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Diagnosis of Melanoma Disease State From Patient Blood Samples Using Photoacoustic Flow Cytometry

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DIAGNOSIS OF MELANOMA DISEASE STATE FROM PATIENT BLOOD SAMPLES USING PHOTOACOUSTIC FLOW CYTOMETRY

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In partial fulfillment of the requirements for

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By

Jacob Salvatore

May 2021

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Approved March 24th, 2021

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ABSTRACT

DIAGNOSIS OF MELANOMA DISEASE STATE FROM PATIENT BLOOD SAMPLES USING PHOTOACOUSTIC FLOW CYTOMETRY

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Thesis supervised by John Viator, PhD

Approximately 1 in 50 Americans develop melanoma in their lifetime. Early detection of melanoma is pivotal to the survival of the patient, with a 99% survival rate for 5 or more years after an early diagnosis. Metastasis, or the spread of cancer, increases the chances of cancerous growth in other parts of the body. In this study, we propose the use of photoacoustic flow cytometry as a diagnostic of patient disease state, by closely monitoring the amount of circulating melanoma cells (CMCs) in a patient's blood before and after chemotherapy treatments. Using this patient data, we were able to make conclusions regarding the progression of cancer in each of the patients. In the future, we hope to use this real time diagnostic as insight to the effectiveness of a patient's treatment and ideally sculpting a more effective patient- by-patient treatment regimen.

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I. Introduction

Cancer is becoming an ever-increasing factor in our daily lives, affecting many people and resulting in the deaths of hundreds of thousands each year,[1] killing more than any other disease with the exception of cardiovascular disease.[2] In 2017, approximately 1.3% of the world, including 5.5% of the American population, was diagnosed with some type of cancer.[3] The United States posted more diagnoses that year than any other country in the world. Cancer is taking a big toll on our health systems with about 1 in 6 deaths being attributed to cancer-related complications.[4] It is very evident that cancer diagnoses are on the rise and will most likely continue to rise for years to come.

The key to treating these cancer diagnoses and preventing deaths in many patients is a quick and effective diagnosis. By diagnosing cancer at an early stage, doctors can hopefully find an effective form of treatment and stop the spread of disease before the cancer metastasizes and turns into more severe problems for the patient. Five-year survival rates vary for different types of cancer but can be as low as 5% in late stage lung cancer diagnoses.[5] It is apparent that early diagnosis is key to providing the most effective and efficient form of treatment possible, saving patients' time and money.

When developing a new cancer diagnostic, it is important to understand the progression of the disease. This study plans to recount cancer in a basic sense of the disease, then focus on melanoma progression and the development of a diagnostic for melanoma with hopes of universal use.

II. Cancer

Cancer can be a debilitating disease that has troubled our health systems for many years. The 5-year survival rate is progressively rising in some cancers; however, other cancers continue to thwart efforts made in diagnosis and treatment. While the rate of cases has continually increased from the 1970's until now, the death rate has fallen due to the aggressive efforts that have been made in science to develop new methods of detection and treatment.[6] To understand the important considerations one must take while developing a cancer diagnostic, they must first understand the progression of the disease, particularly the initial source. This section strives to outline the key source in cancer development, the cell cycle, and the important roles it takes to safeguard itself from cancerous growth and what happens when this goes awry.

The American Cancer Society defines cancer as the uncontrollable growth of cells to crowd out healthy cells.[7] While scientists have not singled out one cause of cancer, a variety of factors have been known to increase the likelihood of developing cancerous cells which may lead to a diagnosis of the disease. Environmental factors including exposure to chemicals, UV rays, and airborne particles have been known to increase chances. Additionally, personal habits, such as smoking and diet, have been known to increase chances and even catalyze cancerous growth. Previous infections may also trigger cancerous activity within cells, potentially becoming harmful. Genetics inherited from family members can also potentially play a role in the probability of developing cancer.[8] With the large range of possible causes, it is important to closely monitor one's health and frequently communicate with physicians regarding the potential risk. As mentioned before, cells have a natural response to immediately stop any sort of cancerous growth; however, in some situations the harmful growth is too overbearing, and the body cannot halt its progress.

a. Cell Cycle

A common theme associated with each of the potential origins of cancer is the influence that each play on the cell cycle. Unchecked errors within the cell cycle, a cell's reproduction sequence, may lead to

uncontrollable growth of cells, otherwise known as cancer. The cell cycle is essential to for cell survival and growth and comprises of several checkpoints to regulate proper growth and cut down on errors that may occur in DNA transcription. The way that a cell responds to cell damage, specific to the type of damage that has occurred, is critical in preventing the development of cancer.[9] In some cases, damage may be severe enough that the cell will halt the cell cycle or even commit cell suicide, apoptosis. Throughout each of these checkpoints, the cell will monitor order, integrity, and fidelity, insuring optimal conditions for cell replication.[10]

Cells remain in an inactive, non- replicative state for most of their life, this state is considered the G0 state, which consumes most of the cell cycle. Pocket Proteins, a group of proteins found in most cells, closely regulate proliferation and cellular response to antimitogenic signals.[11] The group of pocket proteins includes pRB, p107, and p130, all of which closely work together to control mitogenic gene activity. Figure 2.1[12] shows the cell as it transitions from G0 to G1, pRB is increasingly phosphorylated by cyclin D, leading to the release of more E2F transcription factors, and ultimately increased proliferation. pRB will be hyperphosphorylated until late in the mitotic cycle, allowing for the increased release of E2F. Mutation of the RB protein can have severe consequences with E2F regulation and may lead to inappropriate proliferation.

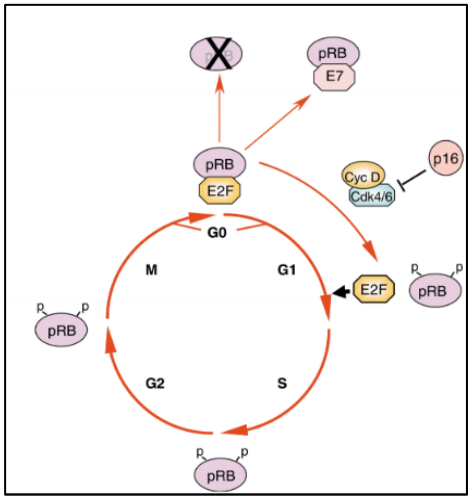

Figure 2.1 pRB- E2F Pathway

The molecular cascade highlights the pRB-E2F pathway. pRB binds and inhibits E2F early in the cycle but is later phosphorylated by cyclin D-Cdk4/Cdk6 freeing E2F. The released E2F induces genes to promote the progression into S Phase. Mutation of the RB gene can lead to cancerous growth due to the overexpression of D cyclins or p16 inhibitor of Cdk4/Cdk6.

The start of a new cell cycle is postmarked by the beginning of a new G1 phase, shown in Figure 2.2.[13] Activation of cyclin- CDK triggers a positive feedback loop resulting in full commitment to a new cell cycle. This positive feedback loop serves as an 'all- or- none switch' to high amounts of cyclin-CDK activity leading to the phosphorylation and inactivation of pocket proteins.[14] D Cyclins are of the first to be transcribed early in the G1 phase along with CDK 4 and CDK 6. Late in the G1 phase, Cyclin E, assists in the further transcription of more genes, including CDK 2. Cyclin D and E monitor and direct the phosphorylation of pRB.[15] E2F, a common transcription factor in this stage of the cell cycle, assists in the transcription of more genes to further the cell cycle. Errors associated with E2F regulation are commonly found amongst cancerous cells, potentially serving as a key observable gene that could be used for cancer diagnosis.[16]

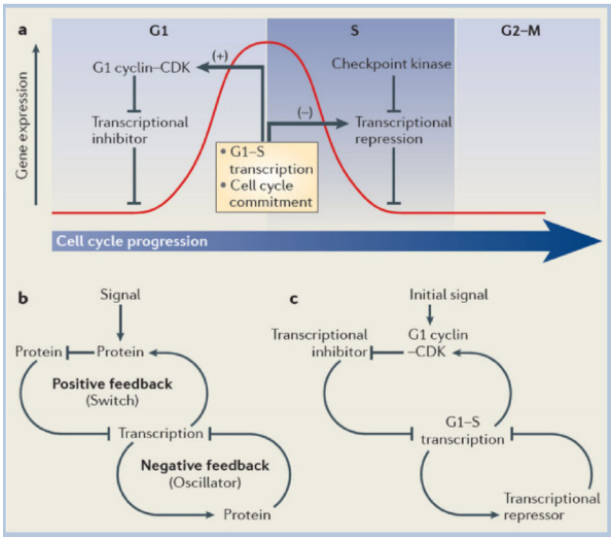

Figure 2.2 G1-S Phase Transition

This graphic denotes the positive feedback loop of the G1-S transcription, continuing until a subsequent negative feedback loop halts gene expression.

E2Fs play a critical role in a variety of cell processes, particularly in the progression from G1 to S phase. The E2F family includes six different transcription factors, E2F1-E2F6, with several implications to the cell cycle, shown in Figure 2.3.[17] The first family, E2F1-E2F3, expression is directly dependent on cell- growth regulation. In the case of 'free' E2Fs, transcription is activated and believed to drastically increase cell growth, forming tumors. The next family, E2F4 & E2F5 bind to pocket proteins to induce S

phase in non-quiescent cells. Lastly, E2F6's role in cells is unknown, but assumed to play some role in E2F- dependent transcription.[18] While it is not completely understood, E2Fs are believed to play numerous other roles in cell maintenance and cycle progression. Effects on E2F through 'oncogenic insults' include viral proteins binding to pRB and displacing E2Fs, excessive pRB phosphorylation due to over expression of D cyclins, loss of p16 inhibitor, and mutation or amplification of CDK4 and CDK6. Ultimately, each of these scenarios result in an inadequate amount of E2F and may result in the development of cancer.[19]

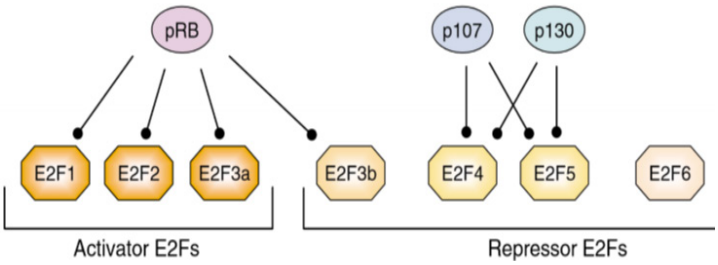

Figure 2.3 E2F Transcription Factors

Shown in this figure is each of the E2F transcription factors, their activation proteins, and the role that they play on cell cycle progression.

