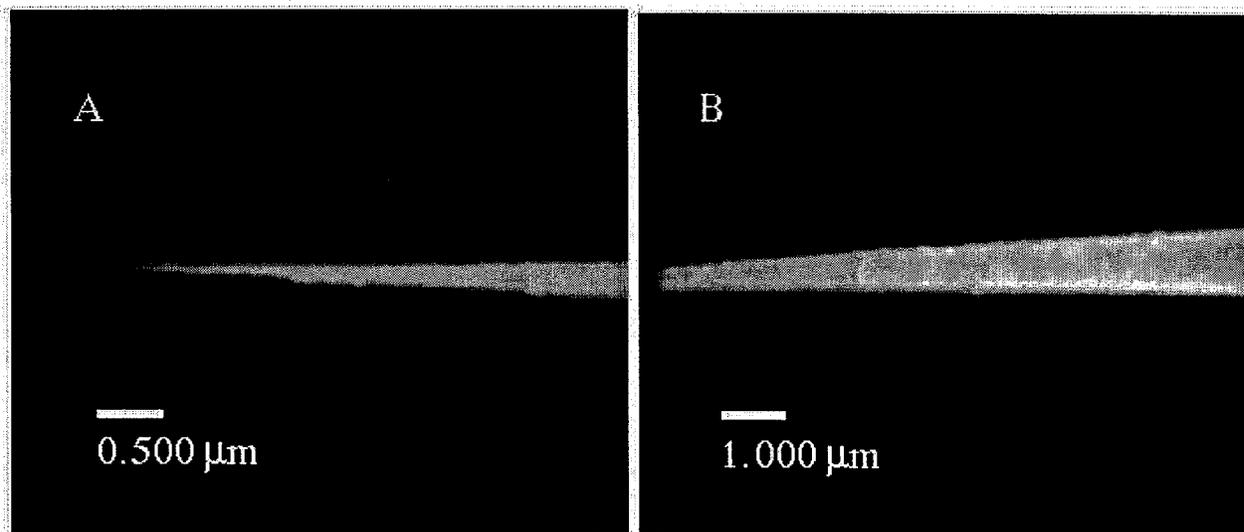


Figure 1: Photograph of an Antibody-based Nanoprobe

over the tapered end is coated with a thick layer of silver, approximately 200 nm thick. The coating process is performed in a thermal evaporator system with the tip of the fiber being angled away from the silver source below. As the vacuum deposition process is begun, the fibers, which are mounted on a rotating stage, are spun to ensure that an even coating of silver is applied to all sides. In addition, by angling the tips away from the silver source below, the tips of the fiber are shadowed from the silver, leaving a free silica surface on the distal end of the fiber. After application of the metallic coating, typical fiber tip diameters of metal-covered bioprobes are less than one micron, on the order of 250-300 nm. This can be seen in Figure 1B.

### 3. ANTIBODY BINDING

The next step in the preparation of the bioprobes for the present work involved covalent immobilization of anti-BPT antibodies onto the fiber tip. Antibody binding to the tip of these fibers follows a simple process that has been described previously.<sup>11</sup> The first step in this immobilization process is to silanize the silica surface on the distal end of the silver coated nanoprobe. Silanization is performed by placing a batch of the silver-coated fibers in a 10% (v/v) solution of glycidoxypopyl trimethoxysilane in water. The temperature of the solution is then elevated to 90 degrees Celsius using a hot plate, and the pH of the solution is reduced to between 2.5 - 3.0 using hydrochloric acid. The fibers are kept emerged in this solution for a period of three hours. Immediately after being removed from this silanization solution, the fibers are placed in a vacuum oven overnight to drive off any excess water that may be present at the tip of the fiber. After drying, the fibers are then placed tip first into a saturated solution of 1,1'-carbonyldiimidazole in acetonitrile. The fiber are kept in this solution for approximately 30 minutes. During this time, imidazole groups begin attaching to the end of the silanized fiber. A cartoon of this binding process is shown in Figure 2. Once the imidazole group is attached

to the end of the flexible linker molecules, they can easily be displaced by the antibody of interest, simply by incubating these activated fibers in a dilute solution of the antibody in phosphate buffered saline (PBS) at 4 degrees Celsius for several days. We have tested fibers that have incubated for periods ranging between 3 days and 3 months, and in all cases found that the activity of the fibers is very similar. During the antibody incubation, the imidazole group is simply displaced by the antibody, and a covalent linkage occurs at the heavy invariable region of the antibody, thereby preventing blocking of the active binding site.

