Chemosensory Evaluation of Prostate Cancer Cells

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Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science
In
Biological Systems Engineering

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December 3, 2010
Blacksburg, VA

Keywords: electronic nose, prostate cancer, in-vitro detection, noninvasive testing

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ABSTRACT

Prostate cancer is the most commonly diagnosed disease and second most commonly caused death among men in America. Although much controversy surrounds the current methods of detection, PSA test and biopsy, no new methods have been approved as an effective method of detection. Biomarkers and non-invasive means of detection are being investigated everyday in hopes of discovering new information that could be of use in the prostate cancer field.

One such non-invasive technology is the use of an electronic nose. The electronic nose technology has been utilized in the agricultural, food, biomedical, and environmental. The objective of this current study is to determine the effectiveness of the electronic nose to discriminate between prostate cancer cells (DU-145 and PC-3) and non-tumor forming cells from the urinary tract (SVHUC). Specific factors that will be investigated are incubation period and cell population.

For all three cell lines, two cell populations of 75,000 and 150,000 cells were cultured and tested after 2, 8, 12, and 24 hours using a conducting polymer based hand-held electronic nose. Multivariate analysis was performed on the data and determined that the greatest discrimination between incubation periods was between 2 hours of incubation and the remaining periods of 8, 12, and 24 hour periods. This presents the idea that by 8 hours, ample volatiles were produced to be detected by the electronic nose. Additionally, when compared to one another, all three cell lines showed distinct differences. The cell lines most closely related were PC-3 and DU-145, the prostate cancer cell lines. However some variation was seen between these cell lines, which may be attributed to the presence of PSA in PC-3 cells or other factors affecting prostate cancer patients. Finally, PCA plots clearly illustrated that after 2 hours of incubation, sufficient volatiles were produced to allow the electronic nose to clearly discriminate the three cell lines from one another, demonstrating the importance of incubation period on successful discrimination.

Based on the findings that the electronic nose was effective at discriminating the three cell lines, testing was completed to determine if cell population or cell maturity had the greatest

effect on discrimination. The cell lines were cultured and tested immediately using an initial cell population of the highest cell population observed after a 72 hour incubation period. The results concluded that when the cell lines were tested immediately after culturing, the Cyranose was able to detect the individual cell lines in culture while also determining a range of detection for each cell line. The range of detection for DU-145 was found to be 26,200 to 262,000 cells based on interclass m-distances of 6.829-9.170 for cell populations lower than 26,200. A range of detection of 51,400 to 514,000 cells was concluded for PC-3 cells based on interclass m-distances of 5.690-7.400 for cell populations lower than 51,400. Finally, the results showed a range of detection of 19,000 to 190,000 cells for SVHUC based on interclass m-distances of 5.520-9.076 for cell populations lower than 19,000. However, when attempting to discriminate the three cell lines against one another immediately after culture, the electronic nose was unable to make clear distinctions between the three cell lines. When testing cancerous and non-cancerous cells, incubation period of the cells should be the only factor considered. It is evident that the cells need time to metabolize and produce volatiles so that the electronic nose can clearly distinguish these cells from one another in culture.

ACKNOWLEDGEMENTS

I would like to thank Dr. Kumar Mallikarjunan for granting me with this wonderful opportunity to pursue my master's degree and for allowing me to be a part of his research group. His assistance throughout the past two years has been invaluable.

I would also like to thank my committee members. Firstly, Dr. Robert Grisso, thank you for your support, flexibility, and suggestions during this process. Secondly, Dr. Elankumaran Subbiah, thank you for allowing me to come into your laboratory to run my experiments and for working with me throughout your hectic schedule. I would also like to thank all of the members of Dr. Subbiah's lab for their patience while teaching me new laboratory techniques and their readiness to assist me with any of the problems I came across.

Thank you to Dr. Saied Mostaghimi, the former department head, and Dr. Mary Leigh Wolfe, the interim department head, for their availability and assistance during my graduate years. To all of the BSE staff, thank you for helping me with all the administrative requests I have had throughout the years.

I would like to thank my family and friends for supporting me throughout the long graduate process, I truly don't know how I would have made it through without you all. I am blessed to have such a wonderful family who offers spiritual, financial, and emotional support and will always be grateful for everything you have done for me. Finally, I would like to thank all of my friends for their love and support. Go Hokies!

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Chapter 1: Introduction

Background

The body is composed of millions of cells that grow, divide, and die methodically. At the beginning of a person's life, normal cells divide rapidly to permit the person to grow, and once adulthood is reached, the cells typically only divide to replace damaged or dead cells. Cancer begins when cells begin to grow irregularly and out of control. Instead of dying, the cancer cells continue to grow and form new cancer cells, which then take over other tissues within the body (American Cancer Society, 2009). Adenocarcinoma of the prostate is the scientific term for a cancerous tumor located on the prostate gland. This masculine gland is located in the pelvis, below the bladder, above the urethral sphincter and penis, and in front of the rectum (Swierzewski, 2007). Prostate cancer (CaP) is the most commonly diagnosed disease and second most prevalent cause of cancer related death among men, with more than 186,000 diagnosis and 28,000 deaths in the United States in 2008. The disease has an overwhelming effect on older men with occurrences increases from 7.8% at age 50 to 20.8% at ages 65 and older (Albertsen, 2010).

In order to effectively treat and diagnose CaP, it is imperative to detect the disease at an early stage. The current techniques employed to detect the disease include analysis of prostate biopsies, assaying prostate-specific antigen (PSA), digital rectal examination, and transrectal ultrasonography, with PSA being the only conventional biomarker accepted by the U.S. Food and Drug Administration (You et al., 2010). Unfortunately, extensive testing for PSA has considerably increased the number of men being diagnosed with CaP because of the diagnosis of small-volume and low-grade disease presence that in most cases is clinically insignificant. For this reason, researchers are diligently looking for alternate biomarkers and means of CaP detection (Albertsen, 2010).

In recent years, volatile organic compounds (VOCs) have been studied for their potential to be used as non-invasive biomarkers of various biochemical pathways present in health and disease (Dragonieri et al., 2007). Research with gas chromatography has shown that tumors from various cancers give off minuscule amounts of formaldehyde, alkanes and benzene derivatives that are not typically found in healthy tissues. Although not commonly used in the present day, the use of volatiles for disease diagnosis has been applied to medicine in the past, with Hippocrates describing the fruity odor of diabetes in the breath and the musty odor of liver disease (McNeil Jr., 2004).

The human nose has more than 300 olfactory receptor types that are sensitive to molecular substances floating throughout the air. Receptors are activated by a substance or odorant and a pattern of signals is then sent to the brain to be recognized as a particular smell. Based on the processes used by the human nose, scientists have created an electronic nose technology to produce distinct patterns in response to different volatile compounds (Friedrich, 2009). This technology offers advantages in regards to mobility, price, and ease of use, with the most promising aspect being its ability to be used as a point of care diagnostic tool (Rock et al., 2008). Furthermore, the hand-held and non-invasive tool will enable medical professionals to provide immediate and accurate diagnosis of chemical elements and microorganisms in breath, wounds, and bodily fluids (Heimerl, 2001).

Justification

The electronic nose technology was initially developed for the use in the food industry to characterize odor patterns from beer, coffee, contaminated water, wine, and many other food products. However, in the past two decades, its use for medical and clinical applications has greatly increased (Pavlou et al., 2000). Although electronic noses have been used to discriminate

between wide varieties of diseases, it is less frequently used to detect cancers. Additionally, when using the electronic nose, studies are typically completed using the breath or urine to detect the diseases.

Few studies have utilized the electronic nose to discriminate cancer cell lines in vitro. Smith et al. (2003) studied the production of VOCs from the head space of two lung cancer cell lines in vitro and determined that the VOCs from the culture of lung cancer cells differed from virgin culture medium as well as control cell cultures. Gendron et al. (2007) performed a similar in vitro study using upper aerodigestive tract tumor cells and received promising results in regards to the ability of the electronic nose to discriminate between different cancer types. The electronic nose has the potential to categorize cancers according to cell type even though the volatized molecules that make each cell line unique have not yet been identified.

With the high occurrence rate of CaP, it is necessary to explore different means of detecting the disease in addition to those currently being used. Researches have discovered that tumor cell lines have distinct response patterns when using an electronic nose and that they may be distinguished from one other (Kateb et al. 2009). With this knowledge, it is promising that the electronic nose will be able to discriminate between CaP cells and non-tumor forming cells from the urinary tract.

Hypotheses

A conducting polymer-based electronic nose can successfully discriminate between CaP cell lines and non-tumor forming urinary tract cells. The cell population will have a greater effect than incubation period on the ability of the electronic nose to discriminate CaP cells (PC-3 and DU-145) from non-tumor forming urinary tract cells (SVHUC).

Objectives

The objectives of this research are to:

1. Determine the effects of cell population on the electronic nose's ability to discriminate between CaP (PC-3, DU-145) and non-tumor forming cells from the urinary tract (SVHUC),
2. Determine the effects of different growth periods on the electronic nose's ability to discriminate between CaP (PC-3, DU-145) and non-tumor forming cells from the urinary tract (SVHUC),
3. Determine the overall capability of the electronic nose to discriminate between PC-3, DU-145, and SVHUC cell lines,
4. Determine which factor, growth period or cell population, has a greater effect on the ability of the electronic nose to discriminate between CaP cells and non-tumor forming cells from the urinary tract, and
5. Determine the range of detection for the electronic nose to successfully discriminate different cell lines in culture

Thesis Outline

This thesis consists of five chapters. Chapter one provides an introduction to the research topic, while chapter two reviews work currently being done in areas related to this research. Subjects discussed include CaP, screening and detection, biomarkers, and sensor technology used for detection. Chapter three presents the research completed to determine the ability of the electronic nose to discriminate between CaP cells and non-tumor-forming urinary tract cells. Additionally, this chapter will discuss the electronic nose's ability to discriminate between these cells lines with varying cell population and incubation period. Chapter four will utilize results

found from chapter three to discuss the importance of growth time and cell population on the electronic nose's ability to discriminate between cell lines. Finally, chapter 5 will summarize the data presented as well as provide conclusions and areas for future studies.

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Chapter 2: Literature Review

Prostate Cancer

Cancer is a very common and difficult to cure disease in which cells grow uncontrollably within the body, attacking and destroying tissues and organs. Prostate cancer (CaP) is a disease that takes over the prostate due to its cells mutating and multiplying very rapidly. The prostate is a walnut sized gland that is necessary for reproduction. It produces a sticky fluid of acids and enzymes that makes up approximately 15% of the total volume of semen and aids in sustaining the sperm cells created in the testicles. CaP is the most common type of cancer in the United States among men over the age of fifty. Although the exact causes are still unknown, research shows that testosterone, age, heredity, genetics, and diets may be contributing factors of the disease (eHealthMD, 2010). While occurring very frequently, only one out of nine men diagnosed with the disease eventually die from it, causing it to be the second highest cause of cancer related deaths in men behind lung cancer (Flaig et al., 2007). Although CaP typically grows slowly and initially stays restricted to the prostate gland where it may mildly cause harm, other forms of the disease are very aggressive and can spread throughout tissues and organs very rapidly (Mayo Clinic, 2010).

The prostate is separated into multiple anatomic zones with the majority of the occurrences developing from the peripheral zone near the rectum and growing outward to invade surrounding tissue. Because the gland is located very closely to several fundamental structures, it can disrupt normal urinary, bowel, and sexual functions (Prostate Cancer Foundation, 2010). Some men with CaP remain asymptomatic but unfortunately die from unrelated causes. The three most important risk factors for CaP in the United States are race, family history, and age. CaP shows great difference in incidence amongst populations, with Asian men showing a very

low incidence, while African American men have the highest incidence rate. Exact reasons for these differences are unknown but are believed to be due to genetic and environmental differences as well as social influences like health care access, which could affect the development and treatment of the disease. Similarly to other forms of cancer like breast and colon cancer, familial grouping of CaP is a frequent occurrence. Five percent to 10% of CaP cases are thought to be predominantly due to inherited genetic factors or CaP susceptibility genes. In addition to race and family history, age has also shown strong correlation to the occurrence of CaP. While CaP is rarely seen in men under the age of 40, 1 in 45 men between the ages of 40 and 59 will be diagnosed with the disease, in addition to 1 in 7 men between the ages of 60 and 79 being diagnosed (National Cancer Institute, 2010).

Screening and Detection Methods

The principle behind screening for CaP is to detect the disease at its earliest stage before any symptoms have developed. While men experience symptoms that they believe to be related to CaP, they can instead be an indication of the presence of other prostate diseases or disorders like benign prostatic hyperplasia (BPH), enlargement of the prostate, or prostatitis. When CaP is detected in the early stages, it can typically be treated the most effectively. Currently, the two tests employed to screen for CaP are the digital rectal exam and the prostate-specific antigen blood test (Prostate Cancer Foundation, 2010).

Traditional Methods

The majority of CaP is discovered by routine screening of the gland. During the digital rectal exam (DRE) a doctor will insert a gloved and lubricated finger into the rectum of the patient to examine the prostate. Because of the close proximity between the prostate and rectum, this method allows for a careful assessment of the texture, shape, and size of the prostate gland

(Mayo Clinic, 2010). The prostate-specific antigen (PSA) blood test is a procedure that measures the production of the PSA protein being produced by the prostate and released into the bloodstream in very small amounts. When problems arise with the prostate, increased amounts of PSA are released and eventually reach a level where it can be detected easily in the blood. During a PSA test, a small amount of blood is taken from the arm and the PSA is measured. Levels under 4 ng/ml are usually considered normal, levels over 10 ng/ml are usually considered high, and levels in between are considered intermediate. There is much controversy surrounding the use of PSA levels as a cancer indicator because levels can be elevated during other problems that arise in the prostate like BPH and prostatitis and in some cases, levels can be lowered when testing overweight or obese men (Prostate Cancer Foundation, 2010).

Prostate Biopsy

After a review of the results from the DRE and/or PSA blood test, the physician will determine if further testing by means of a prostate biopsy is necessary as described by the American Cancer Society (2009). A biopsy is a procedure in which a sample of tissue is removed and examined under a microscope. When testing for CaP, a core needle biopsy is the key method utilized. Using a transrectal ultrasound to locate and observe the prostate gland, a needle is quickly inserted through the wall of the rectum and into the prostate gland. When the needle is removed, a small cylinder of prostate tissue is also removed. This method is repeated an average of 12 times and all the samples are sent to a pathology lab for further examination and determination of the presence or absence of CaP. The samples will be looked at under a microscope and assigned a grade using the Gleason system, with the detection period being anywhere from a minimum of one to three days.

Many pathologists use the Gleason system to grade CaP cells. A scale from 1 to 5 is used to determine how much the cells in the cancerous tissue resemble normal prostate tissue as seen in Figure 2.1. A grade of 1 is designated if the cancerous tissue looks like normal prostate tissue, grade of 5 if the cancer lacks the typical features of regular tissue and the cells are spread irregularly throughout the prostate, and a grade of 2-4 for features in between the two extremes. Because CaPs have areas in the gland with different grades, a collective grade is assigned to the two areas with the greatest cancer content and these two grades are added together to produce a Gleason score between 2 and 10. In addition to determining the Gleason score, the pathologist will also determine the number of biopsy samples that contain cancer, the percentage of cancer in those cores, and the location of the cancer within the prostate (American Cancer Society, 2009).

New Studies

Due to the controversy surrounding the traditional methods for CaP screening and detection, researchers all over the world are studying new techniques for detection of CaP. Although it is uncommon for all physicians to agree on a screening frequency and method, the standard among doctors is that more men will die with their CaP than of it and no current test can distinguish a slow-growing and non-harmful tumor from a tumor that is aggressive and deadly (Edelson, 2009). Seventy to 80% of the estimated 1.2 million patients who undergo a prostate biopsy each year in the U.S. receive negative results, but cannot be fully assured of their diagnosis because of the possibility that the cancer could have been missed by sampling error. With the average of 12 cores being removed from the patient during a biopsy, only approximately 0.04% of the prostate is being examined (Gann et al., 2010). With biopsies being one of the most widely used method to detect CaP, Wright and Ellis (2006) performed a study in which they observed the affects of adding more core samples and anterior apical prostate

biopsies. Their major findings were that the inclusion of the anterior apical biopsy in addition to the traditional lateral cores, improved cancer detection.

Researchers have also been looking at other methods aside from biopsy enhancement to improve the early detection and management of CaP. Cairns et al. (2001) proposed the use of molecular detection of CaP in urine by hypermethylation of regulatory sequences at the glutathione *S*-transferase pi (GSTP1) gene locus, due to the fact that GSTP1 is found in more than 90% of primary prostate carcinomas and not in normal prostatic tissue. They believed that the urine from CaP patients would possibly contain shed neoplastic cells or remains agreeable to DNA analysis. Their results showed that even though 79% of the prostate tumors were positive for GSTP1 methylation, in only 27% of the cases, was the GTSP1 methylation positive in the corresponding urine-sediment DNA. Although less than one third of the urine samples tested positive for the GSTP1 methylation, they demonstrated that molecular diagnosis of prostate neoplasia in urine is possible. Bellorofonte et al. (2005) researched the idea that malignant and normal tumors have different electromagnetic properties with the dielectric constant of growing tumors seen to be approximately ten times larger than for normal tissue. The researchers investigated the possibility of using a nonlinear tuneable oscillator to detect CaP and evaluate the accuracy of its diagnostics. The results provided the first evidence for the non-invasive detection of CaP using electromagnetic interactions. Scans with the oscillator also showed the possibility for identification of patients at risk of CaP as well as patients with an extremely low risk. With any disease, especially one that is aggressive and invasive as CaP, there is an extreme need for effective detection methods that can be reliable and efficient in diagnosing patients.

Biomarkers

When trying to determine appropriate methods for early detection of CaP, it is imperative to recognize the properties and characteristics of the disease so that they can be taken into account when formulating new technologies. One such property of CaP that is frequently considered when planning detection methods is the possible biomarkers that are incorporated into the disease's development and progression. To improve the treatment and reduce the death rate, it is essential to detect the disease as early as possible, which has caused many new technologies to emerge that have been aimed at biomarker discovery and validation. A biomarker can be defined as an in vivo and biologically derived molecule, that can indicate the progress and status of a disease (Zhang et al., 2007). Although there are current methods in place to detect the presence of CaP, there is still a vital need for an accurate prognostic marker that could be used to identify men at high risk for the disease and risk of relapse (Phé et al., 2010).

Prostate-Specific Antigen (PSA)

The serine protease PSA was characterized and named in 1979 and detected in serum the following year. It is secreted into the seminal plasma and responsible for the liquefaction of semen, and very rarely is it released into the bloodstream in healthy men. The main reason PSA may be introduced into the bloodstream is due to destruction of the basement membrane of prostate epithelial cells. This can be caused not only by CaP, but also BPH and prostate trauma, all of which will result in increased levels of PSA. This in turn leads to approximately 60-80% of prostate biopsies that are completed because of the false positive results received from the PSA blood test (Stephan et al., 2007). In 1989, researchers confirmed that serum PSA was directly proportional to increasing stages of CaP. Ten years later, studies described the correlation between serum PSA and CaP volume as reasonably good and not great. Five years following, the

relationship almost entirely disappeared due to an increase in sample population and the main correlation of serum PSA remained solely with BPH (Stamey et al., 2004). Although correlation between PSA and CaP has drastically decreased over the years, PSA remains the only biomarker accepted by the FDA. Fortunately studies have shown that using a combination of PSA derivatives has slightly improved specificity (You et al., 2010). Despite the frequent use of PSA for CaP detection and prognostication, when used independently, it has several limitations for effective detection. Approximately 15% of CaP cases occur in men with very low serum PSA levels, further representing the necessity of identifying and characterizing alternate CaP biomarkers (Makarov et al., 2009).