As a cell progresses to S phase, the nucleus prepares for DNA replication, a vital step to cell reproduction and division which includes a complex cascade of cellular interactions, which can be visualized in Figure 2.4.[20] As DNA synthesis proceeds, cyclin D, which had been previously synthesized in G1 Phase, increasingly blocks p16, allowing the expression of CDK 4 and CDK 6. Once phosphorylated the two CDK molecules block the retinoblastoma (Rb) molecule, in turn freeing additional E2F transcription factors.[21] DNA replication proceeds with DNA helicase separating the dsDNA into two single parental strands. In the leading strand DNA Polymerase proceeds in the same direction as DNA Helicase, synthesizing a new DNA strand with corresponding base pairs. On the lagging strand, DNA Polymerase progresses in the opposite direction as DNA Helicase, also synthesizing a corresponding strand. In an ideal scenario, the two newly synthesized strands are exact copies of one another, but realistically damage is common and will be further discussed.

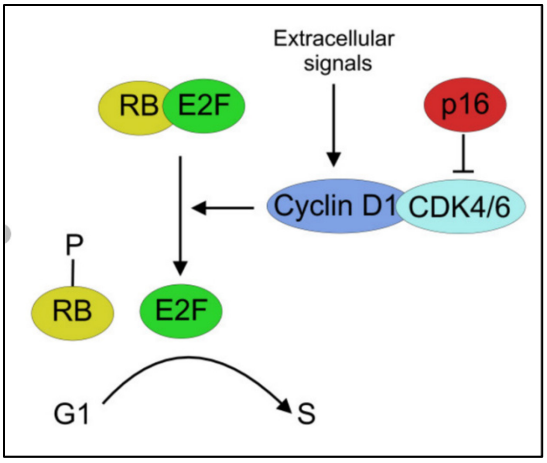

Figure 2.4 CDK 4/6 Role in Transition from G1 to S Phase
CDK 4 and CDK 6 production leads to the blocking of the p16 protein. This in turn frees Cyclin D1 to phosphorylate the Rb molecule, ultimately leaving E2F to assist in the transition from G1 to S Phase.

G2 phase is important in the synthesis of proteins for progression into mitosis. This checkpoint of the cell cycle is the last barrier before the cell fully commits to mitosis and cell division. Cycloheximide, along with puromycin, play an important role in activating p38 and blocking mitotic entry until protein synthesis is fully completed. The synthesis of cyclin B1, Plk 1, Bora, and Aurora proteins lead to the activation of kinases, particularly CDK 1. This mitotic kinase activation occurs through two feedback loops: a double negative loop and positive feedback loop, seen in Figure 2.5.[22] As mentioned, cell division is a complicated process and poses many opportunities for DNA damage and development for cancer. This double switch is important in regulating and limiting DNA damage before a cell progresses into mitosis.

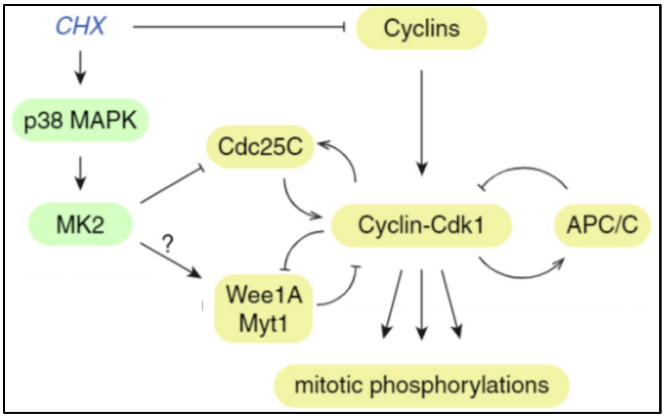

Figure 2.5 Regulation of CDK 1 throughout the G2/M phases

This figure illustrates regulation of CDK 1 throughout G2/M phases of the Cell Cycle. Cycloheximide (CHX) inhibits protein synthesis, blocking cyclin accumulation while activating p38 MAPK, which may delay cell cycle progression through inhibition of Cdc25C, or activation of Wee1A/Myt1. Antigen-presenting cell (APC/C) production helps stabilize Cyclin-CDK 1 production over time.

All protein synthesis and cell division preparation culminate in the final step of cell division, mitosis. This portion of cell division consists of several phases in which a cell oversees the equal distribution of genetic material to each sister cell. Beginning with prophase, genetic material is condensed into duplicate threadlike structures, chromosomes. The cell uses microtubules and the cytoskeleton to centrally align chromosomes. This results in the formation of centrosomes at opposite corners of the cell that serve as foci observing chromosome separation throughout anaphase. Reformation of the nuclei following in telophase. Cytokinesis follows with division of organelles and the remaining cytoplasm.[23] Unequal distribution of genetic material can lead to instability in cells and ultimately increase the potential of harmful or unregulated growth, posing another threat that may lead to cancer development.

b. DNA Damage

As made apparent by the variety of protein syntheses and cell molecule cascades in DNA and Cell Replication, DNA damage can be common. Environmental factors can increase the risk of error, ultimately effecting the production of some proteins and molecules. The slight over- or under- production of these signal molecules can alter the progression of the whole cascade and change cellular production.

Errors may result in unintended genomic sequences that can lead to sequences coding for cancerous growth or harmful development.

Extrinsic factors account for approximately two-thirds of cell cycle alterations that lead to cancer development, with many of those alterations being endogenous mutations. While it is not uniformly accepted, it is believed that many instances of cancer originate from 'malignant transformation of normal tissue progenitor and stem cells.'[24] This transformation is believed to typically be a product of one's environment and extrinsic influences. These influences can vary along with the cancer that they may cause. Data supports that geographic location can lead to and increased extrinsic risk of breast and prostate cancers, while colorectal cancer can result from one's diet. Exposure to diseases has an additional risk as well, particularly HPV with ~90% of cervical cancer cases and H Pylori leading to 65-80% of gastric cancer incidences. The most important to this study being the 65-86% increased risk of melanoma being attributed to sun exposure.[25] There are several additional extrinsic causes that are correlated with increased risk of cancer, many of which varying with the surrounding environment. In a study by Wu et al, some key mutational signatures were able to be traced back to certain extrinsic factors, highlighting the effect that they have on cancerous growth.[26]

Intrinsic factors account for the remaining third of risk factors that contribute to cancer development. These intrinsic factors are believed to alter stem cell divisions, resulting in unhealthy cell division.[27] These intrinsic factors include unhealthy strains on cellular processes that may send a cell into 'unhealthy overdrive.' Some of the most common being rogue intermediates, telomere attrition, oncogene overexpression, or replication errors.[28] The increased strain that these unhealthy cellular products place on the cell may lead to tumorigenesis and cancer development.

Ultimately, DNA damage can alter gene expression and overall cell function. Damage to genes that regulate growth can prove to be the most fatal, resulting in a Loss of Heterozygosity (LOH). Tumor suppressor genes, particularly p53, and amplification oncogenes, MET, are two of the most commonly affected genes resulting from LOH. Genomic loci instability is common amongst cancer cells and may lead to tumorigenesis and common fragile sites (CFS). These CFS are points at which replication stress,

intrinsic or extrinsic factors may lead to DNA damage and ultimately genetic error that could result in cancerous growth.[29]

c. DNA Repair

Damage to DNA is constant due to the numerous constant threats to its vitality, and therefore repair must be constant to protect against genetic errors. Once damage is detected, the cell cycle is slowed, and a cascade response is triggered.[30] Checkpoints, specifically Chk 1 and Chk 2, are built into the cell cycle to catch any damage before too late into the replication cycle. If, however, the damage persists, responses within the cell may be triggered including the ATR and ATM signaling pathways.

The Ataxia Telangiectasia (ATM) gene plays a vital role in in DNA repair, being the first kinase activated in the P13 signaling chain. Initially serving as an inactive dimer, ATM undergoes autophosphorylation and becomes active monomers further triggering downstream targets including p53 and Chk2. While ATM focuses on the double- strand breaks (DSBs) some evidence has shown that ATM will also actively repair other cell damages, which can be important in preventing cancer and other effects from cellular harm. Cells that lack an ATM gene have been found to be extremely sensitive to DNA damaging agents and may fail to delay DNA replication in the situation of harmful cell replication.[31] An absence of ATM function in cells could be threatening to the health of the cell and result in unhealthy cancerous growth, a target for cancer therapeutics.

Similar to the ATM pathway, the Ataxia Telangiectasia and Rad-3 related protein (ATR) coordinates repair related to replication, specifically with replication forks. When triggered by DNA damage, the ATR gene will slow and stall replication forks to prevent further replication origin initiation.[32] By halting replication, the cell has an opportunity to fix any errors before they replicate beyond control.

These chemical agents pose a threat to genetic info and cause an increasing risk of cancer development. Chk 1 and Chk 2 are protein kinases that are activated in response to genotoxic insults. These protein kinases serve as signals to cell infrastructure as to which areas of the DNA and Cell Replication that needs fixing or maintenance. Once receiving checkpoint signals from proximal kinases, particularly ATM and ATR, Chk 1 and 2 relay these signals further to trigger a response.[33]

III. Melanoma

Melanoma is the result of malignant melanocytes, or skin cancer cells that are responsible for the skin pigment, melanin. Cancerous growth may result from increased exposure to UV rays, either from a natural source like the sun or even artificial sources such as tanning beds. As mentioned before, increased sun exposure accounts for an additional 65-86% risk of developing melanoma.[34] This ultraviolet exposure is bad for the melanocyte replication health, commonly causing unintended breaks in the DNA helix. The ultraviolet light may also weaken the strength of genomic loci, important in mitotic chromosomal separation, and lead to common fragile sites (cfs), further increasing replication stress.[35] The cancerous growth will then soon spread to the blood where metastasis occurs and the disease may spread throughout the body, wreaking havoc on other organs and body parts. This quick spread makes detection and treatment vital to a patient's survival. While malignant melanoma incidence has decreased 40% to 20% in recent years, the mortality rate remains high. Only behind pancreatic cancer, melanoma remains the second most dangerous tumor, killing several thousand Americans every year.[36]

a. Melanoma cell markers

It has been hypothesized that cell markers can play an important role in identifying cell function and risk of cancerous growth they may pose. Previous studies conducted by Viator et al, have included *Automated Photoacoustic Isolation of Circulating Melanoma Cells for Molecular Analysis* in which melanoma cells are identified, separated and collected for further analysis of cell markers.[37] Cell markers may not only help us more easily identify high- risk cells but provide additional functions such as validating therapies or providing insight as to how such cells grow or act under certain conditions.

