Biosensors for Olfactory Cell Monitoring

Armstard C. Skipwith Jr. and Pamela Abshire

Department of Electrical and Computer Engineering
University of Maryland
College Park, MD 20742, USA

Abstract –Sensors for detecting the weak extracellular signals produced by electrically active cells were characterized, packged and integrated into a bioMEMS system. In previous work spike signals from bovine aortic cells, similar to those of a neural discharge, were recorded. This project has extended that work by monitoring PC12 cells in preparation for investigating the response of olfactory sensory neurons to various stimuli. Work has encompassed the post-fabrication processing of existing fabricated chips for operation in continuous cell culture, modification of the current test setup for improved data acquisition, and execution of experiments in collaboration with neuroscientists at Johns Hopkins University.

I. Introduction

Systems that combine electronics with living cells could prove invaluable for medical diagnosis, pharmaceutical studies, drug enforcement, scientific research, or homeland security [1]. This technology could lead to the development of devices used as biosensors, monitors for biological cells, or hybrid bioelectric computational engines [2]. Interest in the area continues to grow as researchers are developing integrated systems that detect cellular activity, and better ways to improve the communication between cells and circuits [3, 4].

Methods to detect the electrical activity in cells usually fall into two categories: (1) the patch clamp technique, whereby an electrode is inserted into the cell and (2) extracellular detection. Patch clamp gives an accurate representation of the electrochemical changes across cell membranes, but cell viability is compromised. Extracellular detection is a non invasive method that requires cells to be cultured directly on top of the electrodes, and the cell to be close enough to an electrode to measure any change in potential. There are also optical measurements using fluorescent dyes [3].

The project at the University of Maryland endeavors to create an interface that will detect the weak extracellular potentials that are produced from olfactory sensory neurons, with the goal of characterizing the signals based on the stimuli given (Figure 1). Toward this end a bioMEMS system was developed that has detected the extracellular potentials of spontaneously electrically active bovine aortic cells [2]. In this phase of the project we are extending those results by experimenting with new cells, PC12. These cells exhibit similar characteristics to those of olfactory neurons, but are more robust and long lived *in vitro* than the primary cells. Also we are testing a new chip which was fabricated in a smaller feature size process that includes improved testing capabilities. In this paper we outline

the components of the system, including bench testing of the new amplifier, present our biocompatible post packaging techniques, and discuss experiments conducted on the new chip with PC12 cells.

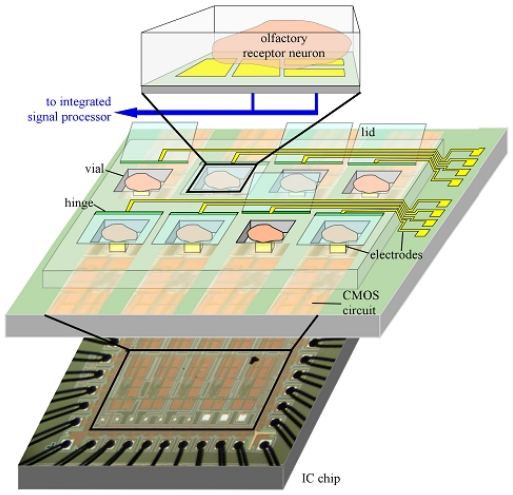


Figure 1. Researchers are developing a system to capture olfactory sensory neurons and detect the weak extracellular signals produced when stimulated

II. System Architecture

A bioMEMS system was created to monitor the responses of cells in continuous culture, and a custom VLSI amplifier was designed to receive the weak extracellular voltages from the stimulated cells and amplify these signals for recording by an external data acquisition system.

A. Bio-amplifier

Our VLSI amplifier is specially designed for low-power, low-noise applications [5]. The circuit is an operational transconductance amplifier in capacitive feedback configuration (Figure 2a), with a midband gain of 100, a cuttoff frequency of 3 kHz, an input-referred noise of approximately 50μV and supply voltages of ±1.25V. Input to the amplifier is taken differentially between two electrodes. The electrodes are 25μm square openings in the top passivation layer, which provides direct contact between metal layers in the chip and the cells cultured directly on top of them. The electrodes are connected as inputs to the amplifier. An array of 17 amplifiers is arranged on the chip with two reserved as test structures (the test amplifiers do not have exposed electrodes). The outputs from the amplifiers are buffered using commercially available opamps in unity feedback configuration with supply voltage ±5V. A new test board was fabricated in order to support experimental investigations using this new chip (Figure 2e).