Emerging Biomarkers

The discovery and characterization of CaP biomarkers continues to be a difficult task. It took more than ten years to convert the discovery of PSA into a functional biomarker for clinical use and a similar period to determine that it was not an ideal biomarker for CaP detection. The discovery of CaP biomarkers is a complicated challenge because there is a great need for biomarkers with the ability to discriminate between indolent and aggressive cancers, in an effort to minimize treatment. Fortunately, success has been made on the discovery of emerging biomarkers in serum, urine, and prostatic tissue, all of which show great promise for cancer detection (Parekh et al., 2007).

The kallikrein gene family, of which PSA belongs, has been widely studied for similar genes that can help in the detection of CaP. For many years, only three human kallikrein genes had been identified, the pancreatic/renal kallikrein (*KLK1)*, the human glandular kallikrein 2 (*KLK2)* and PSA (*KLK3*), and recently twelve new members were characterized. In addition to PSA, KLK2 has shown to add important information for detecting CaP, especially at low values

of PSA. KLK2 was shown to be able to discriminate between low and high-grade tumors as well as more closely correlating with the total CaP volume when compared to other PSA forms. It has been proposed that the gene is a powerful predictor of organ-confined disease and the stage of progression of the localized CaP. Furthermore, at least eight additional genes from the kallikrein family are expressed in high amounts in prostatic tissue. The differential regulation of these kallikrein genes could lead to a successful development of new serum-markers, which could help to reduce the number of unnecessary prostate biopsies (Stephan et al., 2007).

For many of the genes of interest in CaP research, promoter methylation is typically the mechanism underlying their loss of function in the cancer and when function is lost, tumor initiation, progression, and metastasis occurs. Glutathione S-transferase P1 (GSTP1) acts to protect cells from DNA damage and cancer initiation and when this gene is suppressed, there is an increased cancer incidence. A large amount of research has been conducted on the development of GSTP1 methylation as a biomarker to be used in the detection of CaP. GSTP1 promoter methylation represents the best DNA-based biomarker that is currently available for the disease because it is present in up 90% of CaP tissues, giving it a specificity of 85-100%. Results were shown to be very successful when testing for its presence in bodily fluids, with GSTP1 having 75-100% specificity from a biopsy, 82-100% specificity in urine, and 100% specificity in plasma, serum, and sperm. Although further studies are required to validate this set of biomarkers, early results from the use of GSTP1 methylation as a potential biomarker shows great potential for the early detection and risk identification of patients with CaP (Phé et al., 2010).

Novel Biomarkers

The identification of new genes and proteins associated with CaP will assist in the development of a greater understanding of the disease and more targeted approaches for treatment. Although the validation and characterization process is extensive, researchers are working hard to identify genes and proteins that will aid in the detection of the disease because diagnosis and progression prediction are currently being compromised by the lack of specific and robust biomarker assays (Matharoo-Ball et al., 2007).

Due to thorough research, many genes and proteins have surfaced with noteworthy properties that could be used to facilitate the detection and treatment processes for CaP after further research is conducted. Gu et al. (2010) propose the first evidence of signal transducer and activator of transcription 5 (Stat5), which when activated, signals cancer cells to grow and survive, being involved in the induction of metastatic behavior of human CaP cells in vitro and in vivo. In CaP, Stat5 activation is known to be associated with cancer lesions of high histological grade. In this study, the researchers' results show that Stat5 is activated in 61% of distant metastases of clinical CaP and 21% of Stat5-regulated genes in CaP cells were metastases related, with 7.9% being related to proliferation and 3.9% to apoptosis. These results show the capability for Stat5 to be used as a target protein for CaP that has spread.

TRPM8, a member of the transient receptor potential family and melastatin subfamily of these proteins, has the potential to be a valuable diagnostic tissue marker and predictive indicator for the progress of CaP as described by Zhang and Barritt (2006). The over-expression in mostly organ confined CaPs suggests that TRPM8, an androgen-regulated protein, has a role in the development of the cancer from the organ-confined stage to the metastatic stage. The protein may also contribute to the initiation, promotion, and progression of carcinogenesis in pre-

neoplastic cells because of their high susceptibility to apoptosis. Androgen unresponsive CaP is very difficult to treat and there are limited diagnostic markers available. Fortunately, TRPM8 is a potential tissue marker in differential diagnosis and a potential predictive marker for androgen-unresponsive and metastatic CaP.

Steroid hormones and their metabolizing enzymes are frequently studied for their possible role in CaP. Rasiah et al. (2009) discuss the latest interest in the androgen/estrogen inactivating enzyme 17 β-hydroxysteroid dehyrogenase type 4 (HSD17B4). Profiling of gene expression has shown HSD17B4 to be greatly over expressed in CaP when compared to benign epithelial and the researchers believed that the altered expression of the enzyme might contribute to CaP progression through altered hormone balance. Their results concluded that mRNA and protein over expression of HSD17B4 were not only associated with the presence of CaP, but was a major independent predictor of poor patient outcome. Although it could not be used to entirely replace the markers currently being used, the researchers recommend validation using current CaP series to further determine the potential role of HSD17B4 immunostaining as a molecular marker of CaP outcome.

There are many genes and proteins being studied for biomarker identification, due to the distinctive properties of CaP. α-methylacyl-CoA racemase (AMACR) has been identified as a biomarker for prostatic carcinoma and was shown to be present in voided urine samples, while being nearly absent in urine of patients without CaP. It was reported to be one of the few biomarkers that has both high sensitivity and high specificity (Maraldo et al., 2007). Early CaP antigen (EPCA) has also been studied and characterized and showed that immunostaining of this antigen in human prostate needle cores could discriminate men with CaP from those without the disease. The tissue adjacent to the areas of CaP that appeared to be normal was analyzed

histologically and EPCA showed a sensitivity of 84% and specificity of 85%. Although there are several biomarkers with practical operating characteristics, no individual marker is ideal, increasing the possibility for the success of different combinations of biomarkers to provide better results (Makarov et al., 2009). New technologies including genomic microarrays and proteomics have aided the discovery of biomarkers. The process of initially discovering the biomarker to widespread clinical use involves many steps including accurate methods for detection, pilot studies, and rigorous validation, all of which are necessary in order to discover new ways to effectively detect and treat the disease (Parekh et al., 2007).

Volatile Organic Compounds

While many biomarkers are discovered on a molecular and cellular level, the diagnosis of diseases by the analysis of volatile compounds released by specific body compartments has attracted an increasing number of researchers, with the non-invasivity of the method being its most fascinating aspect (Bartolazzi et al., 2010). Biological volatile organic compounds (VOCs) are released by biological samples including olfactive and non-olfactive VOCs and can also be considered as a type of terminal metabolites. Although typically thought to be similar, biological VOCs are different from normal 'odor.' The term 'odor' refers to biological, physical and psychological effects that are caused by the interaction between chemical stimulants like aromas and fragrances and the olfactive system of living creatures. Biological VOCs are comprised of special structures and contain very useful bio-information. Over time the characteristics of the biological VOCs change, which usually reflects the variety of metabolic statuses of biological samples during metabolism. The related bio-information can be widely used in areas such as insect prevention, disease diagnosis, criminal monitoring, fruit quality control, and food safety (Zhang and Li, 2010).

Analytical methods have recently been developed for the analysis of gaseous samples and for the investigation of volatile compounds that are found in the atmosphere surrounding the body, with a main principle of the methods to reduce the invasiveness of the sampling. Researchers are hopeful because with the detection of VOCs, particularly for diseases, a less invasive process can result in a timely diagnosis that can significantly help the prognosis. It is probable that the accelerated metabolism of tumor cells produce volatile compound patterns that can qualitatively and quantitatively differ from those released by healthy patients. Therefore, a pattern of measurable VOCs arising from living tissues could provide a volatile biochemical signature of essential biological processes like cell proliferation, growth arrest, and cell death (Pennazza et al., 2008).

The association between diseases and modifications of the airborne chemicals emanated from the body has been found in a variety of pathologies, particularly in many forms of cancer. The metabolism of cancer cells is greatly altered during their existence, with alterations of chemicals being larger around cancer tissues (D'Amico et al., 2008). Cancer draws out a large amount of complex processes in an organism and traces of the presence of the disease can not only be found in body compartments, but also in the cancer tissue. The accelerated metabolism and the cell death can give rise to non-specific volatile compound patterns. These patterns are easily distinguishable from those released by healthy tissues but unfortunately do not allow for a selective identification of the kind of cancer (Bartolazzi et al., 2010). Pennazza et al. (2008) explains two possible explanations behind the suspected occurrence of specific VOCs with tumor diseases. The first possible explanation is that the VOCs are generated by the emissions of specific volatile compounds from tumor cells and general metabolic compounds produced by the host as a consequence of oxidative stress caused by the cancer. Another alternative is that during

experiments conducted with diseased subjects, the VOCs connected with the disease are likely concealed by vast amounts of endogenous and exogenous factors, for example, drug intake and lifestyle.

Many studies are conducted on the relationship between VOCs and different types of cancers, as wells as their ability for detection. Matsumura et al. (2009) performed research on lung cancer odors based on studies showing that the presence of a tumor could produce specific metabolic or nutritional changes that could modify the production or release of VOCs. Based on a proof of concept study conducted twenty years ago that displayed effective olfactory detection of human bladder cancer by dogs, Balseiro and Correia (2006) investigated the phenomena behind VOCs and cancer detection. They hoped to determine if the volatile compounds produced by malignant tumors were related to alterations in human leukocyte antigen (HLA). It is widely known that there is a profile of volatile components in human odors associated with HLA expression and the authors believed that canine olfaction detection of human cancer is based on HLA molecules expression-dependent odor components. The beliefs were based on the facts that HLA expression is a source of unique odors present in body fluids and human cancer development is strongly related to alterations in HLA expression. Tumor transformation is commonly associated with different levels of the various HLA classes, which suggests that these HLA-olfactory cues of human cancer can be easily analyzed, bringing about a reliable diagnostic of cancer. Another type of cancer typically studied in relation to VOCs is lung cancer. Chen et al. (2007) analyzed lung cancer and determined the use of the VOCs for early diagnosis of the disease. They were able to conclude that VOCs in the culture medium differed from the virgin culture media with no cells. They also determined a correlation between VOCs in the metabolic

products of lung cancer cells and VOCs in the breath of lung cancer patients, hoping to validate these biomarkers for use in lung cancer detection.

Although biomarkers of all types (cellular, molecular, and volatile) are constantly being discovered, there is a large gap between the ability to discover potential biomarkers and the ability to validate these markers for use in a clinical setting (Zhang and Li, 2010). Fortunately, when using VOCs to aid in the detection of diseases, the actual compounds being emitted are not of utmost importance, it is more important to have a detection method with the ability to discriminate between the different forms of the disease.

Sensors for Detection

Health care technologies are currently geared towards non-invasive methods for early diagnosis, with chemical analysis being an effective alternative because of its ability to accomplish both non-invasivity and prevention. The chemical composition of biological materials is reflected in its volatile components and these components in addition to odor have been widely used in the past for medical diagnosis. In the past three decades, instruments such as gas chromatography (GC) and/or GC paired with mass spectrometry (GC-MS) have been utilized for the identification of molecules responsible for producing odors in specific diseases. More specifically, the GC-MS technology has the ability to allow a net separation and identification of compounds in complex mixtures (D'Amico et al., 2008). Although GC-MS technology is advantageous when quantifying and identifying individual volatile chemicals from a mixture, it is unable to indicate whether the compounds contain an odor or not. For this reason, the electronic nose (e-nose) was developed to improve and complement the techniques of GC-MS and to provide a better imitation of the human system for sensory analysis (Dutta et al., 2002).

Electronic Nose Technology

Before discussing the electronic nose, it is important to understand the mechanism behind the human olfactory system, as described by Wang et al. (2007), of which the electronic nose was developed to mimic. The primary sensory cells of the olfactory system, or the sensory neurons of the olfactory epithelium, express more than a thousand different types of chemical receptors. The olfactory receptor neurons can be classified as bipolar nerve cells. "From their apical pole, the neurons extend dendrite to the epithelial surface, where they expand cilia, which are specialized for odor detection. From basal pole of each olfactory receptor, neurons project a single axon to the olfactory bulb, where the axon forms synapses with neurons, such as mitral cells and granular cells, then relay signals to the olfactory cortex".

Electronic noses, or artificial olfactory systems, perform odor detection through the use of an array of broadly cross-reactive sensors in combination with pattern recognition methods. These artificial olfactory systems gained increased recognition in the early 1990s, due to the advances in computational capability, which added significant performance possibilities to pattern-recognition-based sensing technologies. There are assortments of electronic noses (e-noses) available that can differ in how the signal transduction is performed and also in the nature of the algorithms used to analyze the data retrieved from the sensor array. The variety of sensor modules that can be used in the technology include micro-machined cantilevers, conducting polymers, quartz crystal devices, and polymeric dielectric capacitors, to name a few. The pattern recognition algorithms employ statistically based methods like principal component analysis, partial least-squares regression analysis, and neutral network-based methods (Lewis, 2004). Although successful in distinguishing between odors, the comparison between an e-nose and a human nose is similar to comparing the eye of a bee with the eye of a human. The e-noses in use

today cannot replace intricate analytical technologies or odor panels, but supplements them both. It has several advantages when considering its mobility, price, and ease of use, giving it the ability to enter not only well-equipped laboratories, but also our daily lives (Rock et al., 2008).

Since its arrival on the market over twenty years ago, the e-nose has been used for a wide variety of applications. Baby et al. (2000) used an electronic nose to monitor environmental contamination. The nose used was a Modular Sensor System with two arrays of eight tin oxide and quartz microbalance sensors, which was found to be useful in discriminating two water contaminants and odorless insecticides at low concentrations. Naraghi et al. (2010) attempted to use an e-nose as a rapid method for screening the responses of skin disease bacteria to antifungal agents. The complex nose utilized in this study was based on a hybrid array of ten metal oxide silicon field effect transistor sensors and twelve metal oxide sensors, and a capacitance-based relative humidity sensor. The study showed the potential for using qualitative volatile patterns as a quick screening method for antifungal agents against microorganisms. Furthermore, NASA developed an e-nose using a collection of sixteen different polymer films that could detect an electronic change of 1 part per million. The goal of this e-nose was to detect hazardous leaks aboard the space station that humans would typically be unable to detect until the concentration increased, at which point the leak in the closed atmosphere of the space station would become dangerous (Miller, 2004).

In the biomedical field, one of the most important applications of the e-nose is its ability to detect diseases. Researchers all over the world are using different types of e-noses to detect many different types of diseases and illnesses. Thaler et al. (2008) made use of colorimetric sensor arrays compromised of chemo-responsive metalloporphyrin dyes, which are particularly suited to the detection of VOCs produced by bacteria. They believed that this e-nose technology

could be used to distinguish exhaled gas from patients with chronic bacterial sinusitis and those without. The results were encouraging with an accurate classification rate of 90%. Bernabei et al. (2008) performed a preliminary study on the possibility of an early and non-invasive diagnosis of urinary tract cancers using an e-nose. The urine headspace measurements were performed using eight quartz crystal microbalance gas sensors coated with metalloporphyrins. The results confirmed that there was a firm correlation between urine headspace and urological pathologies due to the correct classification of 100% of both healthy and ill patients. Based on the results by numerous authors, the electronic nose technology looks to be very promising for the use of these sensors as detection and screening methods.

Cyranose 320

One of the commonly used electronic noses for biomedical research is the Cyranose 320 (C320). The C320 is a gas detector configured as an array of 32 sensors made from conductive carbon black material that is uniformly blended throughout a specific non-conducting polymer and deposited as a thin film on an alumina substrate. A chemoresistor is created due to the films lying across two electrical leads. Polymers with a wide variety of properties were chosen to permit the array to differentiate between many types of vapors. When exposed to a vapor-phase analyte, the sensor matrix swells up in a way very similar to the olfactory system within the human nose. The increase in volume causes an increase in resistance due to the conductive carbon black pathways through the material being broken. The sensor response is then measured as a bulk relative resistance change. Because each sensor in the array contains a distinctive polymer, there will be a reproducible combination of resistances or ‘smellprint’ for each vapor mixture. The final step after sampling the analyte is a self-purge, at which point the polymer off-

gasses and ‘dries out’ causing the films to shrink. The conductive pathways are then reestablished and the sensors return to their initial resistances (Li, 2001).

Use of the C320 entails a training session when the user exposes the sensors to each type of sample that will be encountered during testing. This training creates a base group so that all future samples can be compared. During the sampling, the tip of the device is placed near the sample and ‘Run’ is pressed. Air referencing, vapor sampling, sensor measuring, and data processing are automatically controlled. If the sample matches one of the pre-trained samples, the device will report the identity of the sample, and if the device is unable to recognize the sample, an ‘unknown’ reading is given (Heimerl, 2001). The former president of Cyrano Sciences describes the approach to their C320 technology as being closely related to the human nose approach. “When you go around smelling, your nose isn’t trying to figure out the components of what you’re smelling. It’s making a decision based on a pattern recognition” (Wayner, 1999).

Current Research

In terms of detecting illnesses and diseases, the C320 has shown great potential for becoming a widely used method in clinical applications. Extensive research has been conducted on the use of the e-nose with patients exhaled breath samples. Using the C320, the exhaled breath of patients with lung cancer was shown to have distinct properties that could be used to help manage and detect the cancer (Machado et al., 2005). Similarly, Dragonieri et al. (2007) used the C320 to sample exhaled breath, however they were testing to see if the e-nose could discriminate asthma from healthy controls and also see if the e-nose could distinguish different degrees of asthma severity. The results showed that the C320 could effectively discriminate

between the breath of asthma patients and the breath of healthy patients. Studies have also been conducted using the C320 and sampling from the headspace of flasks to discriminate illnesses.

Boilot et al. (2002) used the C320 to test the headspace of pure lab cultures and blood agar plates for the bacteria causing eye infections and the bacteria causing ear, nose, and throat infections, respectively. The results showed the potential applications for the C320 when paired with neural network-based predictors, rapid screening and early detection of bacteria associated with the infections, and the development of the C320 system as a tool in medical healthcare. Shykhon et al. (2006) performed similar experiments for the diagnosis of ear, nose, and throat infections. The researches placed swabs collected from infected areas and placed them in culture medium to be tested. The C320 was able to identify specific bacterial pathogens with accuracy and speed, even in small sample quantities. The results were also compared with results from microbiological analysis and the C320 diagnosis was correct in 88.2% of the cases.

Unfortunately, limited research was found on the use of the C320 for the detection and discrimination of CaP cell lines in vitro. However, a study was performed by Gendron et al. (2007) that discussed the discrimination of upper aerodigestive tract tumor cell lines in vitro with the C320. Although this study uses cells from the upper aerodigestive tract, the goal to distinguish between normal and tumor cell lines is similar to one of the goals of this research with CaP. The results show great promise due to the C320's ability to distinguish between several tumor and normal cell lines. Gendron et al. (2007) hypothesized that the C320 had the potential to be a helpful screening tool for a variety of upper aerodigestive tract cancers. Because it does not require the dominant organic compounds released from the breath of cancer patients to be identified, the sensor array technology has the flexibility to discriminate between cancers associated with a broad spectrum of volatile molecules.

With the prevalence of CaP in our country, alternative diagnostic methods are necessary to provide less-invasive and rapid detection of the disease. The current methods of PSA blood test, DRE, and biopsy have been successful thus far; however, there is a need for more effective methods due to the high possibility of misdiagnosis when using the current methods. Researchers have been diligently investigating the disease in hopes of discovering new biomarkers that could help with the early detection of the disease. VOCs are being studied in many types of diseases to determine what type of volatiles are given off during disease progression and how these VOCs can be detected. Studies have shown that the use electronic nose technology has been successful in detecting VOCs in many types of diseases however few studies have been conducted on the use of this technology with CaP cells in culture. If proven to be successful, the electronic nose technology could be studied further to determine its ability to detect a multitude of cancers not only in culture but also from bodily fluids.