Cell Adhesion Molecule 1 (CADM1), one gene amongst many believed to be insightful to melanoma, plays an important role in suppressing cell invasiveness and inhibiting tumors through non-adherent cell death.[38] The CADM1 protein produced by the gene is one of two Epithelial Mesenchymal

Transition (EMT) proteins that regulate tumorigenesis, as displayed in a recent study by Wang et al. This new study investigated CADM1 and microRNA (miR)-214's relationship with melanoma after noticing the two were differentially expressed in cancerous melanocyte cells. MicroRNAs (miRNAs) can promote the occurrence of cancer and inhibit an immune response, while its counterpart, CADM1 is expressed significantly less *in vitro* in melanoma cells compared to healthy melanocytes. The combination of mir-214 promotion and downregulation of CADM1 is believed to result in progression of EMT and the metastasis of malignant melanoma tumors.[39]

While not all cells are tumorigenic, or rapidly grow, those that do are believed to have specific cell markers that can be used to identify and potentially target. These tumor initiating cells may have a differential response to various kinds of therapy. Cancer stem cells (CSCs) are the proliferating cells in a tumor cluster of cancer cells, typically located at the outermost parts of the tumor. The number of CSCs in a tumor varies depending on the tumor type, size, and progression. AC133 is an antibody used to identify CD133, a common receptor on the membrane of many CSCs, and may serve as a valuable tool in identifying cells with increased tumorgenicity.[40]

Some of the main targets for melanoma drug therapy include the BRAF, ERK and MEK genes. These three genes are extracellular signaling kinases, frequent in melanoma, that regulate the G1 cycle proteins: cyclin D1 and p27. Through a cell signal cascade, BRAF activates MEK which in turn activates Cyclin D1 typically through adhesion growth factors and ERK signaling. In melanoma cells, however, integrin and growth factor involvement are bypassed and a mutant BRAF triggers ERK leading to an overexpression of Cyclin D1 in tumors. An increased display of ERK activity is common in melanoma cells, directly impacting the adhesion and growth factors. Seventy percent of melanomas involve a BRAF mutation that lead to ERK activation independent of adhesion and growth factor initiation.[41] Non-adhesion cell growth can lead to an increased production of free-flowing melanoma cells in the blood stream, known as circulating melanoma cells (CMCs).

Melanocytes, non- cancerous cells containing the pigment melanin, typically reside in the border of the two outermost layers of skin, the dermis and epidermis. There the pigmented cells are not highly proliferative, and their main purpose is to pigment the skin. Initially these melanocytes will remain in a radial growth phase, growing perpendicular to the skin surface. As cancerous growth is incited, the melanocytes will begin to penetrate the dermis and subcutaneous layer of the skin in a vertical growth phase. As the melanocytes infiltrate the deeper layers of the skin, they will gain access to the blood stream and spread throughout the body as CMCs.[42]

Two melanoma cell markers that have been analyzed in previous studies include MART1 and SOX10, both of which are common surface markers of melanoma cells. As done in a previous study by Viator et al, after being run through the flow chamber, cells were collected and stained before being viewed under the microscope. Pictured in Figure 3.1[43] below, the staining makes it evident as to which cells possess the melanoma cell markers, as they emit fluorescence under the microscope. This valuable tool can be used as a future diagnostic tool in the detection of melanoma cells.

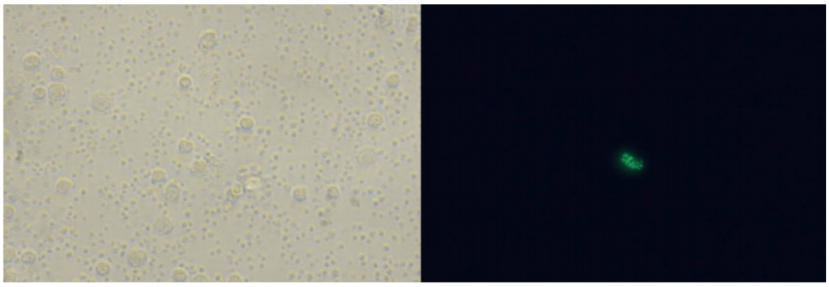

Figure 3.1 Melanoma cells with MART1 Expression
(Left) Brightfield image of captured cells. (Right) Fluorescent image of the same field showing the presence of a melanoma cell with MART1 expression. Images from a previous study by Viator et al.

b. CMCs

At optimal growth, cells will be released from the tumor and flow freely throughout the body's vascular and/or lymphatic ducts.[44] Alterations in the adhesive properties of cancer cells leads to their unusual violation of tissue architecture and the rapid spread of malignant cells.[45] The circulating cells precede metastasis and lead to the rapid spread of disease throughout the entire body, at which point

treatment options quickly diminish, with a survival rate dropping as low as 25%.[46] Once in the blood, CMCs must continue to be able to survive both shear stresses and immune mediated killing initiated by the body's immune system. It is believed that CTCs use blood platelets to 'shield' stresses experienced in the blood stream and attacks from the body's immunes system. 'Metadherin' is a term that has been developed to describe a novel adhesion receptor that is common to metastatic cells.[47]

While an excess of CMCs may be found in the blood, cells must develop the capacity for tumor initiation at another location for secondary tumor establishment.[48] This additional growth continues to place a strain on the body's immune response, leaving it vulnerable for additional infection. It is theorized that the number of CMCs in melanoma patients has a direct correlation with the disease state and patient status, providing potential for real-time assessment.

c. Metastasis

Metastasis is a result of the spread of malignant cancer cells throughout the body as they adapt to the tissue microenvironment distant from the primary tumor. Metastasis serves as a primary hallmark of cancer diagnosis, signaling the progression of the disease that results in 90% of deaths from solid tumors. Effective cancer diagnosis and treatment is critical before metastasis is reached.[49]

Metastasis can be imagined as the evolution of cancer cells and the progression of successful heterogeneities and advantageous cancer cell traits. For cancer cells to successfully metastasize and spread throughout the body, cells must develop a metastatic cascade, while surviving the countless intrinsic and extrinsic tumor suppression attempts from the surrounding environment. This metastatic cascade includes a loss of cellular adhesion, increased motility, entry, and survival in circulation, exit into surrounding tissue, and colonization of a distant organ. Gupta and Massaguè propose that while the evolution through the metastatic cascade is not instantaneous, it is an inevitable product of the countless malignant cells released from a tumor each day.[50] It is key that a diagnostic device, such as the one

proposed in this study, catches the spread of malignant melanoma cells before evolution has been achieved.

The "Angiogenic Switch" as described by Gupta and Massaguè is a vital step in the evolution of solid tumors. This includes the acquisition of an angiogenic phenotype, or the gene to grow blood vessels. The growth of blood vessels throughout a tumor will not only provide tumors with additional nutrients to increase in size, but a highway to further spread the malignant cells. Additionally, 'lymphangiogenesis' a phenomenon observed in advanced primary cancers includes the growth of a network of lymphatic vessels in tumors. These lymphatic networks are more common in melanoma, and increase the spread of malignant cells, particularly because they are 'more leaky' than the vasculature structure of blood vessels.[51]

d. Detection and Diagnostic Methods

Current cancer detection methods are slow and provide somewhat of an unreliable scope of disease state. This drastically effects the time to diagnosis and the treatment that can be administered to the patients. Additionally, unreliable detection methods may lead to ineffective treatment that does not successfully target all the cancerous cells in a patient's body. Ample evidence has led to metastasis serving as the goal post for melanoma diagnosis, with a dim outlook for survival after metastasis has been achieved.[52]

Melanoma diagnostics have drastically evolved through the years with great advancements in technology and methods applied to cancer research, with the most prominent methods highlighted in Table 3.1.[58] In that time however, there has been an increase in clinical diagnoses of melanoma. Basic methods of diagnosis rely on visual identification of gross features and morphological differences. The ABCDE screening method, made popular in the 1980's, is still used as a preliminary melanoma identification method in some clinical settings. This method relies on the identification of: Asymmetry,

Border Irregularity, Color Variation, Diameter and Evolution of skin lesions and nevi. While this method of examination is quick and relatively effective it mainly relies on the discretion of physician, leaving room for error.[53]

As technology progressed and more automated systems were integrated into healthcare, digital dermoscopy proved to be an effective technique in melanoma identification. Multispectral Digital Dermoscopy and Image Analysis are common processes based off the acquisition of several images of the skin at various depths obtained with a series of bands of wavelengths. These methods help differentiate between benign skin lesions and malignant lesions using computer databases referencing previous data and is constantly improving with more reference data always being added.[54]

Laser- Based Enhanced Diagnostics includes several real time imaging analyses for effective melanoma diagnosis. These methods integrate the use of various laser wavelengths to gather images of the skin at consecutive depths. These images can be pieced together to gather a full scope of the subsurface characteristics of the sample. Confocal Scanning Laser Based Microscopy (CSLM) provides a high-resolution option with reproducible results, with newer CSLM methods moving towards the use of fiber optics. Reflectance Confocal Microscopy (RCM) has an even higher resolution than CSLM but has increased sensitivity and is more expensive. Lastly, Optical Coherence Tomography (OCT) provides the greatest depth imaging at 1mm. Light reflectivity in OCT creates a contrast in cellular structures, helping with identification of melanoma cells.[55]

Ultrasound imaging technology has rapidly progressed with application in a variety of health care settings. Utilizing high frequency wavelengths, subtle differences in healthy nevi and cancerous melanoma cells can be noticed. This imaging technology can be paired with mRNA gene expression analysis for profiling.[56] The addition of profiling further highlights the use of genetics and molecular analysis to predict and diagnose cancerous growth.