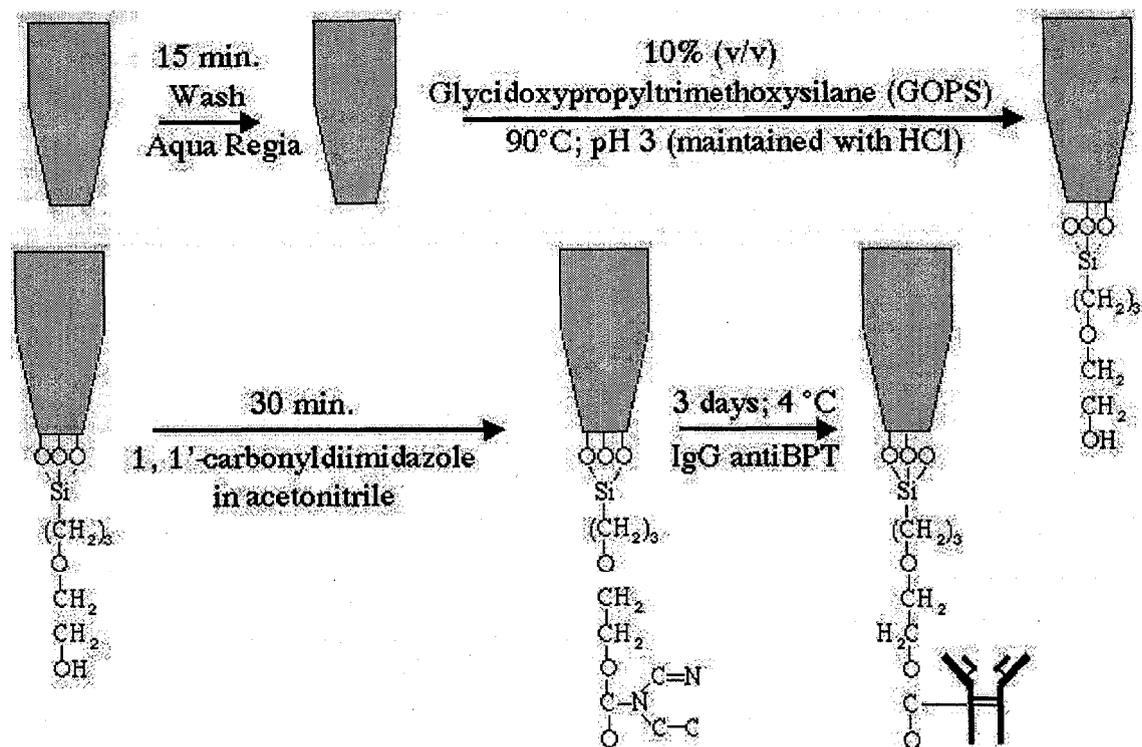


Figure 2: Cartoon Depicting the Antibody Binding Procedure

#### 4. MEASUREMENT SYSTEM

The 325 nm line of a HeCd laser operating at 5mW is launched into a 600  $\mu\text{m}$  (core diameter) silica/silica high  $\text{OH}^-$  content optical fiber using an  $f/2$  fused silica lens. Light transmitted through this fiber is then launched into the proximal end of the nanosensor via an SMA-SMA fiber coupler. The nanosensor is then placed in an X-Y-Z micropositioning system (Narishige Corp.) located on an inverted microscope (Nikon). The fiber is then lowered into a petri dish containing either the cells for measurement or a calibration solution. Once the fiber has been introduced into a cell, it is allowed to equilibrate for five minutes. This five minute incubation period allows the fiber to bind an antigen that may be in the cytoplasm at that location. Once the antigen is bound, the fiber is typically removed from the cell and placed in another petri dish containing neat PBS, while the shutter of the laser is opened. The resulting evanescent field at the distal end of the sensor excites the bound BPT and the resulting fluorescence is collected and collimated with a 60x objective. The collimated light is then filtered with a 400 nm long pass filter (Nikon, Epifluorescence filter UV-2A) and measured by a photomultiplier tube (Hamamatsu) mounted on the front camera port of the microscope. The signal from the photomultiplier tube (PMT) is then 50  $\Omega$  coupled to a universal counter (Hewlett Packard, model 53131A) where twenty measurements are averaged together and recorded.