This chip (Figure 2c) was fabricated in a 0.13μm, 8 metal, 1 poly commercially available CMOS process. The feature size of this process is smaller than earlier chips which were fabricated in a 0.5μm, 3 metal, 2 poly CMOS process and had 24 amplifiers arrayed on the chip (Figure 2b). It was necessary to thoroughly characterize the 0.13μm chip to determine its characteristics. The frequency response was determined using an Agilent 4395A network analyzer (Figure 2d) and follows a first-order low-pass characteristic with midband gain 40dB and cutoff frequency of 4 kHz. We measure three different amplifiers on two separate chips. The resolution of analyzer was set to only resolve frequencies above 10Hz. Noise measurements were also conducted using this network analyzer. The chip was placed in a faraday cage and eight measurements were taken over eight hours. The instrumentation noise was also measured and subtracted from the average total noise measurements in order to determine the output noise contributed by the bioamplifier. Integration over the amplifier bandwidth yields an approximate input-referred noise standard deviation of 50μV.

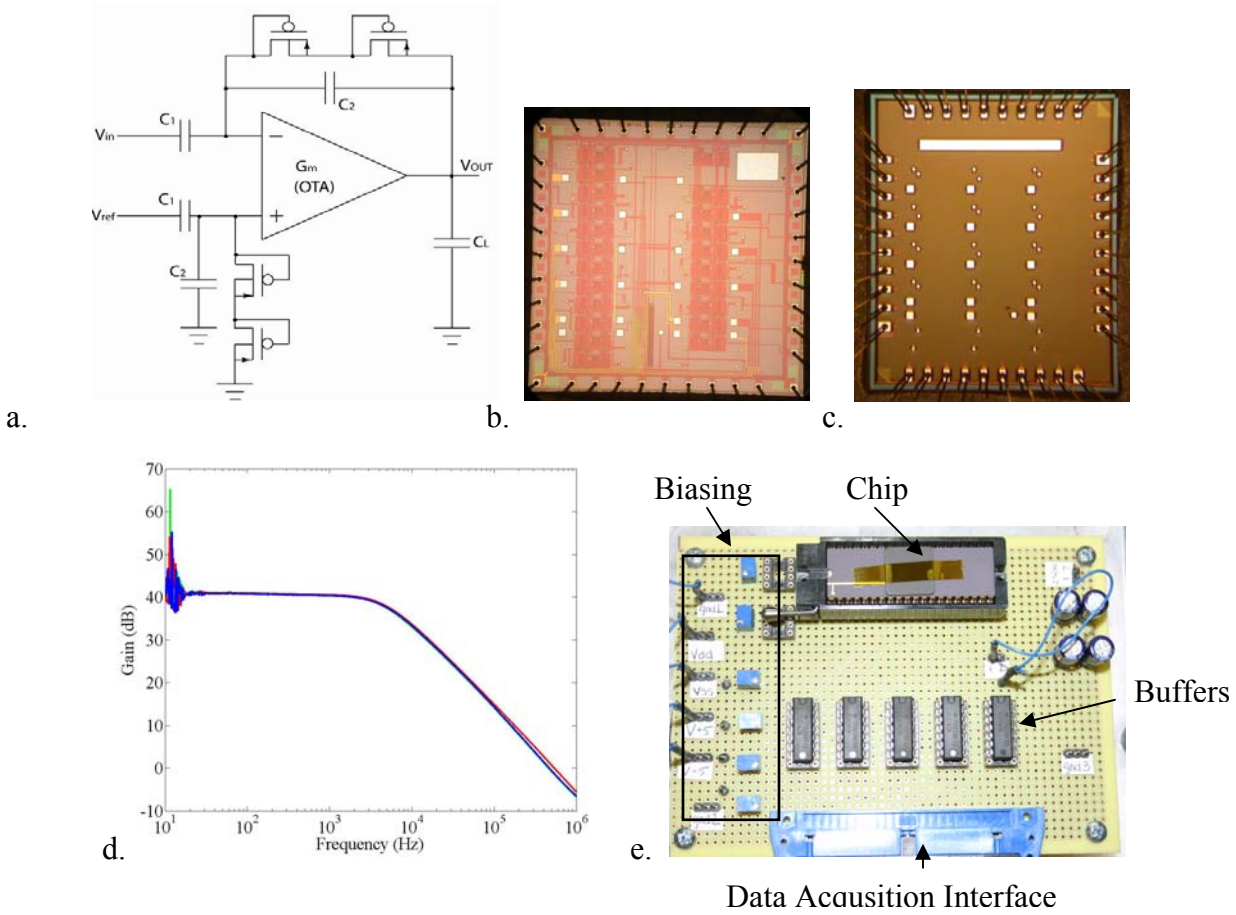


Figure 2.Bio-amplifier (a) schematic [5]. Close up views of the 0.5µm (b) and 0.13µm (c) chips. (d) Three measurements of frequency response of the bioamplifier. (e) New test board designed for the 0.13µm chip.

III. Biocompatible Packaging

A major difficulty that arises when developing an IC system for cell monitoring is that the cells must exist in an aqueous environment which typically disrupts the physical method of data acquisition. Therefore, we must prepare the chips after fabrication to accept the liquid media and provide a suitable home for the cells. Two packaging techniques are implemented: electroless plating and chip encapsulation.

A. Electroless Plating

Aluminum has been, and continues to be the metal of choice for IC fabrication because of its good processing compatibility and conductive properties. Unfortunately aluminum oxidizes and has a low corrosion resistance [6], which makes it unsuitable for electrodes placed in liquid media. Therefore, we cover the electrodes with gold (Figure 3) which is biocompatible [7] and has a high corrosion resistance. Electroless plating is an autocatalytic process that deposits metals through a chemical reaction. It is a low cost and simpler alternative to its photolithographic counterpart, vacuum deposition (sputtering or evaporation) [8] and electroplating which requires an