A new technology referred to as Cellular Electrical Resistance analysis has gained a lot of popularity in recent years. This concept of analysis is based off the measurable electrical resistance, also called bioimpedance, of cells. Bioimpedance is a function of cellular shape, structure, cell membrane, and water concentration. Since cancer cells are shaped differently than normal, healthy cells, they have a quantitively different bioimpedance than healthy nevi. This method only takes a mere 7 minutes for analysis and results, for quick and easy melanoma identification.[57]

Detection Method	**Advantages**	**Disadvantages**
Gross Feature Identification	None	Melanoma not Diagnosed until late stages
ABCDE	None	Based upon physician recognition
Digital Image Capture and Analysis	Close-up Computer Imaging; Ideal for Follow-Up Comparison; Total Body Photography	No Computer Diagnostic Analysis
Computer Augmented Image Analysis	Multispectral sequence of images created in <3 seconds; Handheld Scanner	None
Digital Dermoscopy	Empirical Database for comparison	Requires Oil Immersion
Laser-Based Enhanced Diagnosis	Longer wavelengths allow for deeper penetration; Fiber-Optic imaging allows for handheld devices	Poor resolution; Minimal detection of early stage melanoma
Optical Coherence Tomography (OCT)	High- resolution cross- sectional images; greater depth	Increased image artifacts and overall observation of architectural changes instead of induvial cell observation
Diagnostic Ultrasound	Cost Effective; information regarding skin inflammation	Difficulty Interpreting Images
Tape Stripping mRNA	Rapid and Easy to perform; practical for any skin surface	Small Data Set; Increased gene expression needed
Cellular Electrical Resistance (Bioimpedance)	Quick	Impedance Properties can vary within body location, age, gender, and season

Table 3.1 Advantages and Disadvantages of Various Melanoma Detection Methods

The table (above) highlights some of the most prominent melanoma detection methods used since the 1980s, with advantages and disadvantages to each method.

e. Diagnostic Stages of Melanoma[59]

Like other forms of cancer, the American Cancer Society has developed a 'staging' system in which clinicians may determine spread and severity of melanoma diagnoses. Utilizing this system, those clinicians are then able to determine the best method of treatment and correlate a patient's status with certain survival statistics.

The most common staging system used for melanoma has been adapted from the American Joint Committee on Cancer (AJCC) and is called the TNM system. When utilizing the TNM system, physicians assess the tumor thickness, spread to nearby lymph nodes, and metastasis to distant sites. Ultimately, the greater the numbers of the TNM system correlate with more advanced forms of cancer. Included in Table 3.2 below are a brief description the most recent qualifications of the TNM system, effective as of 2018.

AJCC Stage	Melanoma Stage Description
0	Cancer is confined to epidermis, the outermost skin layer. It has not spread to nearby lymph nodes (N0) or to distant parts of the body (M0). This stage is also known as *melanoma in situ*.
I	The tumor is no more than 2mm thick and might or might not be ulcerated (T1 or T2a). The cancer has not spread to nearby lymph nodes (N0) or to distant parts of the body (M0).
II	The tumor is more than 1 mm thick (Tb2 or T3) and may be thicker than 4 mm (T4). It might or might not be ulcerate. The cancer has not spread to nearby lymph nodes (N0) or to the distant parts of the body (M0).
IIIA-IIID	The tumor has progressed since Stage II and may exceed 4 mm with some ulceration (T4b). The cancer has spread to 4 or more nearby lymph nodes or small areas of nearby skin (N3a or N3b). It has not spread to distant parts of the body (M0).
IV	The tumor can be any thickness and might or might not be ulcerated (any T). The cancer might or might not have spread to nearby lymph nodes (any N). It has spread to distant lymph nodes or to organs such as the lungs, liver or brain (M1).

Table 3.2 Stages of Melanoma Disease Progression

Each of the four Melanoma Disease Stages are described in terms of the progression of the cancerous growth and tumor size and thickness.

i. Need for Improved Detection

While the detection methods described previously have drastically improved since the simple ABCDE identification, they strongly rely on physician identification and a 'good eye.'[60] Visual identification has worked for many years and may prove to be useful in some scenarios, but it relies too heavily on the absence of human error. It is apparent that a new multidisciplinary approach to melanoma detection analysis is needed.

Blood analysis is an overlooked method for cancer identification. When cancer is evolving to achieve metastasis, it must develop the ability to survive in the blood stream and successfully establish growth in a secondary location of the body.[61] An excessive number of cells must enter the blood, as CMCs, to collectively develop the heterogenicity to survive and spread. The cancerous cells at this point provide an unearthed potential for detection and identification. Researchers have found a positive correlation between the number of CMCs and prognosis of a patient. This correlation poses great potential as a quantitative tool to measure diagnosis and progress of therapy.[62]

IV. Photoacoustics

Photoacoustics describes the sound emitted by an object illuminated by a modulated light source. The frequency or wavelength of the sound can be used to gather information regarding a sample's structure,[63] shape,[64] or qualities.[65] Photoacoustics has gained popularity based upon its ease of application, limited costs and the limited damage that it has on samples after analysis. In this section, the various application of photoacoustics will be outlined, particularly Photoacoustic Flow Cytometry and it's use as a cancer diagnostic.[66]

a. Photoacoustic Flow Cytometry

Photoacoustic Flow Cytometry (PAFC) is a groundbreaking technique that can be used for detection of a variety of pigmented cells. An improvement to the Flow Cytometry system, PAFC incorporates photoacoustics, laser- induced ultrasound, to identify pigmented cells for specific detection.[67] This method provides a promising future direction of detection methods for a variety of diseases.

The PAFC system incorporates a variety of lab technologies and techniques, particularly microfluidics and photoacoustics, to provide an accurate detection system, providing rapid results. The system uses a laser to induce photoacoustic response to specific cells. The laser is focused on the sample, which incorporates two-phase flow with oil and is flown through a 10μm quartz tube. When a targeted cell is contacted by the laser beam, an acoustic response is produced and detected by a transducer located at the bottom of the flow chamber. The transducer converts the acoustic response into a computer signal in which the LabVIEW program adds a tally to the total number of targeted cells detected. With the use of high and low pass filters, the response can be tracked and tallied if it meets the criteria of a misshapen melanoma cell. This system can process a 10mL blood sample in a matter of 15 minutes or less, providing real time results.

i. Advantages of Photoacoustic Flow Cytometry to Existing Methods

This newly developed system provides several advantages over existing methods, supporting the argument that this method should be further implemented into healthcare. The highly accurate system reduces the number of false positives, providing physicians with a better idea of a patient's disease state. Additionally, the PAFC system reduces the necessary sample size for testing by an order of magnitude from 10mL to 1mL, lessening the patient sample and ultimately the invasiveness of the procedure. Lastly, an advantage of the described system is the preservation and sorting of the sample for further analysis. Most methods destroy the sample upon analysis, however; with this PAFC system the sample is suspended in mineral oil and can be further analyzed for cell markers or added to the cDNA library which has been done in previous studies.[68] With all of these advantages, it is clear that the PAFC system that has been developed at Duquesne University is a clear option for future diagnosis and disease study.

ii. Photoacoustic Flow Cytometry Applied to Cancer Diagnostics

The potential applications of PAFC could be far reaching which could have drastic change to healthcare diagnosis. As previously mentioned, cancer detection is a very attractive field of research for PAFC, particularly in early detection. Through early diagnosis, cancer treatments may be altered and improved to be more effective and bear less of a financial and physical burden on the patient, cutting costs of treatment in half or quarters.[69]

Cancer detection using PAFC could be developed to be rather standard, with little to no adjustment between cancer type. The detection is based off the acoustic response incited by the laser beam as it contacts the cancerous cell, which differs from that of a healthy cell. Once a baseline response is created for the varying cancer cells, different filters may be used to detect different cancers potentially present in the blood. Melanoma cells, for which the system has been developed, are pigmented and readily incite responses from the green 532nm laser, whereas other cancer cells may incite a better

response from other wavelengths. Using imaging tools, we can observe and assess cancer cell pigmentation and presume which laser wavelength and color could produce the best response. Then by conducting simple sensitivity tests, the best system could be determined for each cancer type. These few hurdles pose little challenge in the development of a universal PAFC cancer detection system.

iii. Other Studies using Photoacoustic Flow Cytometry

With photoacoustics showing such promise in the diagnostic area, many scientists have decided to apply it to their studies, hoping for favorable results. The potential applications of photoacoustics reach far and wide from chemistry to biomedical and everything in between. The field of photoacoustics has really helped scientists learn more about chemical properties, medical imaging, and diagnostic properties of some diseases. Paired with advances in ultrasonic imaging, photoacoustics has begun to allow us a more advanced understanding and interpretation of molecular function in a real time fashion, drastically improving clinical applications.[70]

One study being conducted by researchers at the University of Arkansas analyzes the application of *Circulating Tumor Cell Detection and Capture by Photoacoustic Flow Cytometry in Vivo and ex Vivo*. This application was developed to reduce the invasiveness and labor-intensive process required to prepare samples for other diagnostic tests including RT- PCR, Flow Cytometry, Microfluidic Chips, and Optical Sensors. Instead, this *in vivo* process involves the combination of fluorescence, photothermal and photoacoustic applications. Their method includes the use of one or more laser beams with differing wavelengths to excite melanoma cells in the blood stream. Upon the excitation of melanoma cells, the cells expand, producing both heat and sound. Photoacoustic and photothermal sensors are used analogously to observe the heat and acoustic waves produced by the expansion of the targeted melanoma cell. This study has shown promise in assessing large volumes of blood and enhancing the sensitivity of CTC detection, particularly those of uncommon cancer stem cells. On the other hand, the probes have

shown to be toxic with strong scattering of light, making it hard to assess deep, large vessels.[71] Ultimately, this study has shown some promise in PAFC application *in vivo,* but still needs reproduceable data before further claims can be made about *in vivo* application.

iv. Photoacoustic Flow Cytometry Applied to Chemistry

Additional research being done with Photoacoustic Flow Cytometry furthers the notion that it may be the technology of the future in terms of diagnosis and characterization of biologic and chemical samples. Another study being conducted at the University of Arkansas brings to light chemical applications; the study is titled *Photoacoustic Flow Cytometry for Nanomaterial Research.* This study integrates fluorescent tagging with the PAFC system to be used for nanoparticle (NP) detection and quantification. Using fluorescent tags, cells that do not naturally contain pigments can be detected with the photoacoustic fluorescence flow cytometry (PAFFC) system. This study achieved that detection method through the application of gold nanorods (GNRs) to cultured breast cancer cells. Once the GNRs and cells were attached in a one-to-one ratio, to insure a controlled acoustic response, the cells were imaged and flown through a similar but unique flow chamber. Imaging confirmed GNR attachment to the cell membrane of each of the breast cancer cells. Additionally, GNR attachment incited a photoacoustic response, which dramatically increased after incubation of the breast cells and GNRs. A key finding of the study, the GNRs did not provoke a cell damage or death, which cannot be said about a similar study done with graphene.[72] Ultimately, the study further proved the idea of PAFC and PAFFC systems to be ideal for the future of diagnostics, furthering the potential applications.