## 5. CELLULAR ANALYSES

To demonstrate proof of concept of single-cell measurements with antibody-based nanoprobe, experiments were performed on a rat liver epithelial Clone 9 cell line, which was used as the model cell system. Figure 2 shows a photograph of an antibody-based nanoprobe used to measure the presence of BPT inside a single cell. To test the quantitative abilities of the nanosensors, a series of measurements were performed that would allow a quantitative calibration curve to be constructed for BPT inside the cytoplasm of a single cell. Prior to making any measurements, with our nanofiber probes, a petri dish of Clone 9 cells was incubated with a micromolar concentration of BPT in the culturing media for a five hour period of time. After this incubation period, a single fiber optic nanoprobe was used in a standard addition type of analysis to determine the concentration equivalent of BPT inside of the cytoplasm of an individual cell. First, the fiber was inserted into the cytoplasm of a cell that had not been previously treated with any BPT. The small size of the nanoprobe allowed it to be easily manipulated to specific locations within the Clone 9 cell.

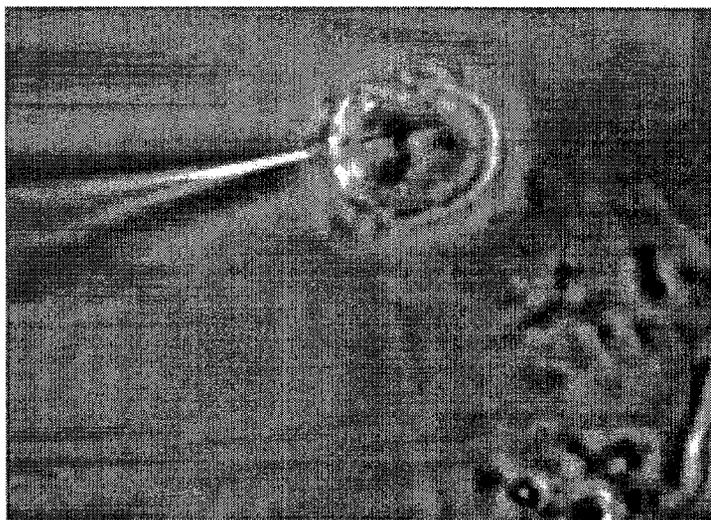


Figure 2: Photograph of Single Cell Sensing Using a Nanoprobe

After the fiber was in place, it was allowed to incubate in the cell for a period of five minutes. This time allowed all of the BPT present in the cytoplasm to bind to the fiber tip. We have also investigated shorter time and found that measurements can be made in as short of an incubation time as 30 seconds in these analyses. However, in order to ensure proper calibration, five minute incubation times were used for all measurements describe in this work. After the fiber had incubated for five minutes, it was typically removed and placed in a PBS solution, to prevent any potential background fluorescence from cellular proteins, during the measurement. In order to complete the calibration, the fiber was then inserted into a very dilute solution of BPT in PBS. It was allowed to incubate again for 5 minutes prior to being removed, placed in a neat PBS solution and making a second measurement. Following this step, the same fiber was then placed into the cell that had been previously treated. Again after a five minute incubation period, the fiber was removed, placed in a BS solution and a fluorescence measurement was made. This measurement was then followed by a series of other measurements of BPT in PBS at ever increasing concentrations. By plotting this data, and fitting the resulting curve with an exponential function to simulate a saturation condition, it is possible to extrapolate out a concentration equivalent for BPT inside the treated cell. Because of the strong affinity of the antibody for BPT, the fiber continues to have more BPT bound with each additional measurement. Therefore, by measuring the change in fluorescence intensity between measurements, it is possible to determine the fluorescence response due to a particular measurement. Using this fact and the exponential calibration function, it was possible to determine that the average concentration equivalent of BPT in the cytoplasm of a single cell (based on many different analyses). In the case of the Clone 9 cells, the concentration that had penetrated into the cytoplasm after five hours of incubation was found to be  $(9.6 \pm 0.2) \times 10^{-11}$  M.