external electrical connection to the plated surface that reduces sensitivity and increases noise during measurement.

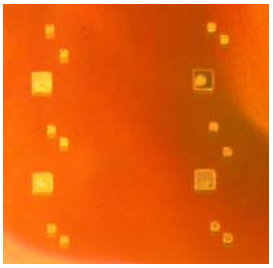


Figure 3. Close up view of gold plated Electrodes

B. Chip Encapsulation

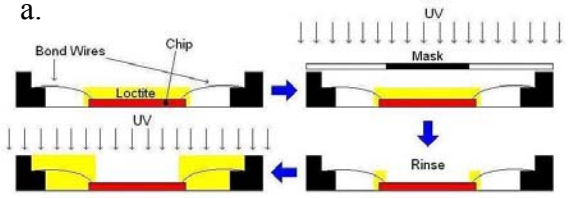
Another challenge while developing these devices is protecting the bond wires on the DIP40 package. Various epoxies and silicones have been previously evaluated. In many instances, after a few days (on average three), the interface of the chip with these products begins to fail. Culture media creeps under the surface, the packaging swells slightly and the bond wires are lifted from the pads causing floating outputs.

For these new experiments, we apply Nuva-Sil®, obtained from Loctite®, to surround the bond wires. This is a biocompatible medical device sealant (a silicone) that is also UV curable. A two step deposition photolithographic process (Figure 4a) patterns an area in the silicone to expose the active surface of the chip. This alignment and rinsing away of silicone is not refined but we are capable of exposing almost the entire surface of the IC, some electrodes are left unexposed. Finally a well is affixed to the chip to hold growth medium and cells. The volume of the well is approximately 500µL. The fully packaged chip is shown in Figure 4b.

Nuva-Sil® was tested for a week to determine package reliability. Culture media was placed in the chip well and the chip was left in an incubator. We checked the two Vdd and ground connections with a multimeter every other day. At the conclusion all Vdd and ground bond wires are intact.

A more robust test of the packaging involves a chip, whose well is filled with culture media, placed on the test board and powered, while continuously monitoring the outputs of the amplifiers for any change from initial bias conditions. This experiment at the time of writing of this paper has not completed but will be done in future work.

a.



b.