V. Experimental Design

A previously designed photoacoustic flow cytometer was implemented in this study to analyze the number of detectable circulating melanoma cells (CMCs) in melanoma patients' blood. The clinical study entailed the collection of blood, approximately 16mL, from previously identified melanoma patients at risk of metastasis. After collection, the samples are immediately sent to Duquesne University for analysis. Through a blood separation procedure, the white blood cell layer is isolated from the remainder of the sample. Any CMCs present in the patient's blood would remain in the white blood cell layer after separation due to similar density. The white blood cells and corresponding CMCs are resuspended in 1mL of Natural Density Solution (NDS) with the addition of approximately 1.6µL DNase.

a. Flow Chamber Design

Our flow chamber design is centered around the idea that a single melanoma cell is the focal point, emitting photoacoustic waves when contacted with the laser beam, with the overall system shown in Figure 5.1 below. Flow Chambers were 3D printed with 100% infill and white PLA filament. The flow

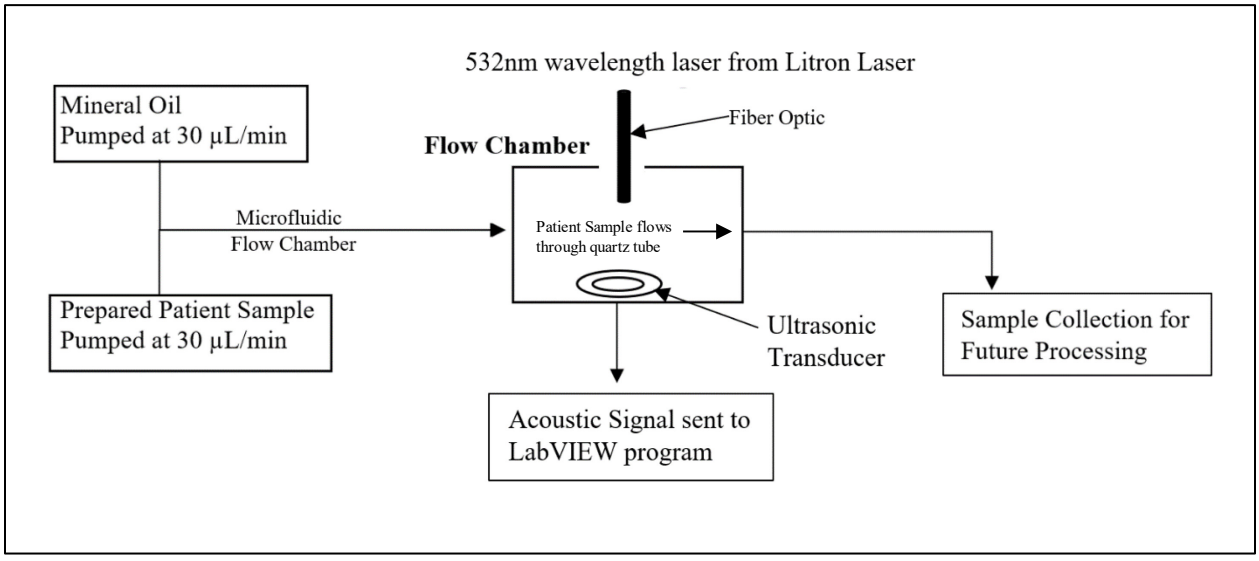

Figure 5.1 Photoacoustic Flow System Diagram

The diagram above shows the process of the photoacoustic flow system used in this study. The sample and mineral oil create a two-phase flow that passes through the microfluidic flow chamber and enters the quartz tube where the laser interacts with the sample and creates a photoacoustic signal detected by a ultrasonic transducer and is sent to a local computer with a LabVIEW program for further analysis.

chambers were made white to avoid the absorption of any light emitted by the laser and interfering with cell detections. The flow chamber, depicted in Figure 5.2,[74] features two holes on opposing sides for the insertion of the quartz tube, and one hole on an adjacent wall for the insertion of the laser optical fiber to emit laser light. The center of the flow chamber remains open at the top and bottom for insertion of the acoustic sensor and to allow a visual of the flow chamber and ensure correct operation of the flow system.[73]

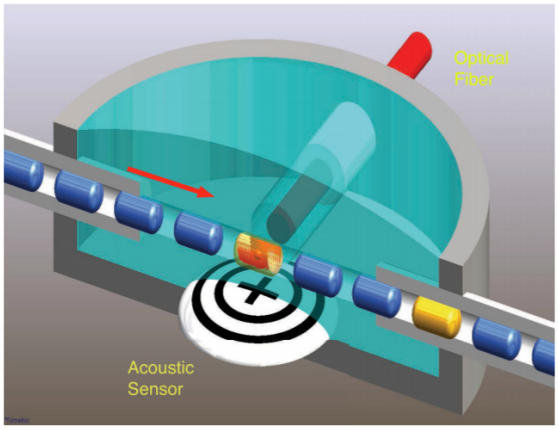

Figure 5.2 Simulated Flow Chamber Design
Pictured is an animated design of the Flow Chamber. The sample flows through the quartz tube, above the acoustic sensor, past the optical fiber.

i. Quartz Tube

Quartz tubes were used in the flow chamber design because they enable maximum energy transfer of acoustic waves to the acoustic sensor located at the bottom of the flow chamber. The quartz tube has a length of 80mm, radius of 0.1mm, and wall thickness of 10µm (Charles Supper, MA), shown with the accompanying packaging in Figure 5.3.[76] The quartz tube is inserted through each of the opposing holes in the flow chamber, passing through the middle of the chamber. The quartz tube connects to a plastic flow tubes on each end to insure a smooth, continuous flow in and out of the chamber. The plastic and quartz tubes are fastened to the flow chamber using glue.[75]

Figure 5.3 Quartz Tube and Accompanying Packaging

Pictured above are the quartz tubes integrated into the Photoacoustic Flow Chambers used in this study. Acquired from Charles Supper, these tubes have a length of 80mm, radius of 0.1mm, and wall thickness of 10µm, suitable for laser penetration.

ii. Laser

We used a Q-Switched Nd:YAG laser operating at 532 nm with a pulse duration of 5 nanoseconds to induce acoustic waves in melanoma cells under flow. Laser light was transmitted using a 1000 µm, multimode optical fiber with a numerical aperture of 0.39 (Thorlabs, Newton, NJ). The laser had a measured energy of 25 mJ. The optical fiber was bent as a means to mix modes, resulting in a near-Gaussian beam. Fluence was calculated to be 0.014 mJ/cm^2.[77] The end of the optical fiber was placed 2-3 mm away from the quartz tube.

iii. Photoacoustic Gel

The entire inner cylinder of the flow chamber, including the quartz tube and optical fiber, was immersed in acoustic matching gel (Sonotech LithoClear; NEXT Medical Products Company, North Branch, NJ). This gel is identical with those used in sonographies and ultrasounds, assisting in transmitting the photoacoustic waves to the acoustic sensor with no interference.

iv. Acoustic Sensor

The acoustic waves were sensed by a focus ultrasound transducer with a focal length of 12.5 mm and a center frequency of 5 MHz (Olympus Scientific, Waltham, MA). These signals were amplified with a gain of 50 using a Tegam 4040B system and was sent to a desktop computer with custom LabVIEW code (National Instruments, Austin, TX) for instrumentation control and data acquisition. By setting a simple threshold, photoacoustic waves could be classified. Irradiation of phosphate buffered solution (PBS) helped determine the noise floor since there is no optical absorption at 532nm. The simple threshold was set at three times the noise floor to designate a signal originating from a CMC. Design and descriptions of a previously used photoacoustic flow cytometer for detection and capture of CMCs is comprehensively outlined in O'Brien et al.[78]

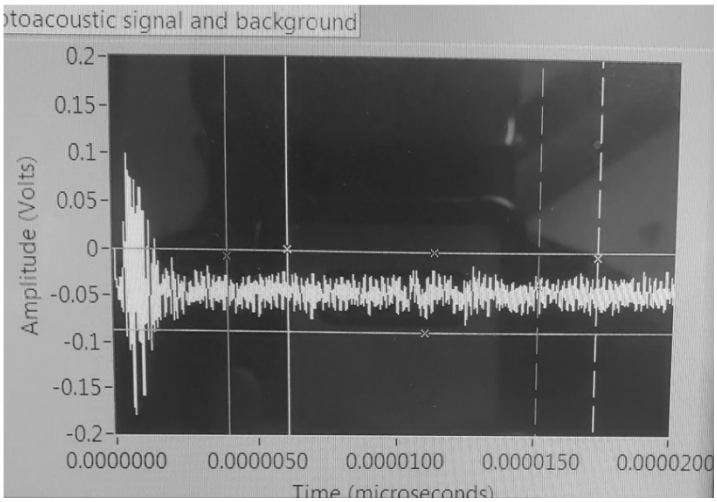

Figure 5.4 LabVIEW laser signal incited by White Blood Cells
Shown here (left) is the normal LabVIEW photoacoustic signal incited by prepared patient sample containing ample white blood cells. The signal does not clear either the upper or lower boundaries.

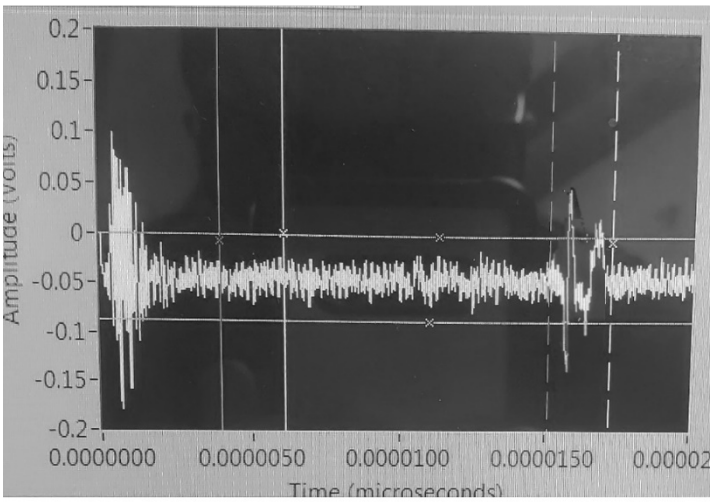

Figure 5.5 LabVIEW laser signal incited by melanoma cell detection
Shown here (left) is LabVIEW photoacoustic signal incited by melanoma cell detection. Upon interaction between the laser signal and a melanoma cell, a spike in the signal is created. The LabVIEW program counts the number of 'melanoma spikes' that clear at least the upper or lower boundary.

The green, 532nm wavelength, laser used in our detection system incites a detectable response from melanoma cells, due to their increased melanin content, while white blood cells and the remainder of the prepared patient sample does not incite a detectable photoacoustic response. The normal photoacoustic signal, shown in Figure 5.4, can be compared to the melanoma- incited signal 'spikes' shown in Figure 5.5. The LabVIEW program is designed to count any signal that passes either of the upper or lower boundaries set.

b. Sample Preparation

Thirty- seven patients from Thomas Jefferson Medical center were included in a blind study blood sample collection. These patients had previously been identified to have developed Stage IV melanoma and were determined to be at risk of further developing metastasis. Patient blood samples were collected pre-treatment. Immediately after the whole blood was drawn from these selected patients, it was shipped overnight to Duquesne University for further processing.