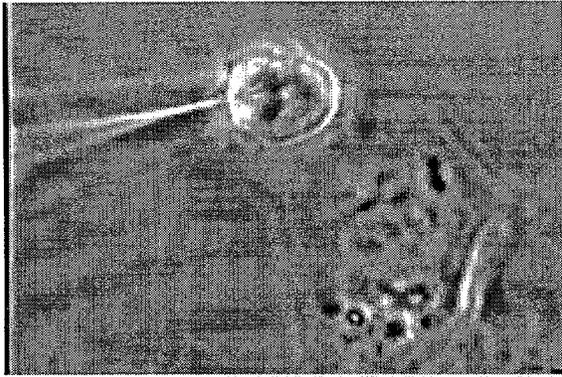
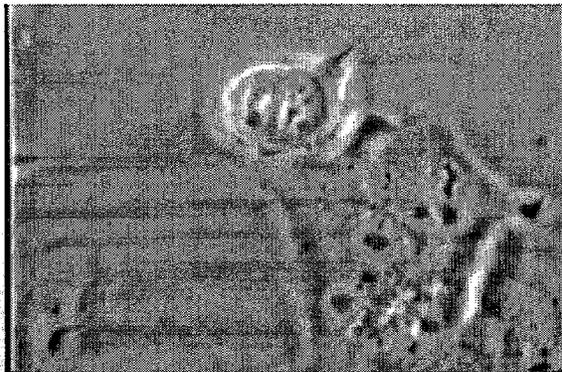
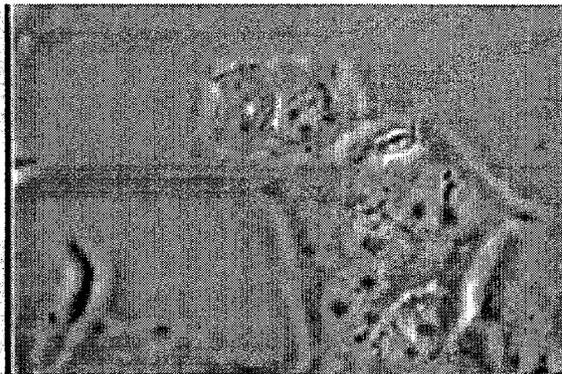


Image Time Relative  
to Measurement

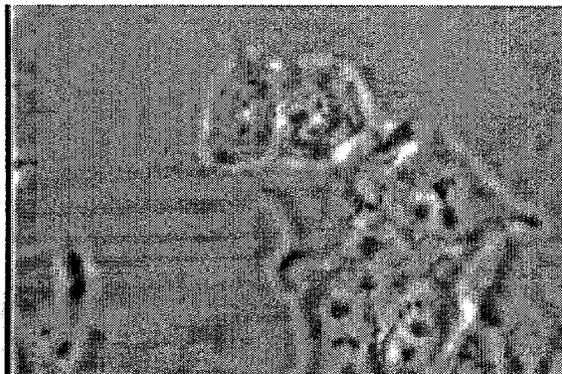
~ 0 minutes



~ 25 minutes



~ 50 minutes



~ 75 minutes

Figure 4: Images of Cell Continuing Through Mitosis After Being Probed

## 6. CELLULAR VIABILITY TESTING

In order to use a technique for the measurement of various chemical concentrations inside of a living cell, it is important to verify that the cell is functioning normally. To test whether or not fiber optic nanoprobe effect the normal functions of a cell, we monitored a cell following insertion and measurement with one of our nanoprobe. Figure 4 shows a series of images taken of that cell during and at various times after being interrogated with a fiber optic nanoprobe. In the first frame of this sequence (top) the fiber optic nanosensor has been inserted into a cell that was beginning to undergo mitosis. As the cell began to release from the petri dish to divide, the nanosensor was inserted into the cytosol for a period of five minutes, and a fluorescence measurement was made. After this, the fiber was removed and the cell was observed for a period of approximately two hours. The remaining frames of this figure show the cell at various times over this period. The second frame shows the cell approximately twenty-five minutes after the fiber was removed. This image shows the cell beginning to reattach to the petri dish and the beginnings of a division in the cell. The last two frames in this sequence show the same cell, approximately 50 and 75 minutes after the fiber was removed from the cell respectively. From these pictures, a clear division of the cell into two daughter cells is apparent. This sequence of images clearly demonstrates the minimally invasive nature of fiber optic nanosensors for single cell analyses, since cell mitosis appears to continue unaffected following a five-minute insertion and measurement.

## 7. CONCLUSIONS

This study demonstrates the application of antibody-based nanoprobe for measurement of chemicals inside of a single cell. Further studies will be conducted to improve the sensitivity and applicability of the technique. Such nanoprobe open new horizons to a host of applications in biotechnology, molecular biology research and medical diagnostics. For instance, different antibodies as well as other bioreceptors such as DNA probes targeted to species of biological and medical interest could be used in nanoprobe arrays to provide highly multiplexed probes for high-throughput drug discovery. In addition, this technique could be extended to non-fluorescent antigen species by using a second, fluorescently tagged antibody in a sandwich type immunoassay, or by performing a competitive binding assay with a fluorescently labeled antigen. These nanodevices could also be used to develop advanced biosensing systems in order to study in situ intracellular signaling processes and to investigate gene expression inside single cells.

## 8. ACKNOWLEDGEMENTS

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