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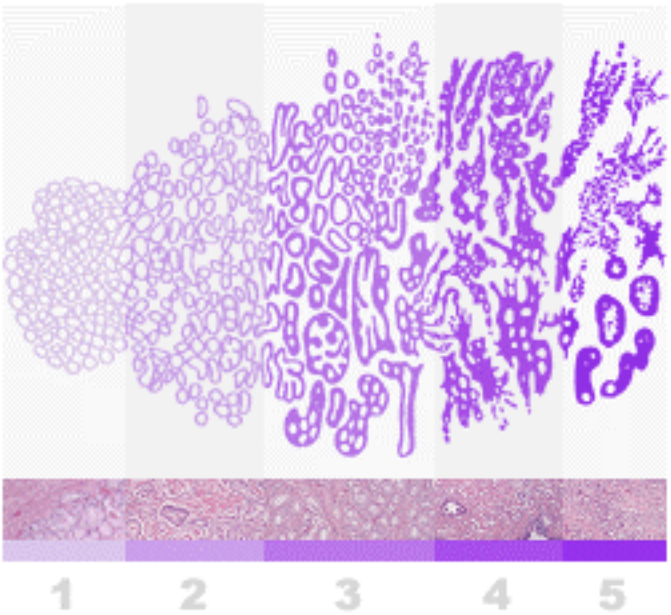

Figure 2.1 Images of cells for each Gleason score (Wetzel and Becich, 2000)

Chapter 3: Electronic Nose discrimination of prostate cancer cells (PC-3 and DU-145) from non-tumor forming cells (SVHUC) with varying cell population and incubation period

Abstract

With prostate cancer being a widespread disease in the United States, it is necessary that new detection methods be investigated due to the controversy surrounding current methods. In this study, an electronic nose was utilized to determine its ability to discriminate between prostate cancer cells (PC-3 and DU-145) and non-tumor forming cells from the urinary tract (SVHUC). Additionally, the effects of cell population and incubation period on the electronic nose's discrimination ability will be investigated. For each cell line, two initial cell populations of 75,000 and 150,000 cells were sampled with the device after different incubation periods of 2, 8, 12, and 24 hours. The results were analyzed using multivariate methods of principal component analysis (PCA) and canonical discriminant analysis (CDA). When analyzing the cell lines individually, taking the incubation periods into account, by 8 hours sufficient volatiles were produced to be detected by the electronic nose for discrimination. When looking at the overall differences between the three cell lines, the PCA and CDA plots showed them to be entirely different from one another. The smallest variation between the three cell lines was seen between PC-3 and DU-145, the two prostate cancer cell lines. Cross validation results showed 0% misclassification when predicting how the data would be classified in the future given the current model. These results demonstrate that tumor cells emit different volatiles than those of non-tumor forming cells, differences that can be distinguished using electronic nose technology. Finally, PCA plots show that after 2 hours of incubation, the cells had produced sufficient volatiles for the electronic nose to discriminate between the three cell lines, proving the importance of incubation period when discriminating cancerous and non-cancerous cells.

Introduction

Prostate cancer (CaP) is the most frequently diagnosed cancer among men in America with approximately 192,000 men being diagnosed in 2009 and 27,000 men expected to die from the disease (Wolf et al., 2010). With such a prevalent disease it is necessary to determine the most effective means of detection to successfully decrease the number of men dying from CaP. Unfortunately, there is little agreement on a universal screening method for CaP. Both the American Urological Association and the American Cancer Society suggest using a combination of the digital rectal exam (DRE) and serum prostate-specific antigen (PSA) levels to screen men who are potentially at risk for the disease (Herman et al., 2009). Although PSA is currently the best CaP biomarker available, it is not perfect. Much controversy has been raised due to the lacking sensitivity and specificity of the serum to correctly detect the presence of CaP. The many issues surrounding the accuracy of PSA have led researchers to investigate and explore additional biomarkers and devices that could be beneficial in detecting CaPs (Nogueira et al., 2010).

One type of biomarker being explored in CaP research is volatile organic compounds (VOCs). VOCs are chemical compounds produced by cell catabolism and found as gases within humans. Studies have shown that changes in malignant cells during tumor development lead to peroxidation of cell membrane components that later leads to the release of VOCs (Bjartell, 2010). To examine VOCs expelled from humans, elaborate sensor technology has been developed in the form of electronic noses to detect and discriminate the production profiles of volatiles compounds. One of the first models of the electronic nose was developed in 1982 in attempt to detect different volatile compounds by imitating the various stages of the human olfactory system resulting in volatile odor recognition (Turner and Magan, 2004). For decades,

many types of electronic noses have been used in the field of medicine to help researchers detect a wide variety of diseases. Fend et al. (2006) used an electronic nose with 14 conducting polymers to detect *Mycobacterium tuberculosis* in the headspaces of culture and sputum samples and results showed that the nose had the ability to successfully complete this task. Additionally, Mazzone (2008) looked into multiple studies that investigated the production of VOCs from the headspace of gas of lung cancer cells lines using mass spectrometry. The researcher determined that the VOCs from the culture of lung cancer cells differed from virgin culture medium and control cell cultures.

Few studies have been conducted with an electronic nose analyzing the headspace from cells in- vitro and more specifically, analyzing CaP cells in-vitro with the electronic nose technology. However, the studies that have been completed suggest that the electronic nose has the potential to discriminate between different cell lines in-vitro. The objectives of this current study are to determine the ability of a conducting polymer-based electronic nose to discriminate between two CaP cell lines and one non-tumor forming urinary tract cell line with different cell populations and incubation periods.

Materials and Methods

The cell lines, materials, and equipment used to maintain and prepare the cells in this study were obtained from Department of Biomedical Sciences & Pathobiology at the Center for Molecular Medicine and Infectious Disease. All testing was conducted in a biosafety level 2 laboratory at the Integrated Life Sciences Building at the Corporate Research Center in Blacksburg, VA.

Cell Lines

This study was conducted using three cells lines, SVHUC, DU-145, and PC-3. One of the greatest ways to study tumorigenesis in cancers related to the human bladder is the use of human urothelial cells. SVHUC is a normal human urothelial cell line that has been immortalized by the simian virus and grows as epithelial layers in vitro but does not form tumors (Wang et al., 1995). DU-145, the first CaP cell line generated in tissue culture, are epithelial cells developed from the brain of a 69-year-old white man with CaP and lymphocytic leukemia. Additionally, this cell line does not express the prostate-specific antigen (PSA). PC-3 cells, which were first accounted for in 1979, were derived from a lumbar vertebral metastasis in a 62-year-old white man and are composed of undifferentiated malignant cells (Sobel and Sadar, 2005). Although current studies have shown contrasting result as to the expression levels of PSA in PC-3 cells, the majority of researchers believe that PC-3 retains the expression of PSA (Dozmorov et al., 2009). This is one of the main reasons that this cell line was included in the study, to be compared to DU-145, which does not express PSA.

Aseptic Technique

All of the laboratory studies were completed using the aseptic technique for cell culture as described by Coté (2001). Before gathering materials, latex surgical gloves and laboratory coat were worn to ensure bodily protection. The majority of the work for the experimentation was completed in a ThermoScientific Forma 1400 Series Biological Safety Cabinet. The workspace was cleared of unnecessary equipment and wiped down with 70% ethanol. When new materials were being brought into the cabinet, they were also wiped down with 70% ethanol. The items in the workspace were arranged in a logical pattern from clean to dirty to avoid passing contaminated materials over clean and sterile items. When disposable items were finished being

used they were properly disposed of in the appropriate Sharps Biohazard Collection Container. Finally, all other materials were returned to their proper location and the cabinet was wiped down with 70% ethanol.

Cell Culture

In order to ensure viability of the cell lines, the cells were cultured on a regular basis until experimental testing began as well as after testing to ensure that the cells would be available if other experiments needed to be performed. SVHUC cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% heat inactivated fetal bovine serum (FBS), and antibiotics (100 μg/ml penicillin and 0.1 μg/ml streptomycin (Invitrogen, Carlsbad, CA). DU-145 cells were grown with Gibco Minimum Essential Media (MEM), 15% heat inactivated FBS and antibiotics. PC-3 cells were grown with Hams F-12 nutrient mixture, 15% FBS, and antibiotics. When not in use, growth media and other solutions were kept in a ThermoScientific REVCO refrigerator and then placed into a ThermoScientific Microprocessor Controlled 280 Series Water Bath at 37°C for at least 15 minutes prior to use by the cells. All cells were grown at 37°C with 5% CO_2 in a ThermoScientific Forma Series II Water Jacketed CO_2 Incubator until it was time for them to be re-cultured or tested.

The proper cell culture methods used in this experiment were obtained from Simon (2004) based on his methods and protocols for cancer cell culture. Corning cell culture flasks of size 25 cm^2, 75 cm^2, and 150 cm^2 were used depending on the cell population.

General Cell Maintenance

To maintain each cell line when the cells were not being tested, the cells were carefully removed from the incubator and examined every 24 hours with a Carl Zeiss Invertoskop 40C Microscope to monitor the cell growth. If the cells had been growing for at least three days and

had not become confluent, the media was changed. First, the old media was removed from the flask with a sterile Corning Incorporated Costar Stripette and discarded. 5ml of phosphate-buffered saline (PBS) was then added to the flask with a stripette and removed and discarded. Washing with PBS was completed twice to ensure the successful removal of any traces of serum from the media that could negatively affect the remainder of the cell culture process. Trypsin EDTA was then added to the flask for the dissociation of adherent cells into a single cell suspension. The amount of trypsin EDTA used depended on the flask size being used as seen in Table 3.1. The solution was swirled across the monolayer to guarantee that the trypsin EDTA effectively reached all the cells. The flask was then returned to the incubator for 3-20 minutes depending on the cell line being used with DU-145 needing approximately 2 minutes, PC-3 needing approximately 4 minutes, and SVHUC needing approximately 15 minutes. The cells were checked throughout the detachment period to monitor the dispersing of the monolayer because if left in trypsin for too long, the amount of viable cells begins to decrease. Once all of the cells detached, fresh media was added to the flask with a stripette using media amounts given in Table 3.1. The flask was then labeled with the passage #, date, and placed back into the incubator.

If during the initial microscopic observation of the cells under the Invertoskop the cells appeared to reach confluency, the cells were transferred into a larger flask for expansion. After detachment with trypsin EDTA, the appropriate amount of media to fill the new larger flask was obtained with a stripette and dispensed into the original flask currently holding the cell suspension. After the flask was slightly shaken to ensure that all of the cells moved into the media, the cell solution was pipetted into the larger flask and properly labeled with the date,

passage #, cell type, and experimenter's initials. The flask was finally placed back into the incubator to continue growth.

Cell Counting

Based on the nature of the experiment being conducted, after the cells detached and fresh media was added, the cells were placed into a Corning 50 ml centrifuge tube in preparation for the cell count. Two methods for cell counting were used in this experiment: hemocytometer and cellometer. The first method utilized the Reichert Bright Line Hemocytometer (Buffalo, NY). The hemocytometer was prepared by carefully placing a clean coverslip over the hemocytometer grid. In the biosafety cabinet, 100µL of cell suspension was removed from the centrifuge tube with a ThermoScientific Finnipipette F1 and placed into an eppendorf tube. 100µL of Trypan Blue solution composed of 5 ml HyClone Trypan Blue Solution + 45 ml PBS was also added to the eppendorf tube, thereby creating a 1:2 dilution. Using a pipette, a small amount of the cell+Trypan blue solution was removed from the eppendorf tube. The pipette was then placed at the edge of the coverslip and some of the contents were slowly expelled filling the fluid into the chamber by capillary action. The hemocytometer was then observed under the Invertoskop. The cells were counted from each of the four sixteen-square corners chambers and the average was taken from those four values. After the cell count was obtained, Equation 1 was used to determine the cell concentration.

$$Average \times 2 \times 10^4 = Cell\ conc.\ (cells/ml) \quad \text{(Eq.1)}$$

The cellometer method utilizes the Cellometer Vision (Nexcelom Bioscience LLC, Lawrence, MA) that combines brightfield microscopy and fluorescence images to generate a cell count. After fresh media was added to the cells and placed into a 50ml centrifugal tube, 20µl of the cell suspension was removed with a pipette and carefully inserted into one end of the disposable

counting chamber as seen in Figure 3.1A. The chamber was then inserted into the cellometer. The cellometer was connected to a computer, which allows the sample to be given a name and then counted as seen in Figure 3.1B. The device then output the live cell count, mean diameter (micron), cell concentration (cells/ml) and the data was recorded.

Sample Preparation

After the cells were properly cultured and counted, they were set up in preparation for testing. The desired cell populations for the experiment were 75,000 cells and 150,000 cells. These cell populations were chosen based on the average seeding densities for all cell types and recommended cell population for a 25 cm^2 flask (ATCC, 2010; Lonza, 2010). Based on the cell concentration determined by the counting method used, the amount of cell suspension used was calculated based on equation 2.

Cell conc.(cells/ml) / Desired total(cells) = Amount(ml) (Eq. 2)

Using a stripette, ten 25 cm^2 flasks were filled with the appropriate amount of media used to total 5ml when taking into account the cell solution that was later added. For example, if calculations determined that 750µL of cell solution was necessary for the 150k samples, then 4.25ml of media would be added to the flasks. The flasks were then labeled accordingly, with five labeled as'75k' and the remaining five labeled as '150k'. The appropriate calculated cell solution amount was then added to the flasks with their corresponding cell total. All ten flasks were then placed into the incubator until testing began.

Electronic Nose

The electronic nose apparatus used for the entirety of this study was a conducting polymer based electronic nose (Model: Cyranose 320, Smiths Detection, Pasadena, CA). The Cyranose 320 consists of a sensor array of 32 conducting polymer sensors and as the array is

exposed to the sample, the volatiles react with each sensor causing them to reversibly swell. On an individual level, each sensor responds to the sample differently, but when looked at as a whole, the response of the array is identified as a unique smellprint.

Cyranose320 Settings

The Cyranose 320 was connected to a computer to utilize the PCnose (Smiths Detection, Pasadena, CA) software. While using the software, parameters for the electronic nose were examined and adjusted. Additionally, the software allowed for a visual representation of how the sensors were reacting to the sample. Figure 3.2 shows the internal settings used by the Cyranose 320 during its sampling of the cells. Before the electronic nose was exposed to the experimental samples, the device was allowed three to five preliminary runs from the air in the biosafety cabinet and purges to allow for any residual volatiles from previous studies to be eliminated. This preliminary data was then deleted prior to completing the experimental sampling.

Testing

The Cyranose 320 was utilized at different incubation periods after the initial culture and trained to determine how varying cell population and maturity affect the sensor's ability to discriminate between the cells. The cells were tested with the Cyranose 320 at 2hr, 8hr, 12hr, and 24hr. The electronic nose was connected to an accompanying computer and all of the proper cords and cables were assembled and connected. At each time period, the flasks were removed from the incubator and placed in the biosafety cabinet for testing. For each of the cell populations studied (75k and 150k), each of the five flasks were sampled once by placing the 'snout' of the electronic nose carefully into the mouth of the flask as seen in Figure 3.3. The flasks were then put back into the incubator until the next testing time. For each of the two cell populations studied, there were five samples taken at each of the four time periods, totaling

twenty samples each in the 24-hour time span. This experiment was performed in triplicate creating sixty samples for both of the cell populations of the cell line being tested. Figure 3.4 illustrates the setup used for all experimental trials with PC-3, SVHUC, and DU-145 cell lines.

Statistical Analysis

Although the Cyranose 320 comes equipped with its own statistical software, PCnose® (Cyrano Sciences, Inc., Pasadena, CA), when analyzing large amounts of data, the software has some disadvantages due to its ability to analyze only six sample classes with a maximum of ten exposures each (Whysong 2009). To avoid the constraints presented with the PCnose® software, JMP® (SAS Institute, Inc., Cary, NC) statistical software was used. The data was classified into classes based on cell type, incubation time, and cell population. The Cyranose's ability to discriminate between different prostate cell lines with different cell populations and maturity were analyzed using JMP's multivariate statistical methods (principal component analysis and canonical discriminant analysis).

Results and Discussion

While testing the different cell lines with the Cyranose320, PCnose® software was utilized to observe the instrument and how the sensors were reacting to volatiles given off by the cells. The scrolling strip chart shows how each sensor is reacting to the volatiles in the flask in terms of time vs. sensor response. The PCnose® software also produces a smell print for the given sample. Taking into account the entire class analyzed, the smell print includes all 32 sensors and shows the variation of the training response based on the change of resistance incurred during sampling. As previously discussed, the PCnose® software is equipped with the ability to complete statistical analyses however, the inability to increase class sizes and arrange data into appropriate classes makes this specific software inappropriate as an analysis tool for the

given study.

Because the PCnose® software was unsuitable for this experiment, the data retrieved from the Cyranose320 was analyzed using JMP® and multivariate techniques consisting of principal component analysis (PCA) and canonical discriminant analysis (CDA). The purpose of PCA is to develop a small number of independent linear combinations (principal components) of a set of variables that maintain as much of the information in the original variables as possible. Similar to PCA, CDA examines the correlation between a linear combination of a set of Y variables and a linear combination of a set of X variables (SAS, 2010). These methods will be used to analyze how the Cyranose320 reacts when exposed to the volatiles from PC-3, DU-145, and SVHUC cells after 2, 8, 12, and 24 hour incubation periods and with different initial cell populations of 75,000 (75k) and 150,000 (150k). The software will also compare the cell lines against one another to determine the Cyranose320's ability to discriminate between the three cell lines.

PC-3

The first CaP cell examined in this study was PC-3 cells, which are believed to express PSA (Dozmorov et al. 2009). Figure 3.5A shows the PCA plot for all three trials of the 75k PC-3 cells after 2, 8, 12, and 24 hours. As seen in the plot, the cells tested after 2 hours form the most distinct cluster. The cells tested after 8 hours also seem to form the makings of a cluster but then disperse into the cells tested after 12 and 24 hours, which appear to cluster with one another. The PC-3 cells tested after 2 hours may have the most distinct cluster because the cells have not began to significantly metabolize due to their recent culturing. As the cells begin to metabolize, they progress farther away from the initial 2 hour cluster. Although the last three incubation periods show little distinction between one another, 96.2% of the variation can be credited to the first three principal components with 89.8% of the variation being attributed to principal

component 1 as seen in Table 3.2. The CDA plot in Figure 3.6A shows similar results with incubation periods of 2 and 24 hours having the most distinctive from one another based on their location in the plot. It is understandable that the clusters for 8 and 12 hours are closely related due to the small time frame in between their testing. Additionally, the analysis showed there was 0% misclassification between the classes illustrating that the Cyranose would be able to re-categorize each class based on the data retrieved during the training.

The analysis of 150k PC-3 cells showed similar results as seen in Figure 3.5B. However, cluster formation for 2 hours and 8 hours are more distinct, with 12 and 24 hours being clustered with one another. The higher cell population used can help explain the differences in cluster formation because with more cells, metabolites could be produced at higher rates. In contrast to the 75k cells, Table 3.2 shows that 92.9% of the variation was accounted for by principal component 1 with the first three principal components accounting for 97.0% of the variation. The CDA plot seen in Figure 3.6B corroborates the results acquired from the PCA plot, with all 4 incubation periods having a unique position on the plot. Similarly to the 75k cells, there was 0% misclassification when analyzing the 150k cells.

It was expected that the two cell populations would exhibit similar responses due to the cells being of the same origin and emitting similar volatiles. The differences between the populations can most likely be attributed to the fact that with different cell populations, different amounts of volatiles are being emitted, thereby causing different sensor responses. For both cell populations, it is clear that by 12 hours of incubation, the cells had metabolized to a distinct level that was sufficient for classification by the electronic nose.

DU-145

The next CaP cell line investigated in this study was DU-145, which does not express PSA. Based on the PCA plot shown in Figure 3.5C, the 75k cells incubated for 2 hours form a clear cluster. Similar to the cells tested after 2 hours, the cells tested after 8,12, and 24 hours tend to gather within their respective group. However, these three classes are clearly distanced from the cells tested after 2 hours and seem to form a larger cluster encompassing all three groups. These results show that after 8 hours of incubation, similar amounts of volatiles are being given off. The majority of the variation within the groups, 88.6%, is accounted for in principal component 1 as seen in Table 3.2 and 95.6% of total variation is accounted for in the first three principal components. The CDA plot shown in Figure 3.6C depicts similar results with all four classes of incubation periods forming unique clusters. Additionally, the clusters representing the 2hour testing are shown to have a distinct difference based on the large distance seen between that class and the remaining three classes. This difference can most likely be attributed to the short time period the cells were given to metabolize and produce sufficient volatiles for effective sensing by the Cyranose. Due to distinct differences between the classes, the analysis was able to perform 0% misclassification when predicting how future classes would respond.