Upon arrival at Duquesne, the blood was immediately processed to preserve the sample and any CMCs. A centrifuge technique, common for blood separation and shown in Figure 5.6,[81] was used to isolate the white blood cells from the remainder of the blood sample, with the addition of Histopaque to facilitate the health of the white blood cells. As mentioned before, all CMCs will be found in the white blood cell layer of the sample, due to the similarity in density. Once the white blood cell layer has been isolated from the rest of the sample, red blood cell lysis buffer is added to kill any remaining red blood cells. After sitting, the sample is centrifuged again to pellet the white blood cells and CMCs. Lastly, the pellet is resuspended in Non- Density Solution (NDS) and DNase is added to prevent the synthesis of any more DNA.[79]

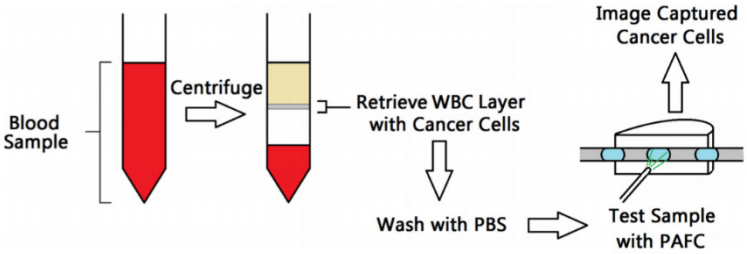

Figure 5.6 Patient Blood Sample Preparation Process

Samples are processed with centrifugation to separate the blood into various layers. The layer of our interest, the buffy coat, contains leukocytes and any possible CMCs present, is extracted, and run through the photoacoustic flow cytometer (PAFC).

c. Two Phase Flow Generation

One important characteristic of our microfluidic system includes Two Phase Flow Generation. Created using a T-Junction, two separate phases are combined into one flow path while the phases remain distinct. Air and water were used in previous studies[80] with limited success. While the air and water combination could easily be extracted without contaminating the sample, the two liquids built up pressure, leading to the purging of the system. Use of surfactants reduced the interfacial tension between phases.

The study discussed in this paper uses a phosphate buffered solution (PBS) and mineral oil. The differing phases create an immiscible interface. The sample, containing the blood cell suspension in a non-density solution, like that of PBS, refuses to mix with the less dense mineral oil. When in simultaneous flow at a rate of 30μL/min, the two liquids form slugs, isolating single cells for easy detection.

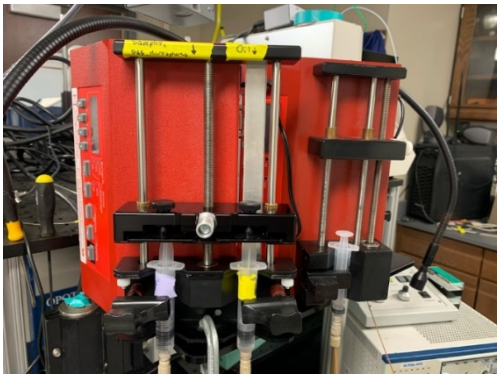

Figure 5.7 Two Phase Flow Generator

Shown here is the two-phase flow generator. Syringes are filled with the desired equal amounts of Oil and PBS, Microsphere Solution, or Sample

The system, pictured Figure 5.7 (*above*) and Figure 5.8 (*below)*, is based upon previous studies conducted by O'Brien et al[81] and Viator et al[82] and differs from most other microfluidic systems because of its size and shape. The system utilizes cylindrical silicone tubing (Masterflex, platinum cured silicone tubing, LIS14, Cole Parmer, Vernon Hills, Illinois), which behave slightly different than square edge systems. Other classic microfluidic systems are typically composed of channel dimensions lower than 100 µm and flow rates less than 1 µL/s, our system used a tubing inner diameter of 1.6 mm and the flow rates used ranged from 100 to 200 µL/min. The slugs that formed were only 3 to 4 µL and the Capillary and Reynolds numbers remained within microfluidic conditions. In addition, high flow rates were used to achiever a greater volume throughput, posting results from an 16mL blood sample in 15 minutes or less.

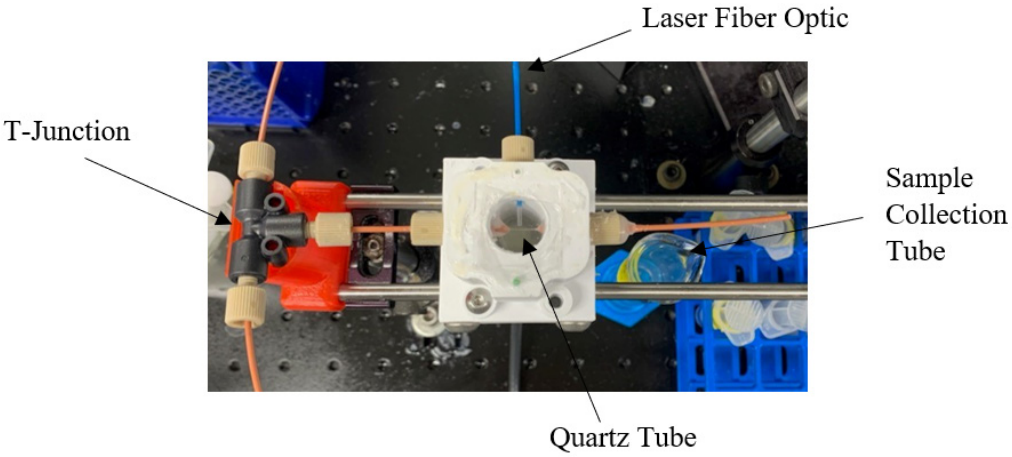

Figure 5.8 Top View of the 3D Printed Flow Chamber

Pictured is a Bird's Eye View of the 3D printed Flow Chamber. The Sample and Mineral Oil flow in two phase flow, generated by the T- Junction. The flow enters the quartz tube and passes by the optic fiber that generates laser pulses. Cancerous cells generate an acoustic response that is sensed by the ultrasound transducer located beneath the quartz tube. The sample is then collected for further testing.

d. Photoacoustic Flow System

Before any samples can be run through the flow chamber, 2 negative tests and a positive test must be run to prep the chamber. Negative tests consist of equal volumes of PBS and Mineral Oil flowed through the tube concurrently in two phase flow. While running the negative tests, 1 mL must pass

through the quartz tube without any detections to effectively prove no air bubbles or microspheres are causing false detections.

A positive test must be run in between each of the negative tests to prove that the laser is working and there will be detections. Black polystyrene microspheres can be diluted and immersed in microsphere solution. Running the immersed microspheres synchronously with mineral oil will create numerous detections and signify that the flow chamber is functioning correctly.

After sitting in a dark hood, the sample is loaded into a sterile syringe following 1 mL of mineral oil. The less- dense mineral oil sits on top of the sample and creates added pressure to ensure the complete sample is pushed through the syringe. Once the flow chamber has been cleared with the second negative test, the sample is run at 30μL/min simultaneously with mineral oil in another syringe. The sample and immiscible mineral oil create two phase flow, isolating the cells in air bubbles and insuring single cell detection.

e. Sensitivity Testing

The PAFC system previously described, used in the testing of patient blood samples, has effectively identified CMCs in both patient blood samples and cultured cells. In an effort to improve the system and more accurately detect the number of cancer cells in a blood sample, it is important to determine the sensitivity of the system. Optimizing the sensitivity will allow for a more accurate measure of the number of CMCs, ultimately giving physicians and patients a more accurate disease- state diagnosis.

Upon confluent growth of three cultured melanoma cell lines (HS936, HTB-71, and SK-MEL-3) the cells were counted in a small area of the culture flask with a microscope and cell counter. That number was multiplied times the whole area of the culture flask, assuming equal distribution of cells. TrypLE is added to each of the three flasks designated to each cell line. Cells, along with the accompanying media and TrypLE is transferred to 15mL Falcon Tubes and spun in the centrifuge for 10

minutes at 1600 rpm. The resulting pellet is resuspended in 3mL of NDS, and vortexed to break apart the pellet. The remainder of 'equally distributed' cells are equally split amongst 3 Eppendorf Tubes (approximately 1 mL each). A dilution series of each of the three Eppendorf Tubes is performed until the concentration is calculated to be about 10 cells per milliliter.

The expected number of cells was determined in each individual cell line from a count performed with a microscope and hemocytometer technique. Due to the arduous process of counting every cell in the flask, we counted a small area, assumed equal distribution, and multiplied the small count by the total area of the flask. Since the dilution series was performed by diluting samples by a factor of ten, the expected cell numbers followed the same calculation, rounding when necessary.

An additional heterogenous mixture of is made by combining 1mL of cell media of each of the strains of melanoma cells (HS936, HTB-71, SK-MEL-3). Using the same preparation procedure, the cells are prepared to be run through the flow cytometer. The expected number of heterogenous cells was determined by summing the counts of each of the individual cell lines and following the same calculations for each dilution.

Each dilution series is run through the Photoacoustic Flow Chamber beginning with the most concentrated sample and proceeding until the least concentrated sample. The Flow Chamber is cleared between each of the samples with 0.5 milliliters of PBS and Mineral Oil, respectively. The number of detections per dilution and sample are recorded (Tables 6.6- 6.8). The results of the heterogenous mixture are recorded (Table 6.9).

VI. Results

a. Patient Blood Samples

i. Stage IV

Patient #	CMCs	Patient #	CMCs	Patient #	CMCs	Patient #	CMCs
1	10	11	3	21	7	31	0
2	10	12	4	22	64	32	9
3	10	13	3	23	12	33	0
4	3	14	9	24	5	34	10
5	5	15	4	25	24	35	4
6	0	16	5	26	36	36	0
7	0	17	3	27	8	37	18
8	2	18	3	28	16		
9	3	19	3	29	2		
10	4	20	9	30	0		

Table 6.1 Stage IV Patient Blood Samples and Accompanying CMC Count

This Stage IV patient information is from 'Photoacoustic detection of Circulating Melanoma Cells in late stage patients' by Viator et al.[83] CMCs are typically present in most patients' blood by Stage IV with 31 of the 37 patients (84%) having 2 or more CMCs present in a blood sample. The gray boxes represent patients with CMC detections.

ii. Late Stage Patients Undergoing Chemotherapy

Patient #	Sample #	Days Since Initial Diagnosis	# of CMC's Detected	Chemo?
1	1	0	61	
	2	44	2	
	3	123	3	Yes
	4	192	22	Yes
	5	268	9	
	6	503	16	
2	1	0	16	
	2	63	11	
	3	74	0	Yes
3	1	0	2	
	2	42	3	
	3	100	63	Yes
	4	176	4	
4	1	0	11	
	2	32	14	Yes
	3	43	35	
	4	82	129	Yes
5	1	0	4	Yes
	2	101	2	Yes
	3	265	0	
6	1	0	0	
	2	39	2	Yes
	3	317	0	Yes
7	1	0	2	
	2	152	1	Yes
8	1	0	26	
	2	16	283	Yes
	3	942	3	
9	1	0	39	
	2	358	100	
	3	366	4	
	4	1687	12	
10	1		2	
	2		0	Yes
	3		0	Yes
	4		20	Yes
	5		50	Yes

Table 6.2 CMC Detections in Patients Undergoing Chemotherapy (cont.)