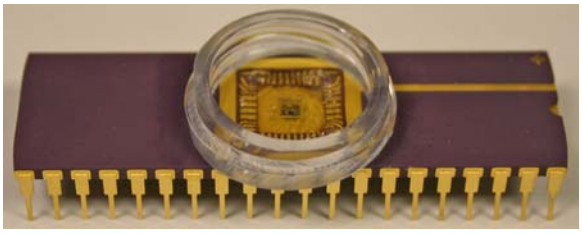


Figure 4. (a) Two step deposition process to expose active chip surface. (b) Fully packaged chip

IV. Test Setup

Once the chips are packaged there are three additional steps needed for an experiment to be executed: cell culture, differentiation, and stimulation. The preparation to run these experiments requires several days.

A. Cell Culture

Growing cells requires a sterile environment and a strict protocol to ensure the cells are

not contaminated by foreign organisms. The handler wears gloves; cells are handled under class II biohazard hoods to protect both the user and the culture; all materials entering the hood must be sprayed with 70% ethyl alcohol, including the handler's hands and no containers are to be opened before entering the hood. All waste materials are disposed in a biohazard container.

Cells are cultured in Petri dishes inside an incubator at 37 °C, 5% CO_2 and 98% humidity to facilitate growth. The cell media contains proteins along with serum and antibiotic and antifungal agents that keep them alive. The media must be changed every two days. PC12 cells are not suspended in solution, but are adhered to the surface of the culture dish. Before transferring the cells to our chip they must be detached from the surface. To do this we use a cell scraper. Once the cells are removed from the surface, the solution is titrated to break up any clumps and suspended cells are deposited onto our chip.

Before plating cells on our chips the chips are cleaned with Hank's Buffered Salt Solution and Sterile water. They also undergo a UV treatment directly before plating to sterilize the chip surface. Laminin in DMEM is then allowed to sit on the chip for 2 hours to aid in interconnectivity between the processes. 24 hours after the cells are plated we add the Nerve Growth Factor.

B. PC12 Differentiation

PC12 cells are used in our experiments (Figure 5a). These cells are a secondary cell line that is derived from a pheochromocytoma, a tumor of the adrenal medulla of rats. PC12 cells respond upon the application of Neural Growth Factor (NGF) causing them to develop characteristics similar to sympathetic neurons. They stop proliferation, develop neurites, and increase electrical excitability (Figure 5b) [9]. Differentiation of an entire population takes about 7 days [10]. By day three 50% of the population has differentiated, we conduct experiments starting on or after day three. These differentiated cells are widely used in neurological studies because they are easier to handle and survive longer *in vitro* than primary cells (neurons).

a.

b.

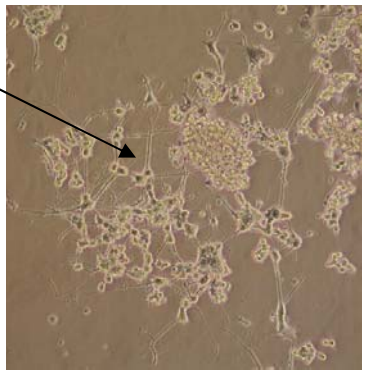


Figure 5. PC12 cells before (a) and after (b) treatment with Nerve Growth Factor

C. PC12 Stimulation

We apply potassium chloride (KCl) to the cellular media to stimulate electrical activity. The potentials that are observed are not potentials in the same sense that we usually think of with circuits. These potentials represent the difference in the concentration of various ions in solution. The application of KCl changes the