When 150k DU-145 cells were observed, comparable results were produced as seen in Figure 3.5D. The cells tested after 2 and 8 hours form unique clusters while although the 12 and 24 hours classes are easily distinguishable, their 2 respective clusters experienced some overlapping. After the 12 hour incubation period, there was a steady increase in volatiles that was recognizable by the Cyranose320. A larger majority of the variation of 95.1% can be attributed to principal component 1 as seen in Table 3.2 while 97.6% of the total variation is encompassed in the first three principal components. The CDA plot in Figure 3.6D successfully

groups each incubation class without any overlapping between groups. Some variation is observed within the clusters of each class however the 8 hour incubation shows the best cluster formation within the group. Moreover, the analysis for the 150k cells was also able to report a 0% misclassification when predicting future responses. All of these plots help to convey the ability of the Cyranose320 to distinguish between cells with different populations and different incubation periods. The plots show an understandable distinction between populations of 75k and 150k as previously observed with the PC-3 cells.

SVHUC

The final cell line tested with the Cyranose 320 for discrimination analysis was SVHUC, which is taken from normal human urothelial cells that have been immortalized by the simian virus and do not form tumors. 75k SVHUC cells were analyzed with a PCA plot seen Figure 3.5E and four separate clusters were formed for each of the classes based on incubation periods. Testing after 2 hours of incubation resulted in a cluster formation exclusive to the other three clusters. The 8 hour incubation class also formed a distinct cluster however; the class was more closely related to the groupings from the 12 and 24 incubation periods as was typically seen when analyzing the other cell lines. Principal component 1 was found to be responsible for 91.9% of the total variation and 97.1% of the total variation is expressed in the first three principal components (Table 3.2). The CDA plot in Figure 3.6E also expresses the distinction between the 75k SVHUC cells tested at the different incubation periods with no overlapping between the four classes. The canonical analysis resulted in 0% misclassification for the prediction of future data sets given the current data.

The analysis of 150k SVHUC showed similar results to those of the 75k cells, which was expected based upon the results of the other cell lines. The PCA plot in Figure 3.6F shows a clear

distinction between the 2hour incubation class and the 8hour incubation class. However, although the 12 and 24 hour classes do not individually form definite clusters, slight separation can be seen between the two classes. Research has shown that tumor cell lines are characterized by a volatile compound pattern (Santonico et al. 2009) and it is possible that in general, non-tumor forming cell lines emit lower levels of volatiles. Table 3.2 shows that only 85.3% of the variation can be credited to principal component 1, which has been the lowest percentage recorded during the analysis of all three cell lines in regards to principal component 1. The principal components represent the sensors of the device and the lower percentage for principal component 1 could be explained by the higher population of SVHUC cells emitting volatiles at a higher level than the other cell lines. In turn, this could have required the use of principal components 2 and 3 to have an increased use in accounting for the variation. Alternatively, 95.3% of the variation was attributed to the first three principal components, which is similar to the previous cell lines. However, when referring to the CDA plot shown in Figure 3.6F, all four classes show distinct variation from one another, with 8 hour incubation having the most unvarying cluster. Additionally, the analysis also resulted in 0% misclassification when predicting future data sets. For both cell populations, the 2 hour incubation period forms the most distinct cluster. By 8 hours, the cells have been able to metabolize and give off enough volatiles for the electronic nose to pick on the differences between the amount after 2 hours and the amounts between 8 and 24 hours.

Cell Type Discrimination

The cell lines were analyzed against themselves to observe how the Cyranose's responses were affected by varying cell line and incubation period. After this analysis, the three cell lines

were compared to one another to determine the ability of the Cyranose to discriminate between CaP cells (PC-3 and DU-145) and non-tumor forming cells from the urinary tract (SVHUC).

The comparison was completed compared all three cell lines as seen in the CDA plot in Figure 3.7. All three cell lines are shown to be independent of one another with no part of the clusters overlapping. This graph illustrates that the Cyranose 320 does indeed have the ability to distinguish between cancerous and non-cancerous cell lines as hypothesized. During the 24 hours of incubation, the cells gave off volatiles that were adequate for the electronic nose to perform a successful discrimination Furthermore, there was 0% misclassification explaining that in terms of predicting future classifications, the three cell lines had such a large variation that there would be no misclassification of future samples. Figure 3.8 shows into the discrimination of the three cell lines and clearly shows the differences between the cell lines. The PCA plot clearly illustrates that PC-3 and DU-145 are more closely related than either are related to SVHUC. Although this observation was thought to be obvious because they are both CaP cell lines, it was uncertain if the Cyranose 320 would be able to detect any differences between the cell lines in culture. With PC-3 containing the prostate-specific antigen, this could be one reason for an evident difference between these two CaP cell lines. Eighty-seven percent of the variation observed can be attributed to principal component 1 as seen in Table 3.3. However, it was necessary to use more principal components to explain a larger portion of the variation, with five principal components accounting for 97.3% of the total variation. The larger number of principal components means that it was necessary to use more sensors to make a more clear discrimination of the cell lines.

Based on results from the hourly incubation of each cell line showing that between 8 and 12 hours, sufficient volatiles were produced for recognition by the device, PCA plots were

developed to determine how effective the electronic nose was at discriminating between the three cell lines after each of the time periods. The principal component analysis began with the 2 hour incubation period and as seen in Figure 3.9 even at the earliest testing period of 2 hours, sufficient metabolites had been produced to discriminate between the cell types. These results show the importance of incubation period in discriminating between cancerous and non-cancerous cell lines.

The overall discrimination results can be corroborated by Gendron et al. (2007) and their findings that an electronic nose was able to differentiate various tumor cell lines from normal cells in vitro. The researchers utilized canonical distributions to determine how the Cyranose320 was able to classify different types of cells in-vitro. The results found that tumor cell lines of adenocarcinoma, squamous cell carcinoma, and mesothelioma were dissimilar from one another while also different from normal fibroblasts and smooth muscle cells. Their results are similar to the results of this study in that CaP cells were shown to be different from one another while also being different from non-tumor forming urinary tract cells.

Conclusions

With CaP being so dominant and widespread in today's society, it is imperative to find alternate methods of detection. The Cyranose was utilized in this study to analyze the volatiles present in two CaP cell lines, PC-3 and DU-145, and a non-tumor forming urinary tract cell line, SVHUC. The instrument was exposed to two different cell populations after four different periods of incubation to determine how the electronic nose would respond to volatiles under these conditions. When looking at how the Cyranose senses cell lines individually, they all demonstrated similar trends with the electronic nose being able to differentiate between the cells tested after 2, 8, 12, and 24 hours. The farthest distinction was seen at the 2 hour incubation time

demonstrating that by 8 hours sufficient volatiles had been produced by the cells to be recognized by the electronic nose. For future studies, in order to see how cell maturity plays a further role in the Cyranose's ability to sense the volatiles, it will be necessary to expand the incubation time periods to 72 hours at which most cell lines are close to reaching confluency. The longer time span would clearly convey how the sensors are reacting to larger cell populations. After investigating how cell populations of 75,000 and 150,000 affect the sensors ability to distinguish between cell lines, it is clear that initially there is a distinction between the two populations. However, after the 2 hour testing, the cells appear to be giving off the same aroma just at different intensities, proving that 2 hours is enough time to produce volatiles to be discriminated by the electronic nose. To determine the true affects of cell population on the Cyranose's ability to discriminate between cell lines, it will be necessary to discard the factor of maturity and sample the cells immediately after being cultured in future studies.

When focusing on the analysis of each cell line compared against one another, the Cyranose was an effective tool for distinguishing types of CaP as well as non-tumor forming cell lines. All three cell lines were shown to be different from one another with the greatest difference being seen between the two CaPs and the non-tumor forming cells. This provides evidence that while in culture, cells give off volatile compounds that are distinguishable by methods such as an electronic nose. The two CaP cells were also shown to be different from one another possibly due to the presence of PSA in PC-3 cells and not in DU-145. Age, diet, or testosterone levels of the patients from which these cells lines were removed could also be an explanation for the differences seen between the two CaP cells. Multivariate analysis shows that after the earliest incubation period of 2 hours, the electronic nose was able to discriminate

between the three cell lines proving the importance of incubation period when performing in-vitro discrimination.

Based on all of the results from this study, the hypothesis can be accepted because the Cyranose320 has the ability to discriminate between the three cell lines tested. This study was one of the first studies to date investigating the use of an electronic nose in conjunction with CaP cells in culture, and the positive results reported warrant more studies to be conducted in this area. Although this study can not report the Cyranose 320 as an effective means of detecting CaP in patients, the ability of the device to differentiate between CaP cells and non-tumor forming cell lines shows promise for future CaP studies, detection methods, and biomarker discovery.

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Table 3.1 Trypsin EDTA and media amounts used depending on flask size

Flask Size	TrypsinEDTA (ml)	Media (ml)
25 cm^2	1	4
75 cm^2	3	7
150 cm^2	5	20

Table 3.2 Variations of tested cell lines shown by principal components 1-3

PC-3	Number	Eigenvalue	Percent	Percent	Cum Percent
	1	28.7463	89.832		89.832
75k	2	1.6570	5.178		95.010
	3	0.3948	1.234		96.244
PC-3	**Number**	**Eigenvalue**	**Percent**	**Percent**	**Cum Percent**
	1	29.7287	92.902		92.902
150k	2	1.1282	3.526		96.428
	3	0.1925	0.602		97.030
DU-145	**Number**	**Eigenvalue**	**Percent**	**Percent**	**Cum Percent**
	1	28.3582	88.619		88.619
75k	2	1.3822	4.319		92.939
	3	0.8760	2.737		95.676
DU-145	**Number**	**Eigenvalue**	**Percent**	**Percent**	**Cum Percent**
	1	30.4218	95.068		95.068
150k	2	0.5588	1.746		96.814
	3	0.2622	0.820		97.634
SVHUC	**Number**	**Eigenvalue**	**Percent**	**Percent**	**Cum Percent**
	1	29.1339	91.044		91.044
75k	2	1.4466	4.521		95.564
	3	0.5068	1.584		97.148
SVHUC	**Number**	**Eigenvalue**	**Percent**	**Percent**	**Cum Percent**
	1	27.3094	85.342		85.342
150k	2	2.8116	8.786		94.128
	3	0.3614	1.129		95.257

Table 3.3 Principal components accountable for variation in discrimination of three cell lines

Number	Eigenvalue	Percent	Percent	Cum Percent
1	27.8487	87.027		87.027
2	1.7346	5.420		92.448
3	0.9903	3.095		95.542
4	0.3432	1.072		96.615
5	0.2143	0.670		97.284