Blood samples from stage IV melanoma patients undergoing chemotherapy was collected at points throughout their treatment regimen Treatment efficacy could be assessed based off the number of CMCs detected.

Patient #	Sample #	Days Since Initial Diagnosis	# of CMC's Detected	Chemo?
11	1		34	
	2		240	
12	1		0	
	2		0	Yes
	3		56	
	4		0	
	5		0	
13	1			Yes
	2		33	Yes
	3		100	
14	1		161	
	2		17	
	3		3	
	4		6	Yes
	5		6	Yes
	6		11	
15	1		34	
	2		2	Yes
	3		423	Yes
	4		1	
16	1		0	
	2		0	Yes
	3		6	Yes
	4		11	
17	1		4	
	2		13	
	3		10	Yes
	4		0	Yes
18	1		2	
	2		4	Yes
	3		0	
19	1		5	
	2		5	
	3		3	
	4		0	
20	1	127	5	Yes
	2	697	3	
	3	730	21	
	4	749	54	

Table 6.3 CMC Detections in Patients Undergoing Chemotherapy (cont.)

Blood samples from stage IV melanoma patients undergoing chemotherapy was collected at points throughout their treatment regimen Treatment efficacy could be assessed based off the number of CMCs detected.

Patient #	Sample #	Days Since Initial Diagnosis	# of CMC's Detected	Chemo?
21	1	2498	0	Yes
	2	2530	0	Yes
	3	2889	0	Yes
22	1	56	0	
	2	88	1	Yes
	3	221	0	Yes
	4	340	11	Yes
	5	434	19	Yes
	6	951	4	
23	1	711	1	
	2	786	0	
	3	2263	7	Yes
24	1	2221	0	Yes
	2	2682	1	
	3	2974	0	
25	1	841	0	
	2	930		Yes
	3	1019	0	Yes
	4	1089	1	Yes
	5	1186	0	Yes
26	1	2010	0	
	2	2480	1	Yes
27	1	2050	1	
	2	2290	0	Yes
	3	2338	0	Yes
28	1	11	0	
	2	83	0	Yes
	3	161	34	Yes
	4	266	5	Yes
29	1	4700	58	
	2	5340	0	Yes
	3	5359	0	Yes
	4	5451		Yes
30	1	35	173	
	2	1335	68	Yes
31	1	353	63	
	2	442	301	Yes
	3	629	122	Yes
	4	723	11	

Table 6.4 CMC Detections in Patients Undergoing Chemotherapy (cont.)

Blood samples from stage IV melanoma patients undergoing chemotherapy was collected at points throughout their treatment regimen Treatment efficacy could be assessed based off the number of CMCs detected.

Patient #	Sample #	Days Since Initial Diagnosis	# of CMC's Detected	Chemo?
32	1	160	243	Yes
	2	547	43	
33	1	89	1	Yes
	2	137	1	Yes
	3	268	0	Yes
	4	358	0	Yes
34	1	2	3	
	2	1670	0	
	3	2240	1	
35	1	1671	1	
	2	1785	0	Yes
	3	1876	0	Yes
36	1	54	1	
	2	93	74	Yes
	3	405	0	
37	1	2710		
	2	5049	0	
	3	5795	1	
	4	5890	0	
38	1	653	1	
	2	2371	0	
39	1	34	1	
	2	102	0	
40	1	1342	2	
	2	1377	1	Yes
	3	1521	0	Yes
	4	1583	0	Yes
41	1	192	83	Yes
	2	624	26	Yes
	3	644	7	Yes
42	1	72	0	
	2	167	2	Yes
	3	251	1	Yes
43	1	2332	1	Yes
	2	2476	1	
	3	2586	3	

Table 6.5 CMC Detections in Patients undergoing Chemotherapy (cont.)

Blood samples from stage IV melanoma patients undergoing chemotherapy was collected at points throughout their treatment regimen Treatment efficacy could be assessed based off the number of CMCs detected.

b. Sensitivity Testing

i. HS936

Dilution Number	Initial	1	2	3	4
Expected # of Cells	2150	215	22	3	0
Cells Detected	2116	546	197	33	4

Table 6.6 Sensitivity Testing Results with Cultured HS936 Melanoma Cells

HS936 melanoma cells were cultured in lab. Upon confluent growth the cells were prepared similarly to patient samples. A dilution series was performed until single cells were able to be isolated in the sample.

ii. HTB-71

Dilution Number	Initial	1	2	3	4
Expected # of Cells	2800	280	28	3	0
Cells Detected	2743	520	77	13	7

Table 6.7 Sensitivity Testing Results with Cultured HTB-71 Melanoma Cells

HTB-71 melanoma cells were cultured in lab. Upon confluent growth the cells were prepared similarly to patient samples. A dilution series was performed until single cells were able to be isolated in the sample.

iii. SK-MEL-3

Dilution Number	Initial	1	2	3	4
Expected # of Cells	2000	200	20	2	0
Cells Detected	1979	397	86	2	0

Table 6.8 Sensitivity Testing Results with Cultured SK- MEL- 3 Melanoma Cells

SK-MEL-3 melanoma cells were cultured. Upon confluent growth the cells were prepared similarly to patient samples. A dilution series was performed until single cells were able to be isolated in the sample.

iv. Heterogeneous Mix

Dilution Number	Initial	1	2	3	4
Expected # of Cells	6000	600	60	6	1
Cells Detected	5776	736	136	45	8

Table 6.9 Sensitivity Testing Results with Heterogenous Mix of HS936, HTB-71, and SK-MEL-3 Cultured Cells

Equal volumes of the three cultured cell lines are were combined and prepared similarly to patient samples. A dilution series was performed until single cells were able to be isolated in the sample.

VII. Discussion

a. Patient Blood Samples

As expected, the presence and the extent of melanoma cells directly corresponds with the patient's stage of melanoma. Previously discussed, melanoma cells do not typically enter the blood stream until late stage II or early stage III.[84] At this point, the CMCs have overcome the shear stress of travelling through the blood stream to populate other areas of the body.

In the 37 stage IV melanoma patients studied, presented in Table 6.1, an overwhelming 31 patients (84%) had detectable levels of CMCs, with a distribution of the number of detected cancer cells per patient shown in Figure 7.1. The patients with detections averaged 10 CMCs, a significant increase compared to the average of 2 cells per mL in stage II patients. The higher detection lines up with the previously discussed idea that melanoma cells are commonly found in the blood by stage IV of melanoma.

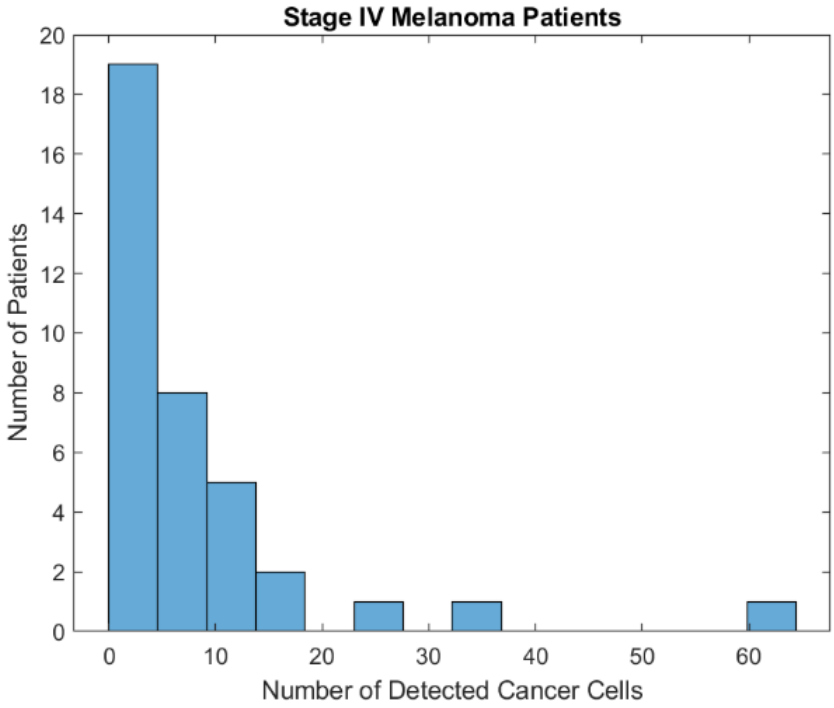

Figure 7.1 Stage IV Melanoma Patient CMC Detection Results

16mL patient samples were collected after their clinical diagnosis of stage IV melanoma. CMC detections were recorded and the distribution of # of detections per patient is shown here.

A reliable diagnostic tool can be vital in helping physicians and oncologists gain an insight as to how well chemotherapy treatments are working. By using the photoacoustic flow cytometry described in this paper, we can gather an idea of the number of CMCs in a patient throughout their therapy. Using this information physicians can adjust the course of treatments, accordingly, increasing or decreasing the time in between treatments. The results of a study closely monitoring the CMC count in melanoma patients following chemotherapy treatments can be found in Tables 6.2- 6.5. After closely analyzing the results of 43 patients, three main conclusions were made regarding the chemotherapy treatments being used.

The first common trend found amongst the melanoma patients undergoing chemotherapy treatment was the effectiveness of primary and secondary rounds of chemotherapy. Of the observed patients, 42% showed a significant reduction in CMCs after the first round of treatment, which can be seen in Figure 7.2. After the second round of chemotherapy, an additional 19% of total patients studied showed a decrease of CMCs. Of the cumulative 61% of patients that saw improvement, after one or both rounds of treatment, there was an average of 72% reduction of their CMC count, with all but one patient seeing their CMC count cut in half, shown in Figure 7.3. This tells us that the chemotherapy treatment is effective for more than half of melanoma patients with CMCs after 2 rounds of treatment.