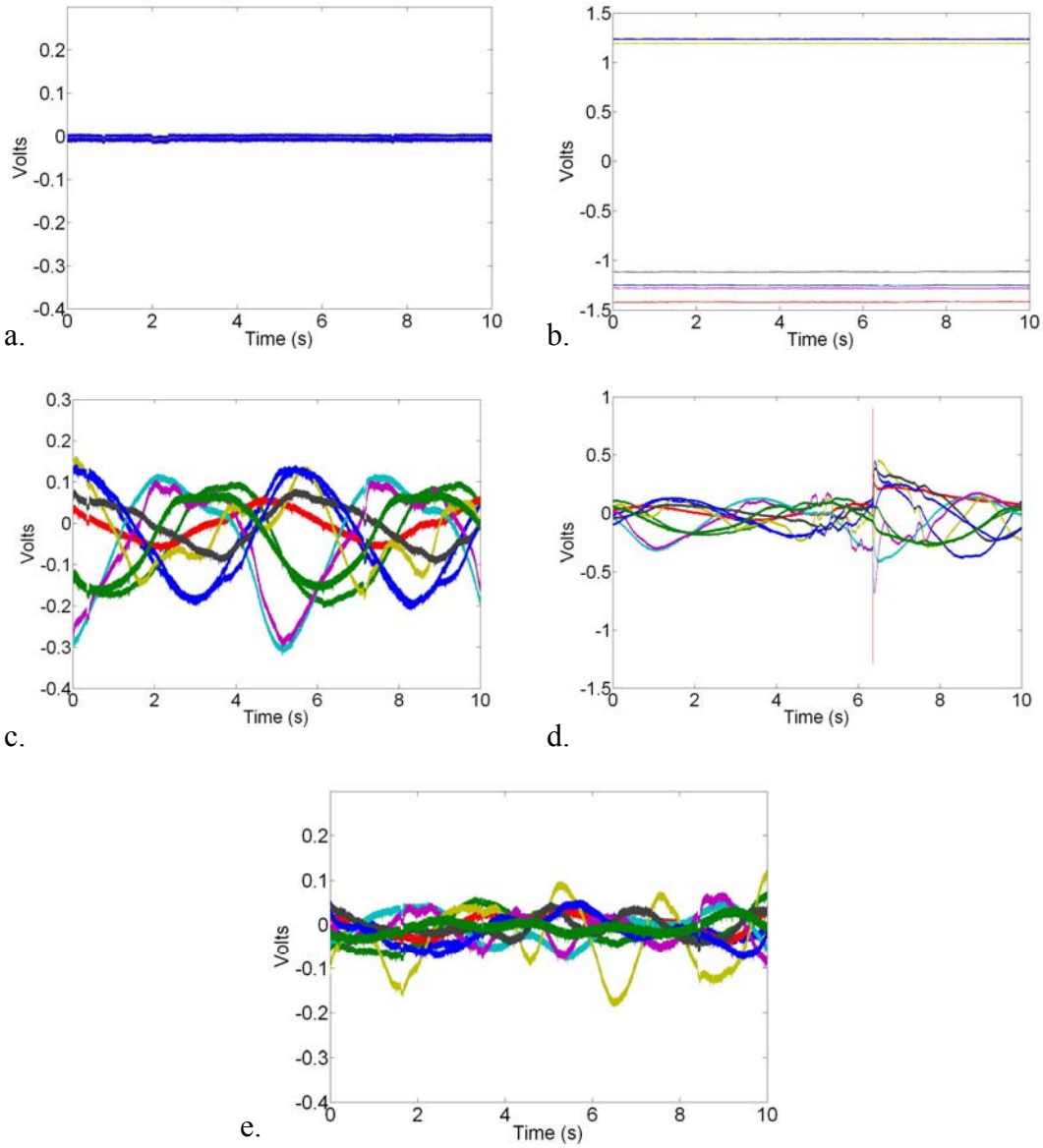


Figure 6. Experimental results from the second experiment done with cells. (a) Output of a dry chip. (b) Output of a chip with packaging failure. (c) Output of amplifiers before stimulation with KCl. Amplifiers are in operating range but have an unexpected oscillation. (d) Output of amplifiers after stimulation with KCl the second time. (e) Output of amplifiers after removing cell media. The amplitudes of the oscillations are reduced relative to the initial in (c).

concentration of ions in the solution, causing the cell to react to stabilize back to the equilibrium state. The change in voltage we will perceive is the movement of ions through the cell membrane in the solution. We continuously monitor the output of the amplifiers sampling at 15 kHz.

V. Experimental Results

We conducted two experiments with cells on the chip. The first experiment did not yield any measurable results. The outputs of the

amplifiers were floating indicating a failure of the packaging. This initial test was done using the 0.5µm process chip which was encapsulated with the previous epoxy Loctite® 3340.

The next experiment was done with the 0.13µm process chip packaged with Nuva-Sil® silicone. These experiments were conducted after six days of culture, four of which the cells were treated with NGF. Two chips were tested, of which one package remained intact. Upon inspection it was found that bond wires had not been fully protected in the failed package.