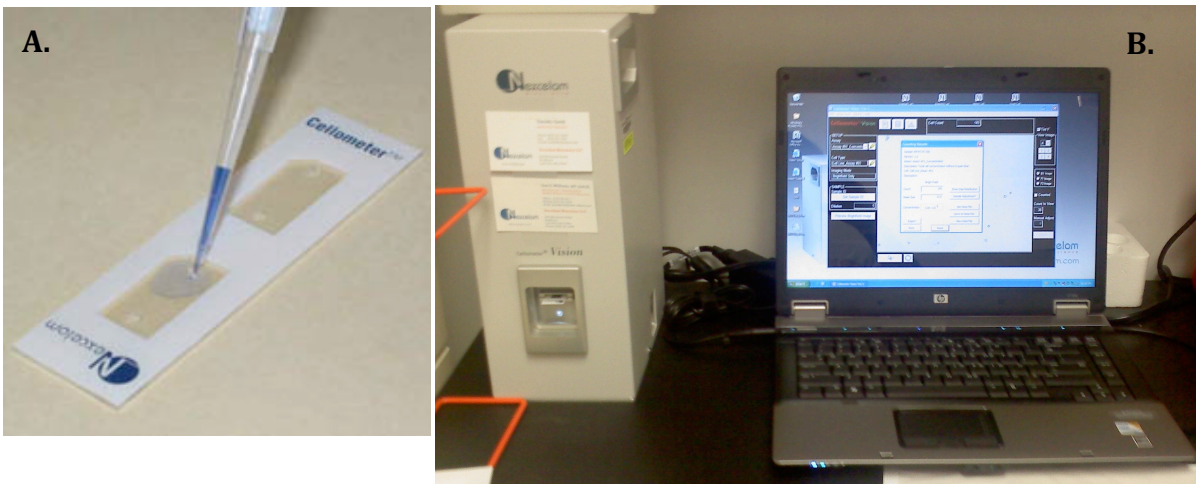

Figure 3.1 Cellometer instrumentation and Computer Setup

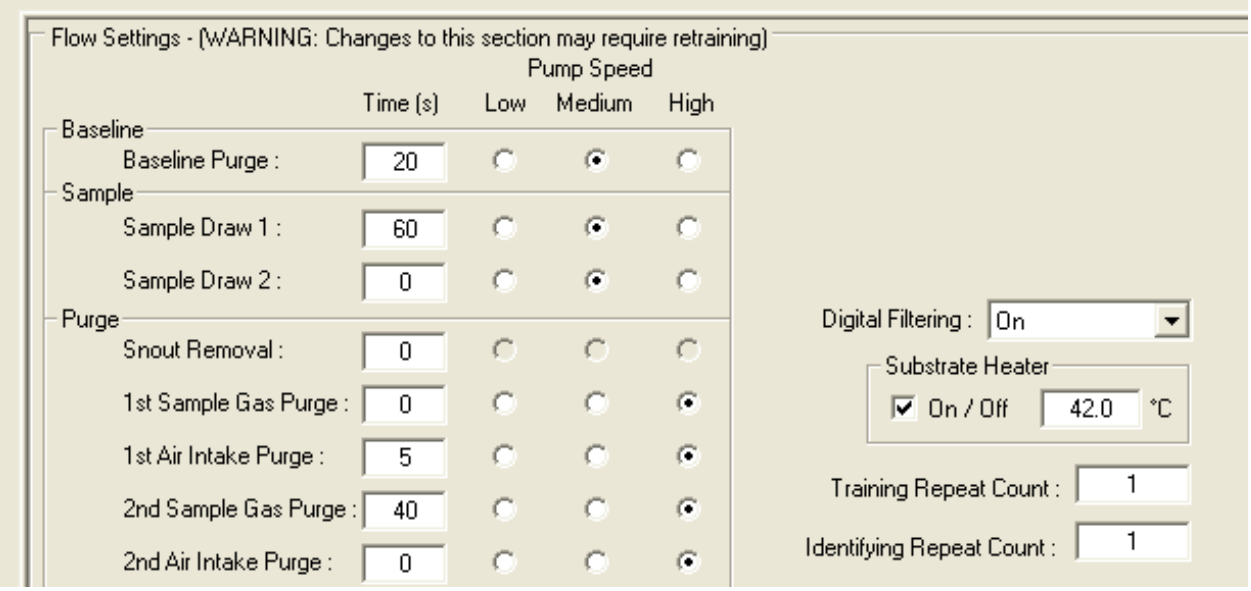

Figure 3.2 Cyranose 320 Flow Settings

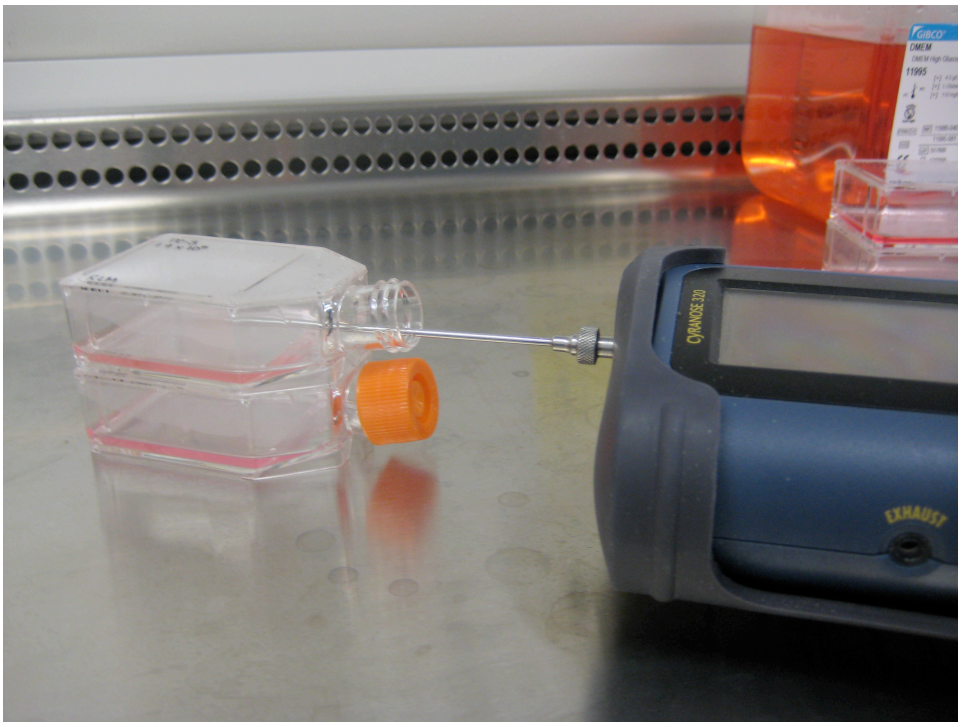

Figure 3.3 Insertion of snout into flask for sampling

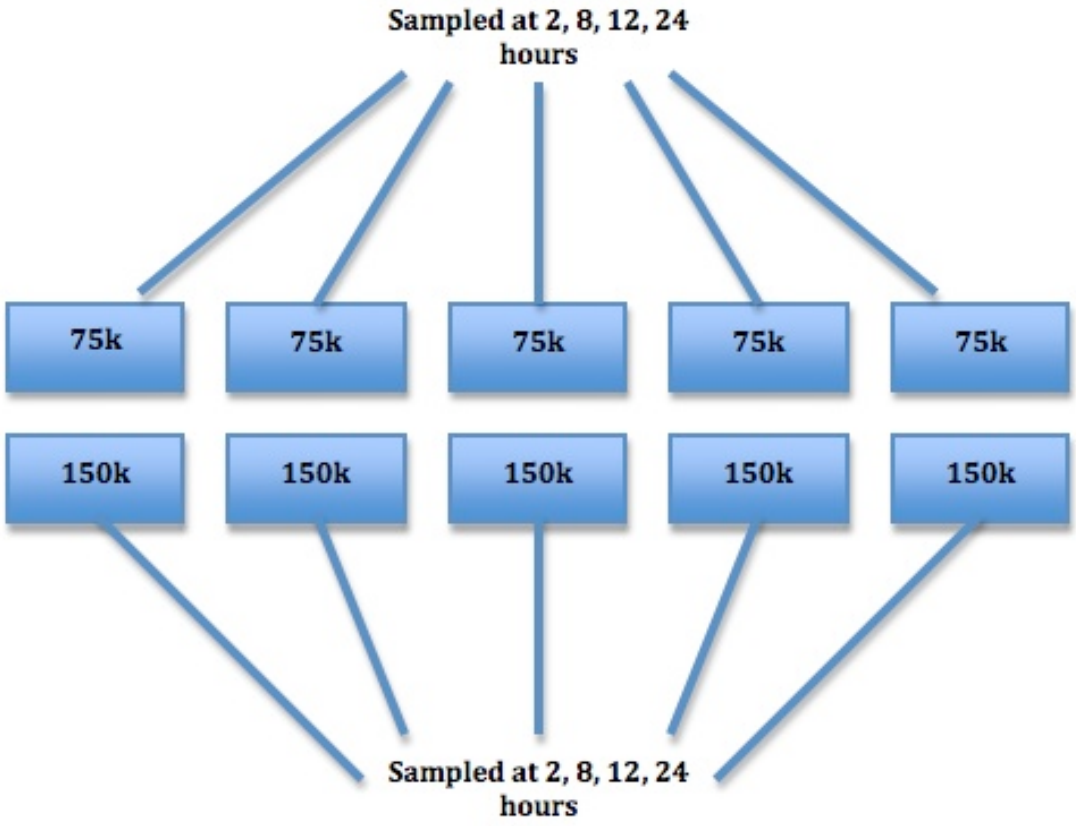

Figure 3.4 Experimental Setup used for each cell line

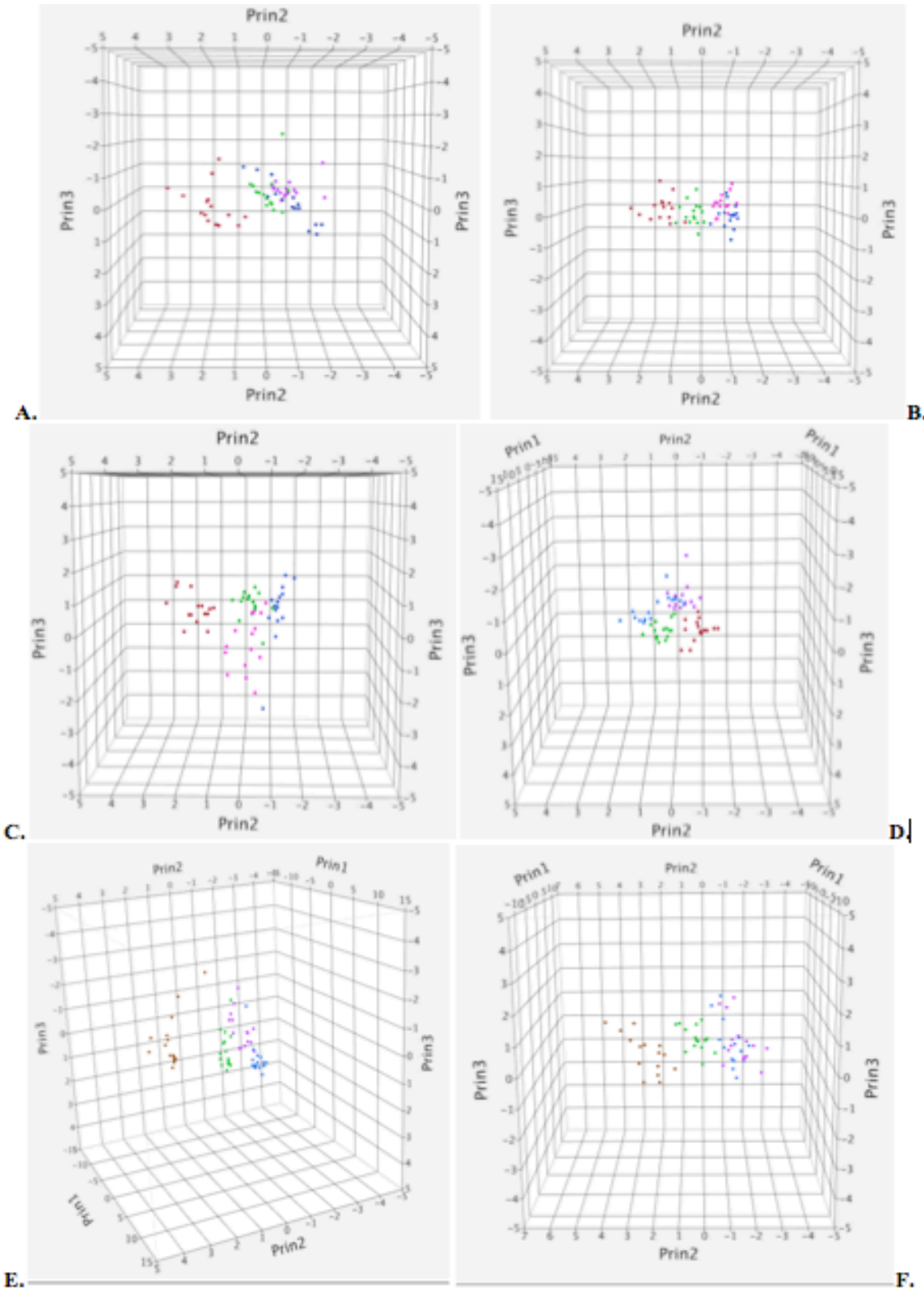

Figure 3.5 PCA plots for cells after 2, 8,12, 24 hours incubation
A) 75k PC-3 B) 150k PC-3 C) 75k DU-145 D)150k DU-145 E) 75k SVHUC F) 150k SVHUC (Maroon-2h, Green-8h, Blue-12h, Purple-24h)

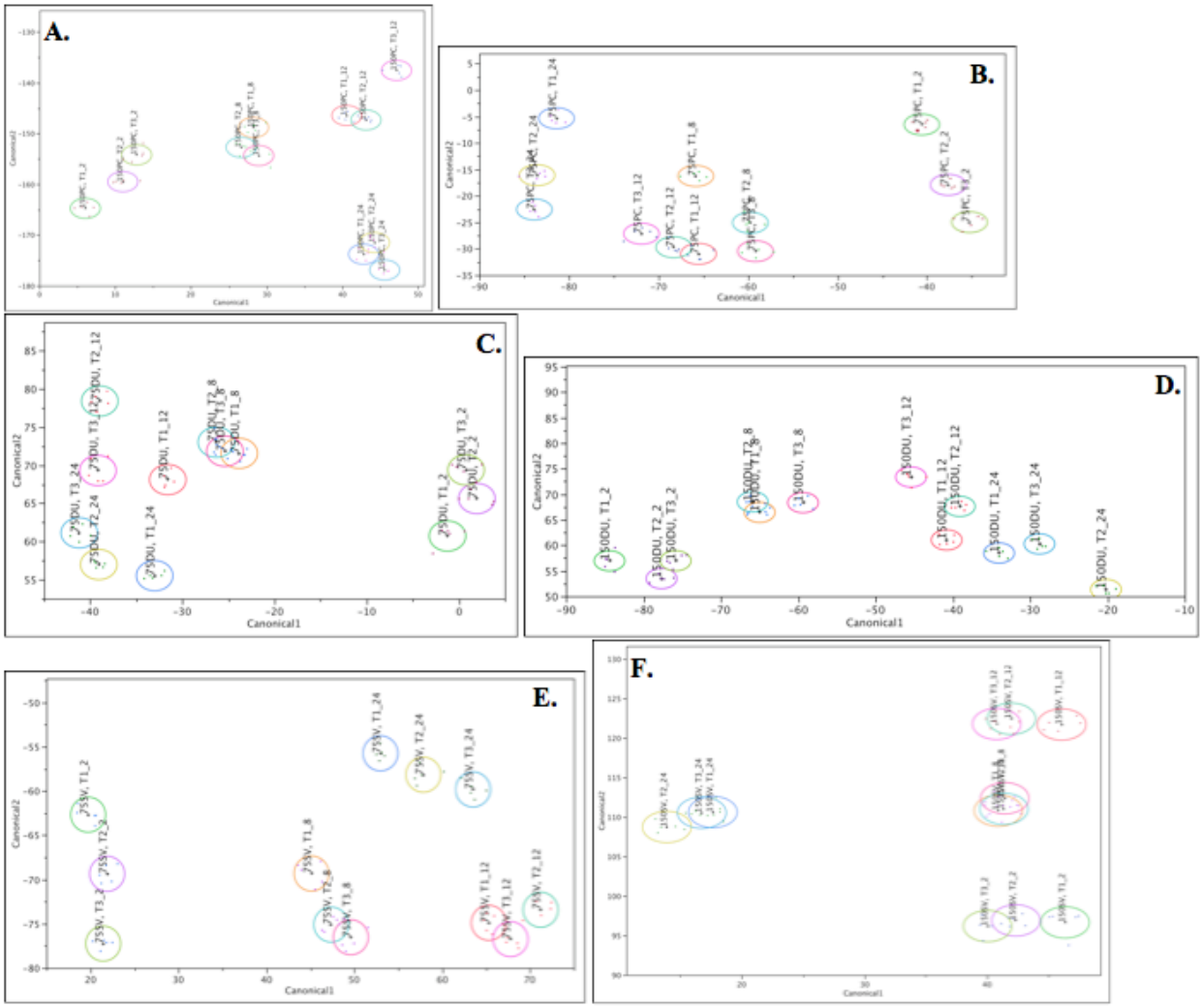

Figure 3.6 CDA plots for tested cell lines based on incubation period
A) 75k PC-3 B) 150k PC-3 C) 75k DU-145 D) 150k DU-145 E) 75k SVHUC F)150k SVHUC

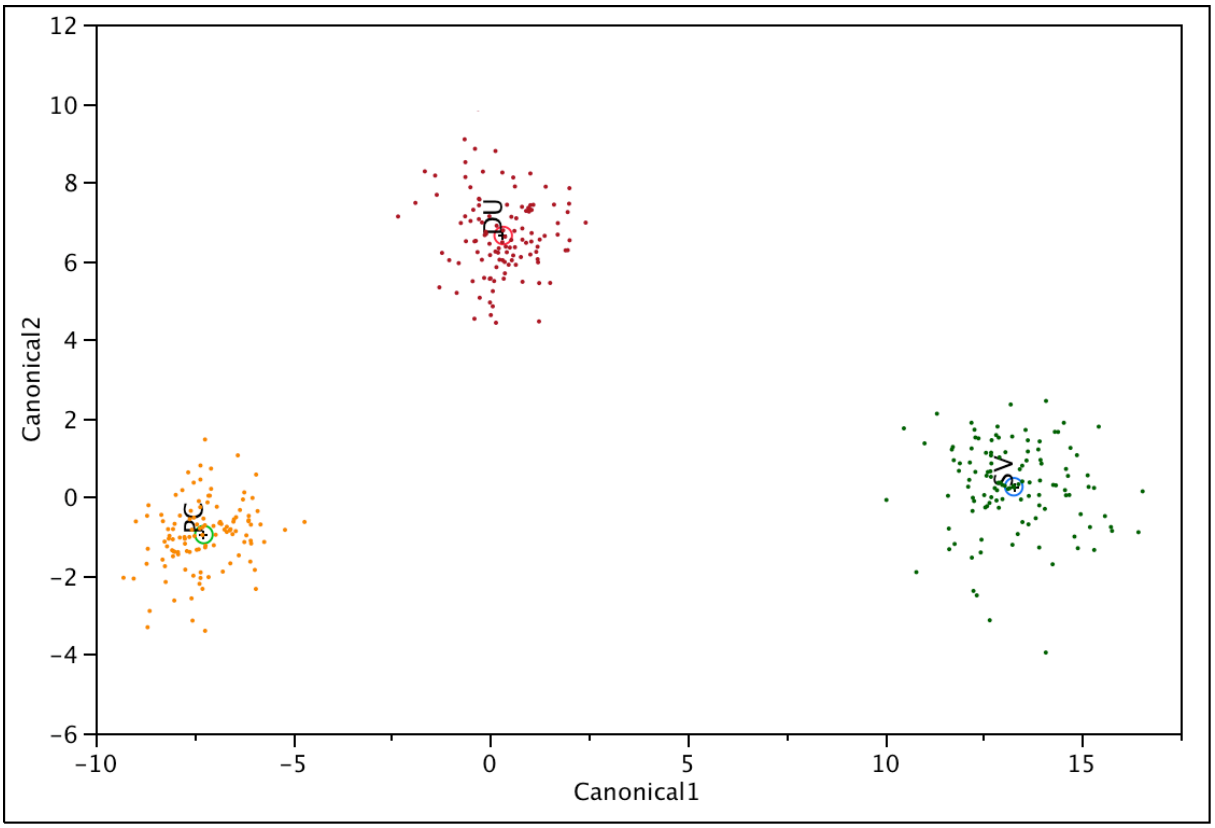

Figure 3.7 CDA plot of PC-3 vs. DU-145 vs. SVHUC cells (Orange-PC3, Maroon-DU145, Green-SVHUC)

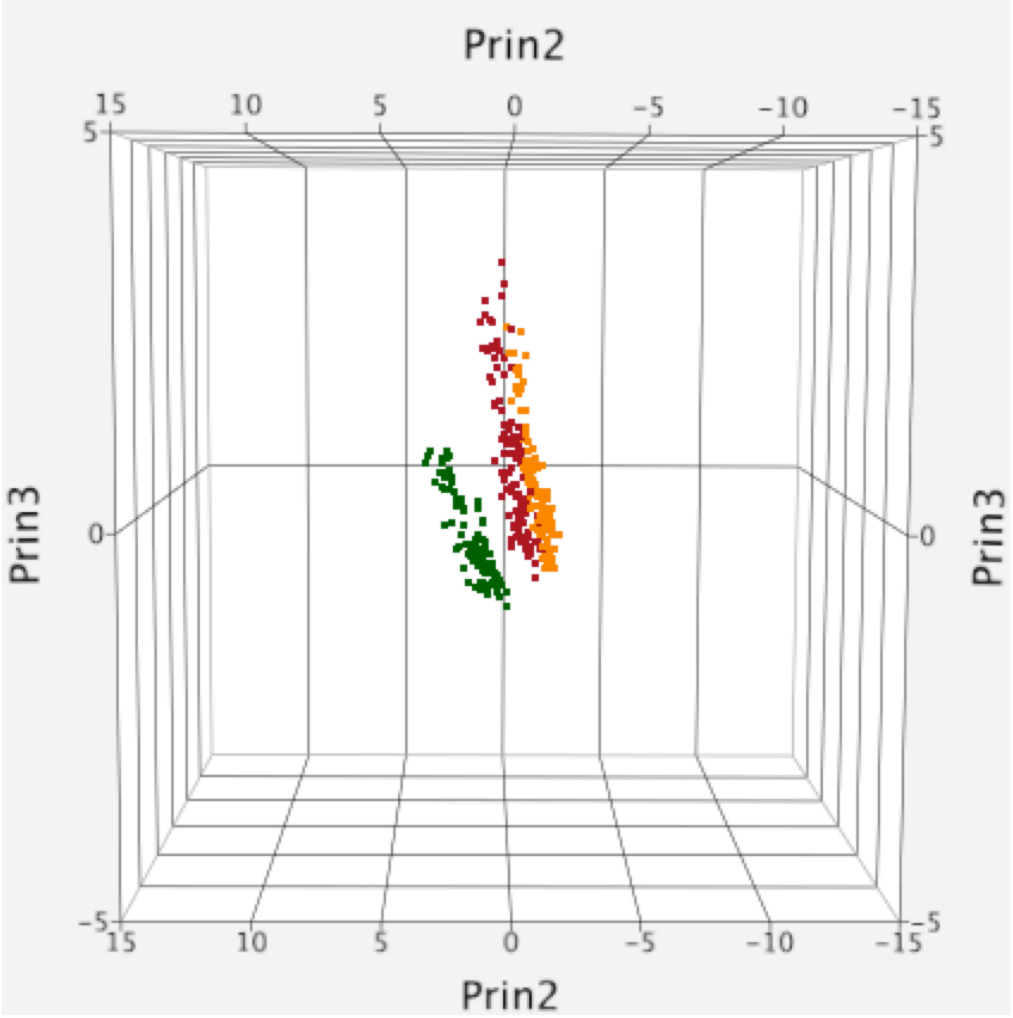

Figure 3.8 PCA plot of SVHUC vs. PC3 vs. DU145 cells (Green-SVHUC, Orange-PC3, Maroon-DU145)

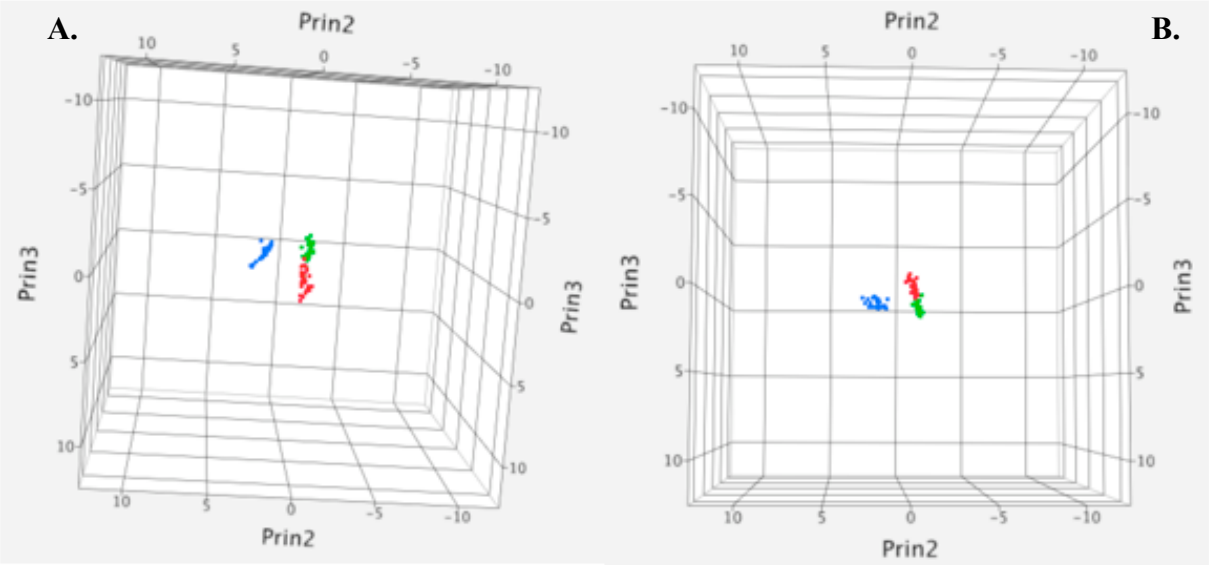

Figure 3.9 Cyranose 320 cell type discrimination after A) 2 hours and B) 8 hours

Chapter 4: Impact of cell population and cell maturity on the electronic nose's sensitivity to discriminate prostate cancer cells (DU-145 and PC-3) from non-tumor forming cells (SVHUC)

Abstract

Electronic nose technology has been employed in many fields to detect and analyze volatiles from different mixtures. A recent study has shown that the Cyranose320 has the ability to discriminate between different prostate cancer cell lines (DU-145 and PC-3) and non-tumor forming cells from the urinary tract (SVHUC). The current study takes the results from the previous study further and determines whether cell population or cell maturity is the most important factor in the device's ability to discriminate between these cells. Additionally, ranges of detection will be determined for each cell line to be successfully detected in culture. Using the cell populations found after 72 hours of incubation, serial dilutions were performed in a five-log dilution and the samples were immediately analyzed with the Cyranose320. For each cell line there was a clear distinction between the cell populations, with the most variation seen between the largest population and the remaining groups. The following ranges of detection were found for each cell line based on analysis performed by JMP® and PCnose® software; DU-145: 26,200-262,000 cells, PC-3: 51,400-514,000 cells and SVHUC: 19,000-190,000 cells. These ranges could be of use if studies warranted the use of an electronic nose to detect the individual cell lines in culture. Additionally, PCA plots help to convey the idea that cell population does not affect the ability of the electronic nose to discriminate between the three cell types based on the lack of clear cluster formation after testing was done immediately after culturing.

Introduction

Of the wide variety of cancers present in our society today, prostate cancer (CaP) is the most commonly detected non-skin cancer in American men. American men have the largest occurrence rate of CaP in the world, which studies believe to be attributed to the United States being one of the few countries with extensive CaP screening. However, many challenges arise in the concept of early detection because of the range of behaviors of CaP (Brawley et al., 2009). Diagnosis of the disease is supported by the use of the prostate-specific antigen (PSA) in combination with a digital rectal examination (DRE). Even though PSA is generated almost exclusively by the prostate, unfortunately it is not cancer specific and may be increased as a result of other factors affecting the prostate such as benign prostatic hyperplasia, acute urine retention, and urinary tract infections. Consequently, a raised PSA level leads to further invasive testing in an effort to make the CaP diagnosis (Osman et al., 2010). Because of the challenges that arise with PSA testing, the American Cancer Society (ACS) has been unable to resolve the issue surrounding the value of testing for early CaP (Smith et al., 2010).

With all the controversy surrounding the use of PSA, researchers have been working hard to determine alternative biomarkers that can overcome the lack of specificity. Biomarkers of the blood and urine have been suggested however none of them are widely utilized. Volatile organic compounds (VOCs) have also been proposed as alternative biomarkers and in the case of CaP, it can be hypothesized that specific VOCs might be present that could expose the presence of a malignant tumor. To detect and analyze these VOCs associated with tumors, sophisticated biochemical techniques are typically used (Cornu et al., 2010). One such type technique is the electronic nose, engineered to imitate the mammalian olfactory system within an instrument intended to acquire measurements that allow for the identification and classification of volatile

mixtures. Unlike other techniques, the electronic nose allows for the identification samples as a whole without having to determine the individual species within the sample mixture (Wilson and Baietto, 2009). The electronic nose technology has been employed in numerous scientific research fields including environmental (Baby et al., 2000), biomedical (Kateb et al., 2009), food (Zheng et al. 2009), and pharmaceutical (Naraghi et al., 2010), to name a few.

When discussing CaP, it is important to look into all possibilities for early detection methods. While the electronic nose has been used for medical studies like analyzing the breath of lung cancer patients (Dragonieri et al., 2009) and detection of *Mycobacterium tuberculosis* (Pavlou et al., 2004), few studies have looked into the ability of the electronic nose to discriminate between cancerous and non-cancerous cells from the prostate. Tokumitsu et al. (2010) did however investigate the ability to discriminate noninvasive CaP cells from normal cells in adherent culture by phase shifts measurement using phase-shift microscopy and determined that CaP cells could indeed be distinguished from normal cells. Furthermore, Santonico et al. (2009) measured the volatile compounds from cultured tumor cell lines and suggest that tumoral cell lines are characterized by a proper volatile compound pattern and these patterns are apt to cluster according to the type of tumor.

In regards to VOCs expelled from CaP, the electronic nose has yet to be investigated. The objective of this current research takes into account the results from the previous study to further investigate the electronic nose's ability to discriminate between different lines of CaP. While the previous study showed that the electronic nose could successfully distinguish between the three cell lines studied even after only 2 hours of incubation, the current study will investigate whether the electronic nose's sensitivity is based on the cell population or the maturity of the cells.

Additionally, a necessary range of detection for the electronic nose to be successful will be examined

Materials and Methods

The cell lines, materials, and equipment used to maintain and prepare the cells in this study were obtained from Department of Biomedical Sciences & Pathobiology at the Center for Molecular Medicine and Infectious Disease. All testing was conducted in a biosafety level 2 laboratory at the Integrated Life Sciences Building at the Corporate Research Center in Blacksburg, VA.

Cell Lines

This study was conducted using three cells lines, SVHUC, DU-145, and PC-3. One of the greatest ways to study tumorigenesis in cancers related to the human bladder is the use of human urothelial cells. SVHUC is a normal human urothelial cell line that has been immortalized by the simian virus and grows as epithelial layers in vitro but does not form tumors (Wang et al., 1995). DU-145, the first CaP cell line generated in tissue culture, are epithelial cells developed from the brain of a 69-year-old white man with CaP and lymphocytic leukemia. Additionally, this cell line does not express the prostate-specific antigen (PSA). PC-3 cells, which were first accounted for in 1979, were derived from a lumbar vertebral metastasis in a 62-year-old white man and are composed of undifferentiated malignant cells (Sobel and Sadar, 2005). Although current studies have shown contrasting result as to the expression levels of PSA in PC-3 cells, the majority of researchers believe that PC-3 retains the expression of PSA (Dozmorov et al., 2009).This is one of the main reasons that this cell line was included in the study, to be compared to DU-145, which does not express PSA.

Aseptic Technique

All of the laboratory studies were completed using the aseptic technique for cell culture as described by Coté (2001). Before gathering materials, latex surgical gloves and laboratory coat were worn to ensure bodily protection. The majority of the work for the experimentation was completed in a ThermoScientific Forma 1400 Series Biological Safety Cabinet. The workspace was cleared of unnecessary equipment and wiped down with 70% ethanol. When new materials were being brought into the cabinet, they were also wiped down with 70% ethanol. The items in the workspace were arranged in a logical pattern from clean to dirty to get away from passing contaminated materials over clean and sterile items. When disposable items were finished being used they were properly disposed of in the appropriate Sharps Biohazard Collection Container. Finally, all other materials were returned to their proper location and the cabinet was wiped down with 70% ethanol.

Cell Culture

In order to ensure viability of the cell lines, the cells were cultured on a regular basis until experimental testing began as well as after testing to ensure that the cells would be available if other experiments needed to be performed. SVHUC cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% heat inactivated fetal bovine serum (FBS), and antibiotics (100 μg/ml penicillin and 0.1 μg/ml streptomycin (Invitrogen, Carlsbad, CA). DU-145 cells were grown with Gibco Minimum Essential Media (MEM), 15% heat inactivated FBS and antibiotics. PC-3 cells were grown with Hams F-12 nutrient mixture, 15% FBS, and antibiotics. When not in use, growth media and other solutions were kept in a ThermoScientific REVCO refrigerator and then placed into a ThermoScientific Microprocessor Controlled 280 Series Water Bath at 37°C for at least 15 minutes prior to use by the cells. All cells were grown at 37°C with 5% CO_2 in a

ThermoScientific Forma Series II Water Jacketed CO_2 Incubator until it was time for them to be re-cultured or tested.

The proper cell culture methods used in this experiment were obtained from Simon (2004) based on his methods and protocols for cancer cell culture. Corning cell culture flasks of size 25 cm^2, 75 cm^2, and 150 cm^2 were used depending on the cell population.

General Cell Maintenance

To maintain each cell line when the cells were not being tested, the cells were carefully removed from the incubator and examined every 24 hours with a Carl Zeiss Invertoskop 40C Microscope to monitor the cell growth. If the cells had been growing for 94 hours and had not become confluent the media was changed. First, the old media was removed from the flask with a sterile Corning Incorporated Costar Stripette and discarded. 5ml of phosphate-buffered saline (PBS) was then added to the flask with a stripette and removed and discarded. Washing with PBS was completed twice to ensure the successful removal of any traces of serum from the media that could negatively affect the remainder of the cell culture process. Trypsin EDTA was then added to the flask for the dissociation of adherent cells into a single cell suspension. The amount of tryspin EDTA used depended on the flask size being used as seen in Table 4.1. The solution was swirled across the monolayer to guarantee that the trypsin EDTA effectively reached all the cells. The flask was then returned to the incubator for 3-20 minutes depending on the cell line being used with DU-145 needing approximately 2 minutes, PC-3 needing approximately 4 minutes, and SVHUC needing approximately 15 minutes. The cells were checked throughout the detachment period to monitor the dispersing of the monolayer because if left in trypsin for too long, the amount of viable cells begins to decrease. Once all of the cells

detached, fresh media was added to the flask with a stripette using media amounts given in Table 4.1. The flask was then labeled with the passage #, date, and placed back into the incubator.

If during the initial microscopic observation of the cells under the Invertoskop the cells appeared to reach confluency, the cells were transferred into a larger flask for expansion. After detachment with trypsin EDTA the appropriate amount of media to fill the new larger flask was obtained with a stripette and dispensed into the original flask currently holding the cell suspension. After the flask was slightly shaken to ensure that all of the cells moved into the media, the cell solution was pipetted into the larger flask and properly labeled with the date, passage #, cell type, and experimenter's initials. The flask was finally placed back into the incubator to continue growth.

Cell Counting

Based on the nature of the experiment being conducted, after the cells detached and fresh media was added, the cells were placed into a Corning 50 ml centrifuge tube in preparation for the cell count. Two methods for cell counting were used in this experiment: hemocytometer and cellometer. The first method utilized the Reichert Bright Line Hemocytometer (Buffalo, NY). The hemocytometer was prepared by carefully placing a clean coverslip over the hemocytometer grid. In the biosafety cabinet, 100µL of cell suspension was removed from the centrifuge tube with a ThermoScientific Finnipipette F1 and placed into a eppendorf tube. 100µl of Trypan Blue solution composed of 5 ml HyClone Trypan Blue Solution + 45 ml PBS was also added to the eppendorf tube, thereby creating a 1:2 dilution. Using a pipette, a small amount of the cell+Trypan blue solution was removed from the eppendorf tube. The pipette was then placed at the edge of the coverslip and some of the contents were slowly expelled filling the fluid into the chamber by capillary action. The hemocytometer was then observed under the Invertoskop and

the cells were counted from each of the four sixteen-square corners chambers and the average was taken from those four values. After the cell count was obtained, Equation 1 was used to determine the cell concentration.

$$Average \times 2 \times 10^4 = Cell\ conc.\ (cells/ml) \quad \text{(Eq.1)}$$

The cellometer method utilizes the Cellometer Vision (Nexcelom Bioscience LLC, Lawrence, MA) that combines brightfield microscopy and fluorescence images to generate a cell count. After fresh media was added to the cells and placed into a 50ml centrifugal tube, 20µl of the cell suspension was removed with a pipette and carefully inserted into one end of the disposable counting chamber which was then inserted into the cellometer as seen in Figure 4.1. The cellometer was connected to a computer, which allows the sample to be given a name and then counted. The cellometer then output the live cell count, mean diameter (micron) and cell concentration (cells/ml) and the data was recorded.

Sample Preparation- Hourly Cell Count

After investigating the electronic nose's ability to discriminate between cells after different time periods and with different cell populations, it is important to determine the cell population after each of those time periods. In order to determine the cell population of the flasks that were tested in the previous study, the study was repeated. However in this study, at each time period investigated, the cells were counted using the cell counting methods previously discussed. The previous study investigated time periods up to 24 hours, however the cell populations after 48 and 72 hours were also investigated to show how the cell population increases with maturity for PC-3, SVHUC, and DU-145 cell lines.

After the cells were properly cultured and counted, they were set up in preparation for testing. Based on the cell concentration determined by the counting method used, the amount of

cell suspension used was calculated based on equation 2 with the desired cell populations being 75,000 and 150,000 total cells, consistent with the previous study.

Cell conc.(cells/ml) / Desired total(cells) = Amount(ml) (Eq. 2)

Using a stripette, twelve 25 cm^2 flasks were filled with the appropriate amount of media used to total 5ml when taking into account the cell solution that was later added. For example, if calculations determined that 750µl of cell solution was necessary for the 150k samples, then 4.25ml of media would be added to the flasks. The first six flasks were labeled with '75k' and hours 2, 8, 12, 24, 48, and 72. The remaining six flasks were labeled with '150k' and the appropriate hours that they would be tested. The calculated cell solution amount was then added to the flasks with their corresponding cell total. All twelve flasks were then placed into the incubator until testing began.

At each of the six time periods, a flask for the 75k and 150k cells were removed from the incubator and placed in the biosafety cabinet for testing. The cells were properly detached from the flask using Trypsin EDTA as described during general cell maintenance. Once full detachment was achieved, 20µl of the cell suspension was removed from the flask with a pipette and carefully inserted into the disposable counting chamber for use in the cellometer as described during the cell counting procedure. Each flask was tested twice and the cell populations for the six time periods were recorded. The experiment was duplicated resulting in four cell population values for each time period in both the 75,000 and 150,000 initial cell populations used.

Sample Preparation- Sensitivity

As discussed in the previous experiment, the cell populations were determined for each cell line at the different time periods investigated. The highest cell population recorded in the 72 hour time period for each cell line was then used to determine whether the Cyranose320 had a

higher sensitivity when exposed to larger cell populations or cells that were more mature. In addition to determining which factor had a greater affect on the sensitivity, the detection threshold of the Cyranose320 was also investigated.

In order to examine the sensitivity and range of detection of the Cyranose320, serial dilutions (Schneegurt, 2004) were performed with each cell line based on the highest cell population observed during the hourly cell count experiment. To prepare the cells for the dilutions, the cells were detached and counted as described in the general cell maintenance and cell counting procedures. While the cells were detaching, five 50ml centrifuge tubes were placed in the biosafety cabinet and labeled appropriately in relation to the 5-log dilution that was going to take place during the dilution as well as with numbers 1-5 to avoid later confusion. Once the cells were counted using the cellometer, the amount of cell solution to be used to achieve the highest population observed was determined using equation2. The calculated amount of cell solution was then added to the first 50ml centrifuge tube in addition to an amount of media to total 10ml. The mixture was thoroughly mixed by gently pipetting the solution five times. Nine milliliters of media was then pipetted into each of the remaining four tubes as depicted in Figure 4.2. To perform the dilutions 1ml was carefully pipetted from tube1, added into tube2, thoroughly mixed with the pipette, and the screw cap was tightly placed back onto tube1. One milliliter was then taken from tube2, added into tube3, thoroughly mixed, and the screw cap was tightly placed back onto tube2. This process continued with tube 3 and tube 4 and ended after 1ml was added to tube5 and thoroughly mixed. It was very important to ensure that the screw caps were placed back on the tubes after use so that the tube could begin to collect necessary volatiles that would later be tested. After all the tubes were inoculated, they remained in the biosafety cabinet for immediate testing with the electronic nose.

Electronic Nose

The electronic nose apparatus used for the entirety of this study was a conducting polymer based electronic nose (Model: Cyranose 320, Smiths Detection, Pasadena, CA). The Cyranose 320 consists of a sensor array of 32 conducting polymer sensors and as the array is exposed to the sample, the volatiles react with each sensor causing them to reversibly swell. On an individual level, each sensor responds to the sample differently, but when looked at as a whole, the response of the array is identified as a unique smellprint.

Cyranose320 Settings

The Cyranose 320 was connected to a computer to utilize the PCnose (Smiths Detection, Pasadena, CA) software. While using the software, parameters for the electronic nose were examined and adjusted. Additionally, the software allowed for a visual representation of how the sensors were reacting to the sample. Figure 4.3 shows the internal settings used by the Cyranose 320 during its sampling of the cells. Before the electronic nose was exposed to the experimental samples, the device was allowed three to five preliminary runs from the air in the biosafety cabinet and purges to allow for any residual volatiles from previous studies to be eliminated. This preliminary data was then deleted prior to completing the experimental sampling.

Testing

Once all five tubes were inoculated with the proper cells they were allowed 5-10 minutes to metabolize while the Cyranose320 was being connected to the accompanying computer and prepared for testing. The first tube, which consisted of the highest population observed during the hourly study, was then tested three times by placing the ‘snout’ of the Cyranose320 carefully into the tube as seen in Figure 4.4. The remaining four tubes were tested in the same manner,

each being tested three times. Fifteen total samples were taken from the dilutions and the experiment was performed in triplicate creating a total of 45 samples for each cell line.

Statistical Analysis

The Cyranose 320 comes equipped with its own statistical software, PCnose® (Cyrano Sciences, Inc., Pasadena, CA) and although it may have limitations when analyzing large data sets (Whysong 2009), the software was applicable for the current study. The software produced interclass mahalanobis distances (m-distances) as well as cross validation results. In addition to the PCnose® software, JMP® (SAS Institute, Inc., Cary, NC) statistical software was also used. The Cyranose's sensitivity to different cell populations were analyzed using JMP's multivariate statistical methods (canonical discriminant analysis and principal component analysis) to determine a necessary range of detection for the device to discriminate between the three cell lines.

Results and Discussion

The results of the previous study determined that the Cyranose320 was capable of discriminating between CaP cells and non-tumor forming cells. Based on this information, it was important to determine the sensitivity of the device based on the hypothesis that cell population would have a greater effect on the ability of the device to discriminate between cancerous and non-cancerous cells.

Hourly Cell Count

The previous study investigated incubation periods up to 24 hours for initial cell populations of 75,000 and 150,000 cells, but based on the findings, it was clear that it would be necessary to let the cells mature for a longer period of time. The cells in this study were incubated for up to 72 hours and the previous study was re-done with all three cell lines to

determine the total cell population after 2, 8, 12, 24, 48, and 72 hours of incubation. The results were plotted to show the increasing cell population over time for DU-145, PC-3, and SVHUC cells as seen in Figure 4.5. All three cells lines showed similar trends in that up until 24 hours, the cell population was slightly inconsistent but still increasing. The reason for this could be because due to the shorter incubation periods, the entire population was not able to properly attach to the flask and were discarded during the cell count preparations. After 24 hours, the cells appeared to stabilize in their growth pattern and increased consistently over the next 48 hours. The largest cell population for Du-145, PC-3, and SVHUC were recorded as 262,000, 514,000, and 190,000 respectively. The highest cell population recorded was the quantity of greatest interest in order to establish the largest amount of cells grown after 72 hours. Once the highest amount of cells grown for each cell line after the 72 hours was determined, this amount was used to determine how the Cyranose320 responds when exposed to cells immediately versus cells that have matured for various time periods. This information will help to decide if cell maturity or cell population is more important for the Cyranose320 when distinguishing between different cell lines.

Electronic Nose Sampling

While testing for the sensitivity of the Cyranose320 to the three cell lines, PCnose® software was utilized to observe the instrument and how the sensors were reacting to volatiles given off by the cells. The scrolling strip chart shows how each sensor is reacting to the volatiles in the flask in terms of time vs. sensor response. The PCnose® software also produces a smell print for the given sample. Taking into account the entire class analyzed, the smell print includes all 32 sensors and shows the variation of the training response based on the change of resistance

incurred during sampling. The data was also analyzed using JMP® multivariate techniques consisting of canonical discriminant analysis (CDA) and principal component analysis (PCA).

Since we have already learned that the Cyranose320 can effectively discriminate between Du-145, PC-3, and SVHUC cells, it was now important to determine how many cells would be necessary to make that appropriate discrimination in the future. The highest cell population retrieved from the hourly cell count was used to complete serial dilutions with a five-log dilution, expecting to determine at what population level the Cyranose320 is an effective device.

DU-145

A serial dilution starting with the highest cell count achieved after 72 hours of incubation was performed on DU-145 cells. A five-log dilution of 262,000 cells generated total cell population amounts of 262,000, 26,200, 2,620, 262, and 26.2 cells. Figure 4.6A shows the CDA plot for the five cell populations tested with an evident distinction between the higher populations and lower populations. All five populations form distinct clusters with 262,000 cells showing the most variation between the groups. The closest cell population to the 262,000 cells is the 26,200 cell populations, which suggests that a range of 262,000 to 26,200 is an effective amount of cells for the Cyranose320 to detect the cells. Figure 4.7B shows the statistical analysis produced by the PCnose® software. The cross validation results show that the classes with the best classification are the 262,000 class with 8 of the 9 samples correctly identified and the 26,000 class with 6 of the 9 samples correctly identified. These results are consistent with the CDA plot showing that the 262,000 and 26.2 cell populations have the most distinct odor because of the increased and decreased amount of cells tested. The m-distances, also shown in Figure 4.7B help to convey the same results with the highest m-distance values, above five standard deviations, being between the 262,000 cell population and the remaining four classes.

Research shows that m-distances of five standard deviations and higher show the most variation and least overlapping between groups. (Deventer 2001).

PC-3

PC-3 cells were sampled using the same serial dilution methods. The highest cell population after 72 hours of incubation was 514,000. The five-log dilution resulted in samples of 514,000, 51,400, 5,140, 514, and 51.4 cells. Figure 4.6B shows the CDA plot of all five cell populations sampled with the Cyranose320 immediately after cell culture. The plot shows the greatest variation between the 514,000 cells, the 51,400 cells, and the remaining three groups forming a large cluster between them. Based on the clusters formed by the classes, it can be determined that a range of cells between 514,000 and 51,400 would be the most useful in detecting the presence of the PC-3 cells in culture because of the greatest variation between the two classes in comparison to the remaining classes. PCnose® was used to produce cross validation and m-distance results as seen in Figure 4.7B. The cross validation shows similar results to those for DU-145. The classes with the most samples correctly identified were the 514,000 and 51.4 cell classes with 8 out of 9 and 7 out of 9 samples identified, respectively. Again, these two classes have the most distinct odor because of the large population of cells present or lack thereof. When referring to the m-distance results, the largest distances of five standard deviations or more between groups, are seen between the 514,000 class and the 5,140, 514, and 51.4 group (Figure 4.7B). These results help to corroborate the results found by the CDA plot and cross validation in that a range of detection of 514,000 and 51,400 cells would be most effective for detection of the PC-3 cells in culture.