A packaged chip containing no cellular media has outputs that are centered on zero indicating the amplifiers are within the proper operating range (Figure 6a). A chip with a failure of packaging shows output that are floating or near the voltage rails (Figure 6b). During this experiment the amplifiers were operating in the desired range indicating that the packaging had survived, but the initial outputs were periodic unlike a dry chip (Figure 6c). We continued with the experiment and stimulated the cells with KCl three times. There were some fluctuations in the amplifiers output but these cannot be convincingly attributed to cellular activity (Figure 6d). This is because upon stimulation with KCl the test amplifiers output changed. This should not occur because these test amplifiers have no connection to the chip surface and presumably to the media. Also upon removing the cellular media the amplitudes of those initial oscillations decreased (Figure 6e). This causes us to question several factors and only further experiments and research will give a complete understanding of our observations. In addition, after the experiment, cell viability was confirmed using florescence imaging with Calcein staining.

VI. Conclusions

The newly fabricated bioamplifier chip was successfully bench tested and operates according to designed specifications. PC12 cells were successfully cultured on chip and cell viability was confirmed by florescence imaging. The results obtained from experiments push the research into new areas and further test will provide information to fully evaluate the experimental results obtained.

VII. Future Work

Future work will involve conducting experiments so we can characterize the waveforms in our second experiment with plated cells, refining our packaging techniques to enable full reliability and full exposure of active chip area, and testing with primary olfactory sensory neurons. In Addition we are developing ways to control direct placement of cells.

VIII. Acknowledgements

Thanks to my research adviser Dr. Pamela Abshire, the graduate students of the Integrated Biomorphic Information Systems lab, especially Nicole Nelson and Somashekar Prakash, our collaborators at Johns Hopkins University Amy Kleman and Dr. Gabriele Ronnett, fellow undergraduate Emily Sosin, MOSIS, and the 2008 MERIT program.

IX. References

[1] Reeves, N.; Liu, Y.; Nelson, N.M.; Malhotra, S.; Loganathan, M.; Lauestein, J.-M.; Chaiyupatumpa, C.; Smela, E.; Abshire, P.A., *Integrated*

MEMS structures and CMOS circuits for bioelectronic interface with single cells Proceedings of the 2004 International Symposium on Circuits and Systems, 2004. ISCAS '04. Volume 3, 23-26 May 2004 Page(s): 673-6

[2] Abshire, P.; Lauenstein, J.-M; Liu, Y.; Smela, E., *Cell Clinics for Bioelectronic Interfac with Single Cells*. Proceedings of the 2003 International Symposium on Circuits and Systems, 2003. ISCAS '03. Volume 3, 25-28 May 2003 Page(s):618 -621

[3] Heer, F; Hafizovic, S; Ugniwenko, T; Frey, U; Franks, W; Perriard, E; Perriard, J.-C ; Blau, A; Ziegler, C; Hierleman, A. *Single-Chip Microelectronic System to Interface with Living Cells* Biosensors and Bioelectronics. Volume 22, Issue 11, 15 May 2007 Page(s) 2546-2553

[4] Huys, Roeland et al., *Novel concepts for improved communication between nerve cells and silicon electronic devices*. Solid-State Electronics Volume 52, Issue 4, April 2008 Page(s) 533-539

[5] Harrison, R.R.; Charles, C., *A low-power low-noise CMOS amplifier for neural recording applications.* IEEE Journal of Solid-State Circuits. Volume 38, Issue 6, June 2003 Page(s):958 – 965.

[6] Kovacs, G.T.A., *Micromachined Tansducers Soucebook.* WCB McGraw-Hill, Boston, 1998.

[7] Geddes, L.A., *Electrodes and the Measurement of Bioelectric Events*. Wiley-Interscience, New York, 1972.

[8] Datta, M; Merritt, S.A; Dagenais, M, *Electroless remetallization of aluminum bond pads on CMOS driver chip for flip-chip attachment to vertical cavity surface emitting lasers (VCSEL's).* IEEE Transactions on Components and Packaging Technologies. Volume 22, Issue 2, June 1999 Page(s):299-306.

[9] Greene, L.A.; Tischler, A.S., *Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which responds to nerve growth factor.* Proc. Natl. Acad. Sci. U.S.A. Volume 73 1976 Page(s):2424-2428.

[10] Das, Kaberi P; Freudenrich, Theresa M.; Mundy, William R., *Assesment of PC12 cell differentiation and neurite growth: a comparison of morphological and neurochemical measures.* Neurotoxicology & Teratology. Volume 26 Issue 3 May 2004, Page(s):397-406