SVHUC

After determining the highest cell population of SVHUC cells after 72 hours, serial dilutions were performed. The highest population was 190,000 cells and after the five-log dilution the remaining samples were 19,000, 1,900, 190 and 19 cells. A CDA plot was used to determine the variation between the groups as seen in Figure 4.6C. 190,000 and 19,000 cells show the most variation between the groupings with the remaining three groups showing less variation. Based on the cluster formation of the classes, it can be determined that a range of 190,000 to 19,000 cells would be an effective cell population for immediate detection of SVHUC cells in culture. When cell populations smaller than 19,000 were used, little variation were seen between the groups. Figure 4.7C shows the cross validation and m-distances reported from the PCnose® software. Similarly to DU-145 cells, the first two classes and the last class show the greatest identification abilities. 7 of 9 samples were correctly identified for 190,000 cells and 8 of 9 samples correctly identified for 19,000 cells. This data confirms the results seen in the CDA plot because the two 190,000 samples that were incorrectly identified, were identified as 19,000 further verifying that cell populations between these two amounts would be sufficient. Figure 4.7C also verifies the variation between the clusters by means of m-distances. Similar to the other two cell lines tested, the m-distances were greater than five standard deviations when comparing 190,000 cells to 1,900, 190 and 19 while a m-distance of three standard deviations was seen between 190,000 and 19,000. This shows that these two groups were more similar than the others, validating that the Cyranose320 has a sensitivity of between 190,000 and 19,000.

Studies conducted with the electronic nose technology typically use canonical discriminant analysis and mahalanobis distances to determine the differences between the classes tested. Based on their studies Gendron et al. (2007) determined that m-distances of greater than 3

indicated that the classes were distinct from one another and an m-distance of greater than 5 implies that the unknown might be identifiable according to the model. Their study also investigated the use of a Cyranose320 to discriminate between tumor cells and normal cells. The majority of the m-distances from their study were greater than five suggesting that an electronic nose trained with the proper cell lines may be able to identify individual tumor lines. In this current study, all of the m-distances outside of the range of detection were greater than five, showing that in future studies, the Cyranose320 may be able to identify these individual cell lines in culture.

Based on the chosen ranges of detection, PCA plots were created to determine if the Cyranose has the ability to discriminate the three cell lines in culture after being testing immediately after culture. As seen in Figure 4.8, no distinct clusters are formed. This shows that when examining the cell lines in-vitro after immediate culturing, the Cyranose320 does not have the ability to discriminate between the three cell lines. Reasoning for this could be explained by the lack of metabolic reactions that occurred because testing was completed immediately after culturing thereby producing insufficient volatiles for detection by the Cyranose320 to discriminate these three cell lines.

Conclusion

Once it was determined that the Cyranose320 had the capability of discriminating between CaP cells and non-tumor forming cells, it was important to determine whether cell maturity or cell population was more effective in helping the device to be successful. The maximum number of cells grown after 72 hours of incubation was also used as a base point for determining the sensitivity of the device. The cellometer tested DU-145, PC-3, and SVHUC cells and determined the cell populations after 72 hours to be 262,000, 514,000, and 190,000,

respectively. Once the serial dilutions were carried out, the Cyranose320 was immediately exposed to the samples. CDA plots were used to analyze the data and for all three cell lines and the first two population classes were the most distinct from the group. For DU-145, the CDA plots helped to determine that the Cyranose320 has a range of detection of 262,000 to 26,200 cells when detecting these specific cells in culture. For PC-3, the plot determined that the device has a range of detection of 514,000 to 51,400 cells. Finally, for SVHUC cells, the CDA plot concluded that the Cyranose has a range of detection of 190,000 to 19,000 cells when detecting these specific cells in culture. For all three cell lines, these results were verified by the statistical analysis performed by PCnose® software. The m-distances between the highest population and the lowest three populations were all greater than five standard deviations, showing great variation between those groups. These results show that if it were necessary to detect an individual cell line in culture, these ranges of detection would be sufficient. Unfortunately, it is necessary to reject the hypothesis that cell population has the greatest effect on the ability of the Cyranose 320. PCA plots showed that after being tested immediately after culture, no clear distinction could be made between the three cell lines when compared to one another, proving that incubation period is a more important factor when using the electronic nose technology.

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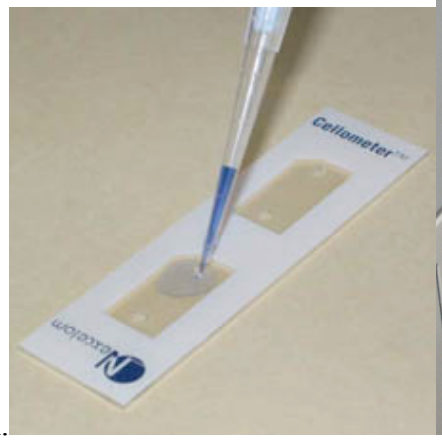
Table 4.1 Amount of Media and Trypsin EDTA to be used depending on flask size

Size of Flask	TrypsinEDTA (ml)	Media (ml)
25 cm^2	1	4
75 cm^2	3	7
150 cm^2	5	20

A. 

B.

Figure 4.4 Disposable slide and Cellometer used for cell counting

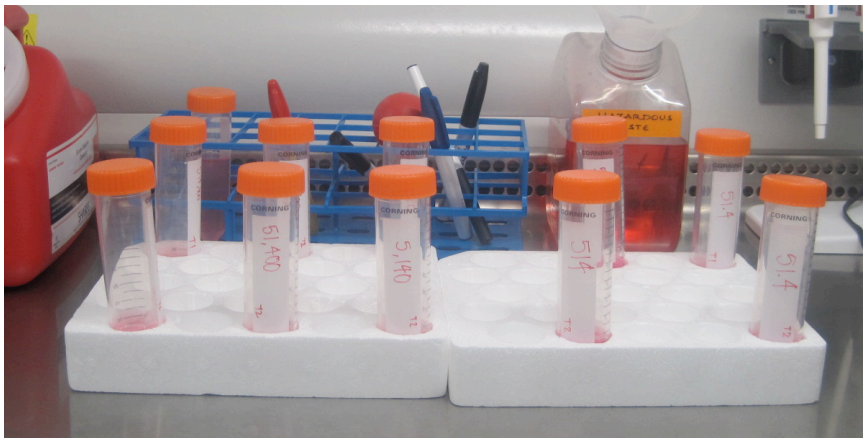


Figure 4.2 Tube setup during serial dilutions

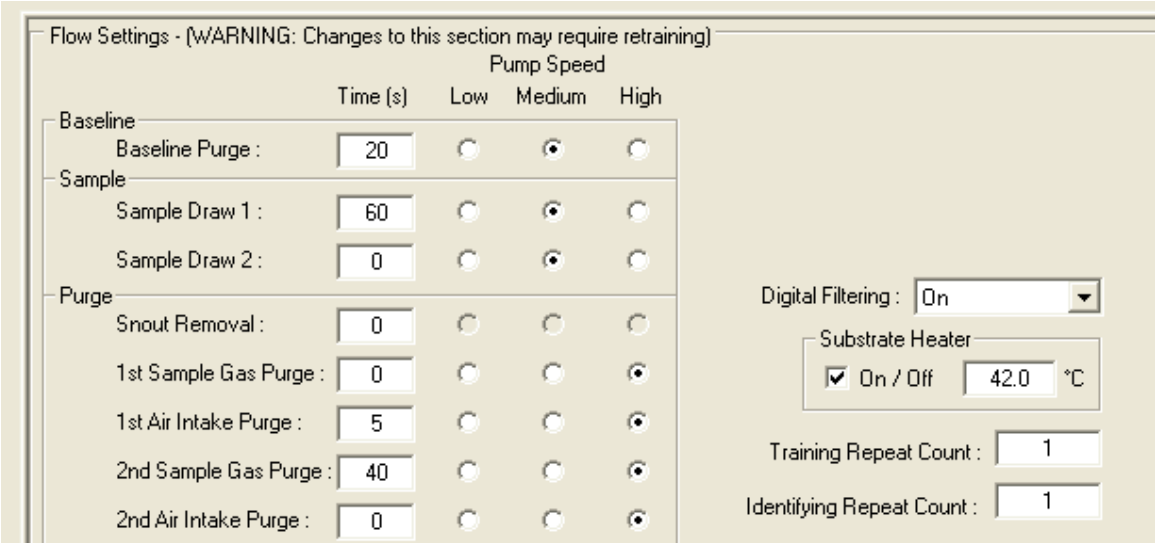


Figure 4.3 Cyranose 320 Flow Settings

Figure 4.4 Insertion of snout into tube for sampling

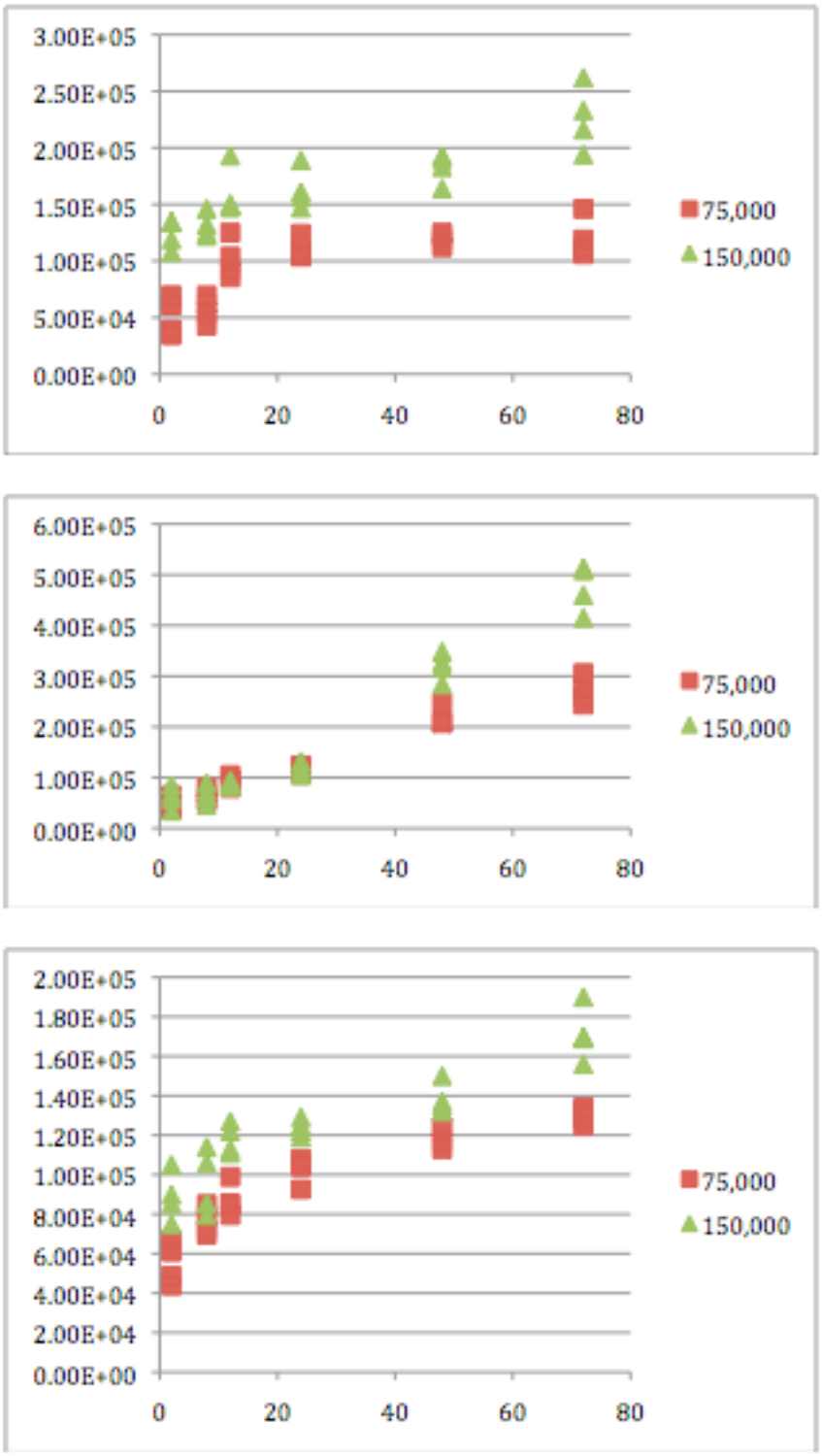


Figure 4.5 Cell Growth after 2, 8, 12, 24, 48, 72 hours of incubation A) DU-145 B) PC-3 C) SVHUC

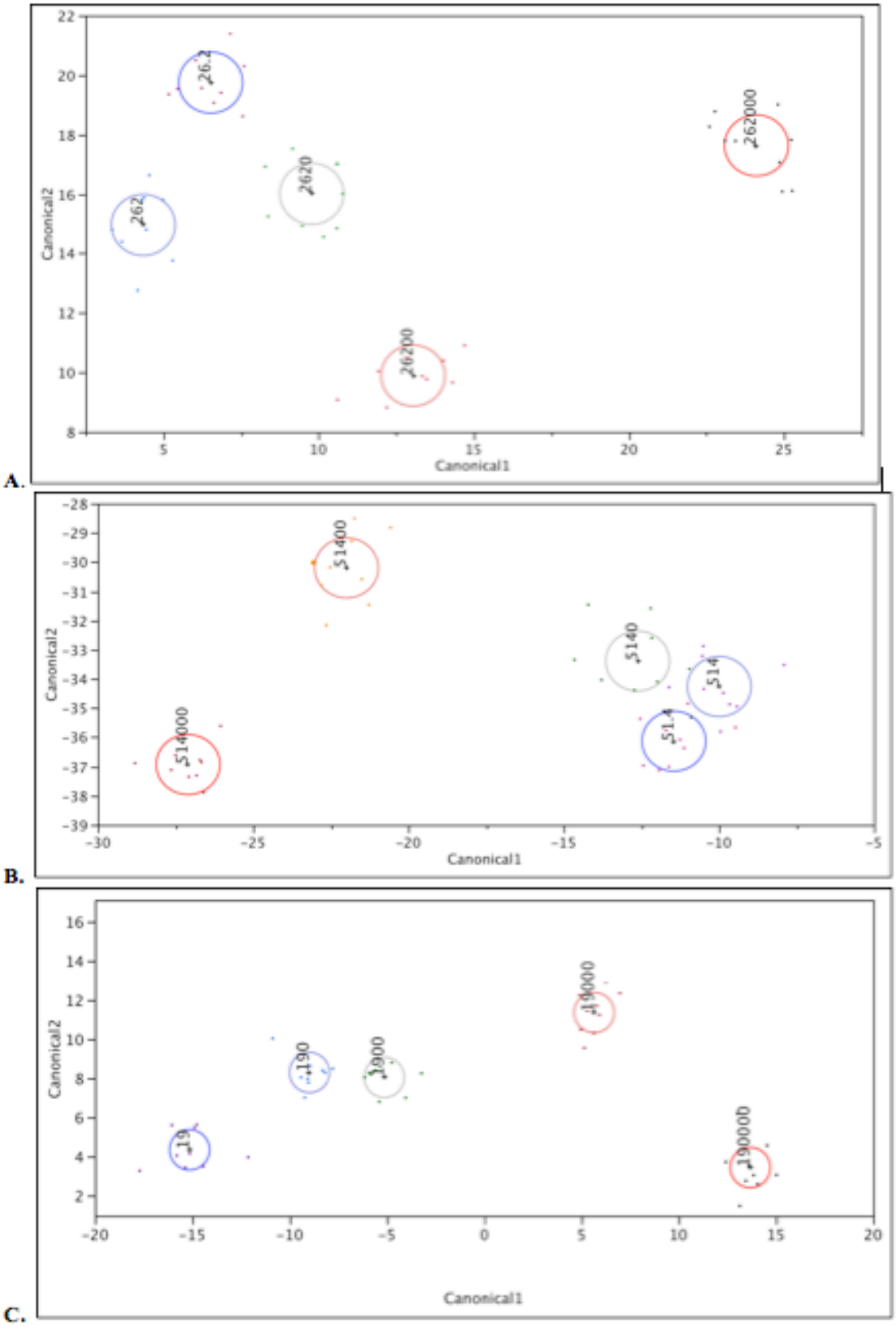


Figure 4.6 CDA plot based on five-log dilution of cell lines to determine Cyranose320 sensitivity A) DU-145 B) PC-3 C) SVHUC

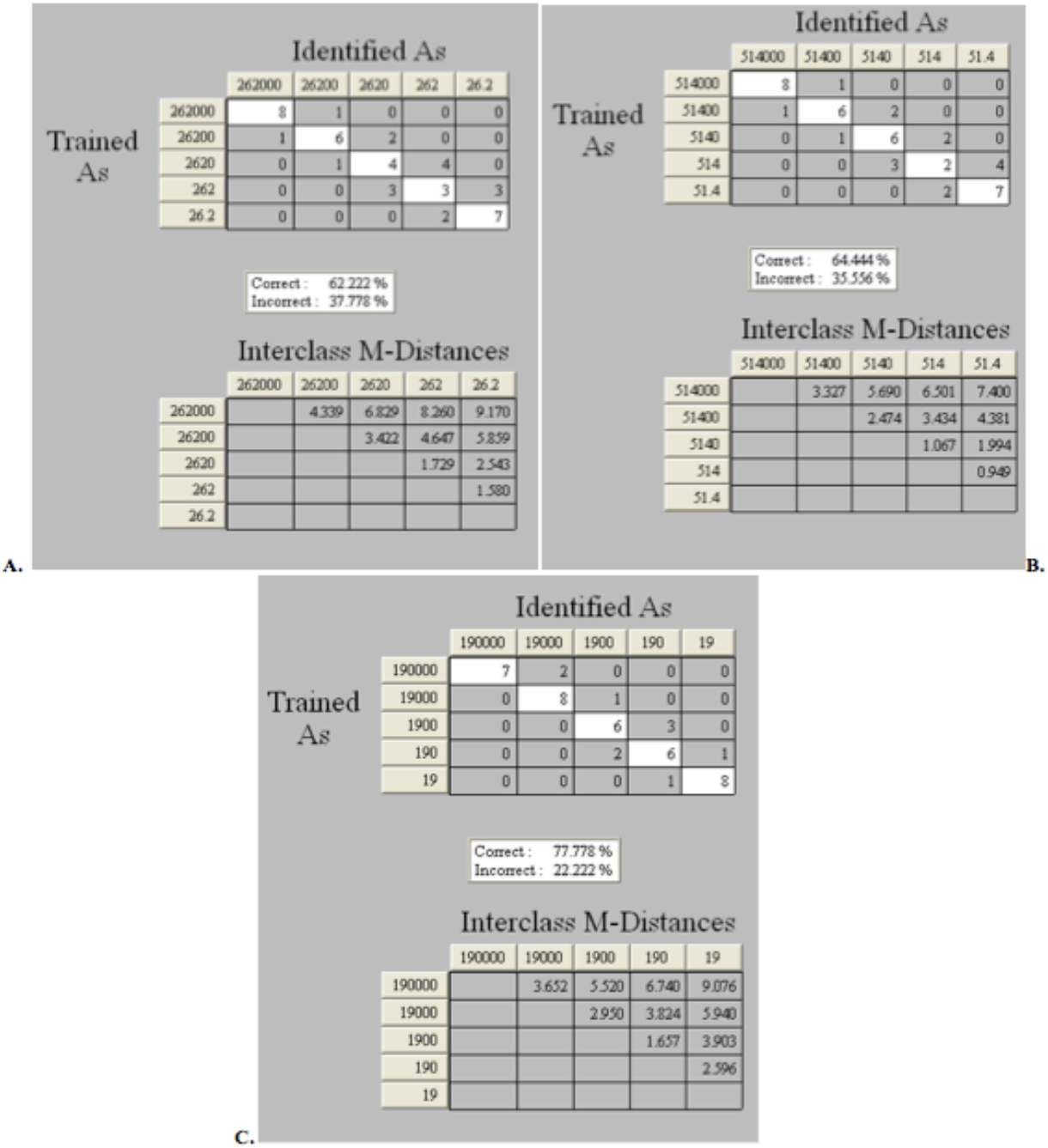


Figure 4.7 Cross Validation and M-distances of each cell line produced by PCnose® A) DU-145 B) PC-3 C) SVHUC

A. B.

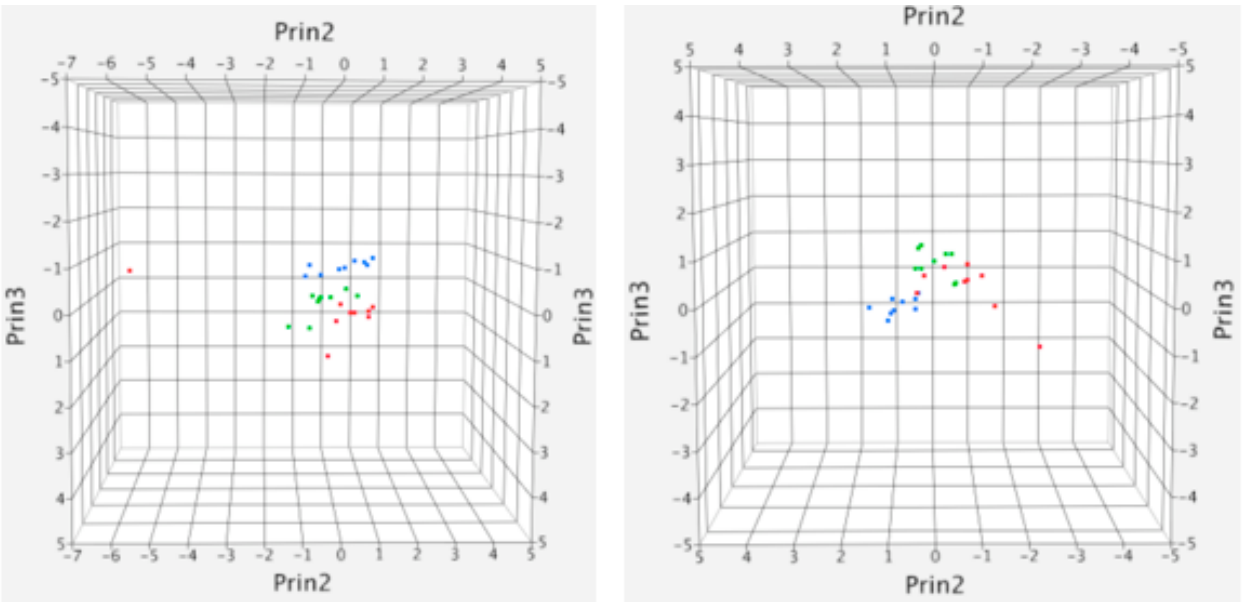


Figure 4.8 Cell type discrimination with different cell populations (SVHUC-Red, DU145-Green, PC3-Blue) A) 514,000 PC3, 262,000 DU145, 190,000 SVHUC B) 51,400 PC3, 26,200 DU145, 19,000 SVHUC

Chapter 5: Project Summary

This research found that a conducting polymer based electronic nose, Cyranose320, has the ability to discriminate between prostate cancer cell lines (DU-145 and PC-3) and non-tumor forming cancer cells from the urinary tract (SVHUC). The overall project was split into two studies. The first study determined the ability of the device to distinguish the cell lines from one another when taking into account different incubation periods and cell population. The second study looks further into the findings of the first study to determine whether cell population or cell maturity had a greater effect on the device. Ranges of detection for the device were also determined for each cell line.

Conclusions

In the first study, two initial cell populations of 75,000 (75k) and 150,000 (150k) cells were cultured and incubated for time periods of 2, 8, 12, and 24 hours. At each of the incubation periods for both cell populations, the Cyranose320 was used to test the volatiles present in the flask. Each cell line was analyzed individually to determine the variation between cells with various populations and various incubation periods. All three cell lines exhibited similar results illustrated by means of canonical discriminant analysis (CDA) plots and principal component analysis (PCA). When investigating the effects of the two cell populations (75k and 150k) on the ability of the Cyranose, the two cell populations showed minimal differences, which is the result of the cells being from the same origin. Some distinction was shown between the 75k and 150k cells however; it was evident that as time increased, the cell count of the 75k reached similar levels to those of the 150k cells, causing less variation. When investigating the effects of incubation periods, the largest distinction was seen between the cells tested after 2 hours, with smaller variation seen between the 8, 12, and 24 hour incubation periods. As time progresses, the

cells are able to expel more metabolites, creating a distinct volatile fingerprint to be recognized by the Cyranose 320.

Finally, when looking into the ability of the Cyranose320 to discriminate between all three cell lines, CDA plots show the device to be effective. All three cell lines formed distinct clusters with wide variation between the groups. The groups with the smallest variation were the two prostate cancer cell lines, PC-3 and DU-145. Some of the variation could be caused by the PC-3 cells expressing the Prostate-Specific antigen (PSA) while DU-145 cells do not. These differences could also be attributed to the differences between the patients from which these cell types were removed. PCA plots were developed discriminating the cell lines from one another after each of the four incubation periods. These plots showed that even after the earliest incubation period of 2 hours, the device was able to discriminate between the three cell types. Therefore, incubation period is critical when using the electronic nose technology so that the cells have enough time to produce volatiles in the headspace of the flask.

The second study performed takes into account the successful results from the first study and looks into the ability of the Cyranose320 to discriminate cells with high cell populations that were tested immediately after cell culture. Based on these findings, ranges of detection were determined to obtain the necessary cell level, or sensitivity, needed to successfully detect each cell line. After the first study was completed, it was noted that 24 hours was not a sufficient time period to observe ideal cell growth. Cells from each cell line, beginning with the initial populations of 75k and 150k were incubated for 2, 8, 12, 24, 48, and 72 hours and then counted to determine how many total cells were present after the 72 hour period. 262,000 cells for DU-145, 514,000 cells for PC-3, and 190,000 cells for SVHUC were recorded after the 72 hours period. Based on these quantities, five-log serial dilutions were performed in order to determine

if the electronic nose was capable of detecting each cell line after immediate culture. It was also determined at which cell population there was a clear distinction.

The cell lines were all analyzed individually using JMP® and PCnose® software. For all three cell lines there was a clear distinction between the highest cell populations used and the lowest. This shows that increased cell population is an integral aspect in the ability of the device to be successful in detecting the individual cell lines in culture. For DU-145 cells, CDA plots showed that a range of 26,200-262,000 cells would be ideal for proper detection of this cell line in culture. For PC-3 cells, the CDA plots showed that a range of 51,400-514,000 cells would be ideal for proper detection. Finally, for SVHUC cells, the CDA plots showed that a range of 19,000-190,000 cells would be effective when detecting this specific cell line in culture. All of these results were verified by the cross validation and mahalanobis distances (m-distances) presented for each cell line. The m-distances reached values of greater than 5 when comparing the largest cell population to the smallest three populations, thereby explaining the large variation between these groups.

The values determined as a range of detection were then used to develop PCA plots discriminating the three cell types against one another. These plots showed that no clear distinction could be made between the three cell lines when tested immediately after culture. The hypothesis was incorrect due to the fact that results show that incubation period is more important than cell population in the discrimination of these three cells in culture. It can therefore be concluded, that the cell population does not need to be taken into account when using the electronic nose technology. This electronic nose technology is successful based on the volatiles present in the headspace. To produce sufficient volatiles for discrimination, it is imperative to allow the cells to mature so that proper metabolic reactions can take place to produce these

volatiles. In cell culture, typically 1-3 days of incubation is necessary for cell experiments. However, the findings of this study show that regardless of the amount of cells used, the Cyranose320 can discriminate between cancerous and non-cancerous cells after at least two hours of incubation.

Future Work

Few studies have been conducted using the electronic nose technology in conjunction with prostate cancer. Bases on the results of this current study, a plethora of new studies could be conducting to determine new biomarkers or detection methods for prostate cancer. Since it is clear based on multivariate analysis that the Cyranose 320 can discriminate between the three cell types, the Cyranose 320 could be used to identify the cell types in culture. The Cyranose320 comes equipped with an identification feature that would allow the device to identify the samples based on the training that occurred in this study. Many factors such as age, testosterone, diet, race, and genetics are believed to have an effect on the incidence of prostate cancer. These factors could be investigated to determine how they affect the success of the Cyranose320 to discriminate between cancer cells. Research has shown that the prostate-specific antigen (PSA) blood test loses its accuracy due to other prostate traumas causing elevated PSA levels. For future studies, benign prostatic hyperplasia (BPH) could be tested to determine how the electronic nose can discriminate this prostate trauma from actual prostate cancer cells. Additionally, PSA could be added to the cell lines tested to determine how the presence of this protein affects the ability of the Cyranose 320 to be successful. To determine the specificity of the device, the cells could be grown for 72 hours, cultured, and then allowed to mature for 2 hours before testing, based on the results that 2 hours was a sufficient incubation period for the device. The cell lines could also be combined in one flask to determine the ability of the

Cyranose 320 to detect specific cell lines in mixed cultures. Principal component analysis (PCA) was used for statistical analysis in the first study, with the principal components being the 32 sensors in the Cyranose320. The analysis reveals the principal components that account for the most variation between the groupings. Future studies could determine what specific sensors are responsible for the variation and create a new prostate cancer-specific electronic nose device that could be used for future detection studies. Finally, since it is now clear that these cell lines are giving off certain volatiles in culture, urine and blood of prostate cancer patients could be examined. One of the main goals of prostate cancer research is to find a non-invasive method of detection. Using a non-invasive, hand-held electronic nose to analyze different bodily fluids would be an ideal means of detection that could open the door to promising areas of research in the prostate cancer field.

Appendix A: Raw Data used for PCA plot of Overall Cell Type Discrimination-Chapter 3

Cell Type	Prin 1	Prin 2	Prin 3
SV	-10.347912	2.7015351	-0.0689137
SV	-10.111437	2.7603552	0.75261108
SV	-9.5449945	2.61695837	0.919551
SV	-9.3176936	2.61040405	0.82288834
SV	-9.4118772	2.46352468	0.84561677
SV	-10.962607	1.04302003	-0.31356
SV	-9.2509202	1.20247996	-0.9831712
SV	-9.2754895	1.08101802	-0.4858665
SV	-8.5446495	1.37918242	-0.3136201
SV	-7.716638	1.06651301	-0.4084156
SV	4.27672502	2.2477426	-0.9335572
SV	3.32941705	1.45256627	-0.9362643
SV	3.37601665	1.27590943	-1.0987759
SV	2.05899752	1.10479701	-1.1344681
SV	1.42632802	1.0549561	-1.11127
SV	-5.156414	1.36783995	0.0940606
SV	-5.4276659	1.31035399	0.03789494
SV	-5.8224476	1.35333203	-0.0004342
SV	-6.4790671	1.12008257	-0.1235085
SV	-6.7412877	1.06529007	-0.4058339
SV	-5.0917027	3.3851407	0.79883978
SV	-5.9605931	2.83652298	0.71386835
SV	-5.563128	2.69529664	0.47329496
SV	-6.5769701	2.43253819	0.4584043
SV	-6.7783584	2.42158845	0.41992554
SV	-3.3490421	1.52900102	-0.627929
SV	-4.4651324	1.43823712	-1.1636136
SV	-4.457082	1.59649871	-0.716789
SV	-4.7678234	1.25597817	-0.6111034
SV	-4.4849862	1.55619473	-0.5900755
SV	8.50879388	1.63929419	-1.2782091
SV	5.74886456	1.36952797	-1.2339031
SV	3.9498151	1.21939244	-1.0940511
SV	3.41861854	0.95951894	-1.207573
SV	0.39070563	0.91741306	-1.0361388
SV	0.37786944	1.91795429	-0.2190876
SV	-0.5437659	1.08435342	-0.3802965
SV	-1.6011446	1.29411608	-0.5146863
SV	-3.1244471	1.08046041	-0.5230089
SV	-4.6313329	0.76842073	-0.6927553
SV	0.10537097	3.58214126	0.58198887
SV	-1.5601218	2.98540592	0.36864231

SV	-1.5677512	2.81526382	0.32129249
SV	-2.9088943	2.68709336	0.42176899
SV	-3.4532311	2.55570196	0.45670825
SV	1.21273037	1.81091757	-0.7666604
SV	-0.6211424	1.7815658	-1.287077
SV	-1.2257497	1.46186975	-0.8595033
SV	-0.9932451	1.45394934	-0.7230344
SV	-2.9058178	1.36911651	-0.782612
SV	8.76273178	1.24926534	-1.3628057
SV	5.34976702	1.23259473	-1.1636887
SV	4.47097588	1.06712288	-1.2605408
SV	3.48619493	0.86871936	-1.3120563
SV	2.15368859	1.03706416	-1.1519859
SV	4.65801073	1.63320045	-0.991822
SV	-0.4758736	1.19681987	-0.8231937
SV	-3.1527413	1.09840173	-0.7617642
SV	-5.339268	0.666254	-0.7848309
SV	-4.4856541	0.42139826	-1.054914
SV	0.72911517	3.65731996	0.45214239
SV	-1.3330488	2.834384	0.35697278
SV	-2.5111549	2.46053077	0.30796491
SV	-4.3968634	2.38530234	0.20831846
SV	-5.2524742	1.99729693	0.1180759
SV	3.49178027	1.75186905	-1.0195044
SV	0.69012222	1.98258713	-1.1352656
SV	-0.6738396	1.3334103	-1.0301352
SV	-0.1013255	1.42808976	-0.7654911
SV	-1.3206607	1.21732154	-0.9297416
SV	7.51233043	1.37380077	-1.3127434
SV	4.79441259	1.1358654	-1.3398854
SV	1.82750939	0.77358926	-1.1123227
SV	0.80720731	0.67071425	-1.2045386
SV	-0.3567692	0.49941635	-1.175129
SV	2.77903376	1.05254425	-1.4155473
SV	-0.2615085	0.77411873	-1.0824165
SV	-0.7699633	0.91863688	-0.9829455
SV	-1.6916536	0.75846797	-1.0701204
SV	-3.913636	0.65073441	-0.9693401
SV	3.38413711	3.34196074	0.0847894
SV	0.4964122	2.78267862	0.05610333
SV	-0.5005987	2.57560134	0.11421802
SV	-2.0652621	2.21206089	-0.0004205
SV	-2.9703263	1.95695532	-0.1342699
SV	5.63767625	2.27224068	-1.0254957
SV	2.68250559	1.58151929	-1.0965847

SV	2.04187889	1.53614062	-0.8910918
SV	1.68400777	1.29866239	-0.9054512
SV	0.31237055	1.29837037	-0.8628911
SV	7.80901909	1.02166074	-1.5117045
SV	4.41926905	0.61552573	-1.5119741
SV	2.81539346	0.69468651	-1.4770946
SV	1.02037452	0.48543444	-1.4799759
SV	-0.3196551	0.5528462	-1.2682978
SV	4.76458335	1.10617794	-1.3179272
SV	1.41431832	0.98331549	-1.3921353
SV	-0.2250546	0.43480746	-1.3582344
SV	-2.3822639	0.53051947	-1.1528931
SV	-4.2177019	0.28781717	-1.1449427
SV	3.53828353	2.9517901	-0.0021039
SV	0.14608097	2.50931823	-0.4166312
SV	-0.9737591	2.22162341	-0.140899
SV	-1.7208426	2.08310065	-0.0614015
SV	-4.0051519	1.89711723	-0.2802614
SV	7.49124819	2.14295346	-0.8691006
SV	4.51633587	1.56198098	-0.9557039
SV	3.71307846	1.62153576	-0.8718238
SV	0.93064319	1.40005847	-0.8153723
SV	-0.2715227	1.11411986	-0.9178203
SV	7.20696015	1.16244115	-1.6529227
SV	4.30642341	0.87490564	-1.6917235
SV	2.20835893	0.63498969	-1.225509
SV	1.03706301	0.56175461	-1.3960069
SV	-0.3617984	0.36884398	-1.5052577
SV	2.84672716	0.89049217	-1.4994512
SV	-1.466209	0.49585448	-1.2326399
SV	-2.7862076	0.62763241	-1.182855
SV	-5.0097412	0.09748406	-1.2084077
DU	-5.6524506	0.15372424	-1.38274
DU	-2.451808	-0.0713872	1.90991514
DU	-1.4814059	0.36101236	3.44167614
DU	-0.9055353	0.15871795	3.12927214
DU	-1.3750049	0.29820628	2.95260534
DU	-3.0429282	-0.0634387	2.49004645
DU	-3.867581	-0.9321102	0.19485498
DU	-2.1354708	-0.4864684	-0.2194065
DU	-1.8899892	-0.5189063	0.91463334
DU	-1.0424219	-0.4322288	1.04977969
DU	-0.3141978	-0.656349	0.91236375
DU	2.81640651	0.2401747	-0.1014175
DU	2.89357036	-0.5730978	-0.3322872

DU	1.65070839	-0.7507352	-0.1219311
DU	0.30766069	-0.7685692	-0.2623903
DU	-1.0481277	-0.7771503	-0.1904137
DU	-6.6513924	-0.2680542	0.20612173
DU	-6.5528884	-0.1147539	1.0183822
DU	-6.2175197	0.10875536	0.93284364
DU	-7.0987158	0.23969671	0.82122832
DU	-7.5526179	-0.0750617	0.63056253
DU	4.66198291	1.03495049	2.8609283
DU	3.61835117	0.7624042	2.55770304
DU	2.83368397	0.72797368	2.54173592
DU	2.16765893	0.51937258	2.24804236
DU	1.40992334	0.33940227	2.12232301
DU	5.59743225	-0.6329369	0.51499048
DU	2.93565571	-0.488099	0.49617809
DU	3.5320975	-0.5119066	0.65336362
DU	3.80636177	-0.3688522	0.67807694
DU	2.88560489	-0.2013026	0.72357507
DU	8.16050304	-0.5503303	-0.2924767
DU	5.43001905	-0.8615114	-0.3270657
DU	4.85532786	-1.1239353	-0.3512588
DU	4.26173575	-1.4569139	-0.5780374
DU	1.93334741	-1.4225011	-0.3788468
DU	1.30899169	0.56478611	0.47700568
DU	-2.695334	0.28212364	0.41128165
DU	-4.6574543	-0.2608638	0.29335551
DU	-5.5112813	-0.342315	0.19264146
DU	-6.9332533	-0.4434955	0.12917389
DU	9.26096853	0.97565391	2.608303
DU	6.8572231	0.840889	2.03147791
DU	6.2558351	0.6213228	2.13690817
DU	5.31283969	0.51517586	1.94852319
DU	5.04919196	0.48067262	1.81230583
DU	9.24333817	-0.1854297	0.61528574
DU	6.83700203	-0.3037322	0.6731203
DU	6.57601134	-0.1239466	0.83341896
DU	5.48010145	-0.4160073	0.74879229
DU	4.01348647	-0.599293	0.51601615
DU	9.31929419	-0.8863065	-0.3822839
DU	3.69119499	-0.5497215	-0.4581925
DU	0.63733327	-0.6127019	-0.3970266
DU	-1.172593	-0.480683	-0.3974623
DU	-3.0314943	-0.6617215	-0.5467958
DU	3.57701432	0.0892366	0.10185473
DU	0.86722849	-0.0588444	0.30289029

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DU	0.11146157	-0.1737525	-0.6653125
DU	-0.6077471	-0.2777833	-0.4403247
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DU	-3.5804331	-0.7732767	-0.5508394
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DU	-0.5152583	-0.1945566	0.1267206
DU	-1.3666879	-0.501927	-0.1650414
DU	-5.7518334	-0.3959362	-0.2933761
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DU	4.61773669	-0.3981743	-0.6133322
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DU	-10.351378	-1.0067625	-0.7572415
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