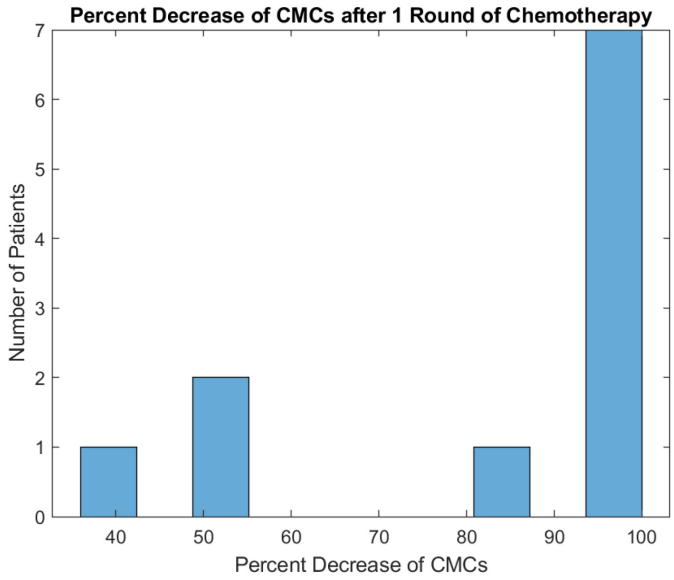

Figure 7.2 Percent Decrease of CMCs After 1 Round of Chemotherapy

This distribution shows the percent decrease of CMC detections after Round 1 of Chemotherapy in patients that responded well to treatment.

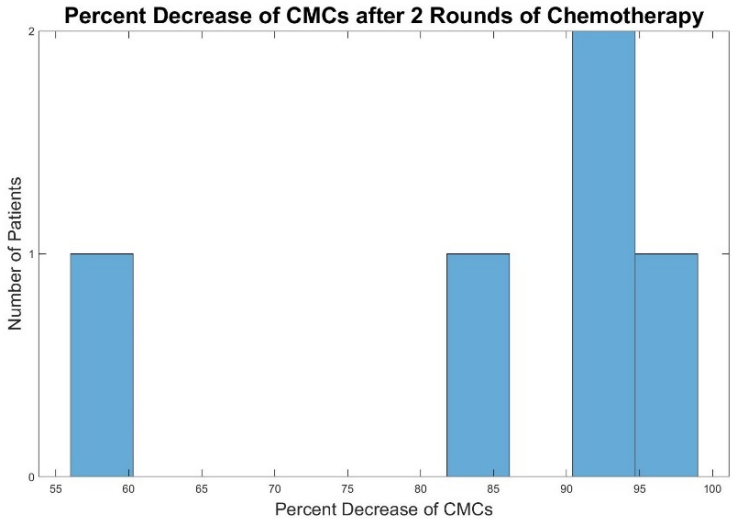

Figure 7.3 Percent Decrease of CMCs After 2 Rounds of Chemotherapy

This distribution shows the cumulative percent decrease of CMC detections after Rounds 1&2 of Chemotherapy in patients that responded well to treatment.

For those patients that did not see an improvement after 2 rounds of chemo, 23% underwent successful reduction of CMCs through sustained chemotherapy. Those patient's CMC count can be seen in Figure 7.4. Sustained chemotherapy refers to three or more rounds of treatment. These repeated rounds of treatment were helpful in not only reducing the CMC count in patients, but also keeping a reduced level of CMCs. Additionally, in some cases patients with 0 initial CMCs continued to have no CMCs throughout the treatments. This is helpful in telling us that sustained chemotherapy may be a helpful option in preventing cancer patients from ever developing CMCs and ultimately preventing metastatic growth.

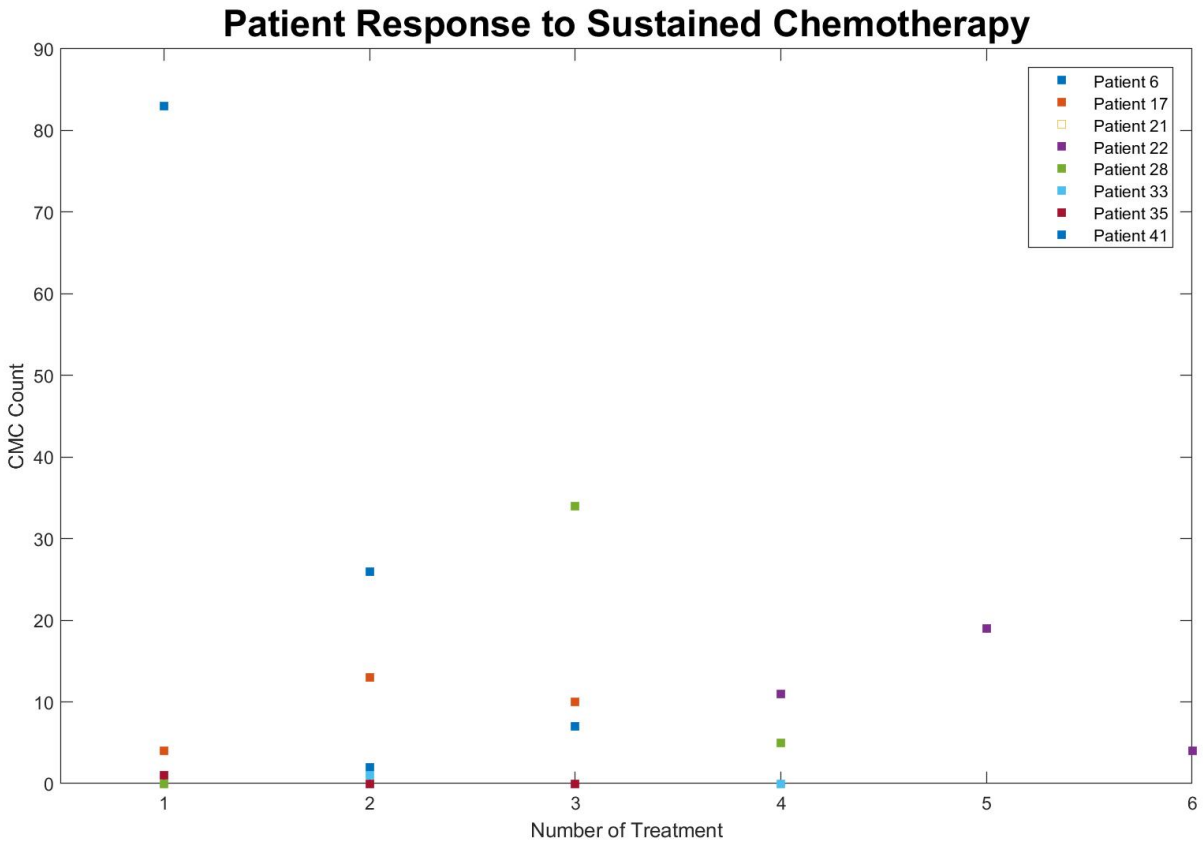

Figure 7.4 Patient Response to Sustained Chemotherapy

Shown in the plot above is the results of the sustained chemotherapy treatment in 8 patients, after processing the patients' 16mL blood sample and running it through the detection system. While patients 6, 17, 22, and 28 all increased after the initial treatment, the sustained treatment led to the eventual decrease in CMCs.

The last conclusion to be made from this initial study is that there will be some patients that do not respond at all to chemotherapy. Unfortunately, 16% of patients in this study did not show any signs of improvement after undergoing chemotherapy, some patients even regressing. While this is discouraging, the 84% improvement rate is a vast improvement compared to the 2-16% 5-year survival rate of metastatic patients in previous studies.

b. Sensitivity Testing

The sensitivity tests produced expected results with each of the three cell lines producing reasonable detection numbers, and the heterogenous mix of all three reflecting just a little less than three times the detections of each individual cell line. The four transcendental curves can be found in Figure 7.5 below.

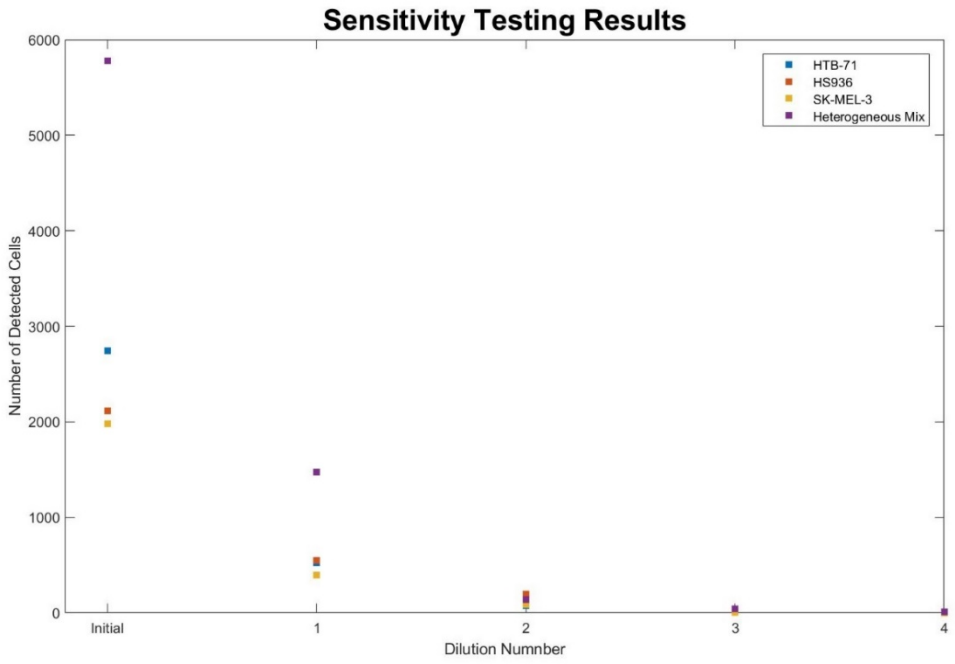

Figure 7.5 Sensitivity Testing Results

Shown here are the # of Detections per each dilution of each of the cultured cell samples and a heterogenous mix of each of the cell lines.

These results reflect the accuracy of our Photoacoustic Flow Cytometry System, closely aligning with the expected number of cells. By being able to isolate and detect single digit quantities of cells, we can confidently say that the system is accurate and does an effective job in detecting individual cells. Moving forward, we can positively conduct diagnostic tests and identify small numbers of CMCs in a patient blood sample, ideally catching any sign of metastatic spread before late stage diagnosis.

VIII. Conclusion

Cancer, a rapidly evolving disease that science still has much to learn about, takes an insurmountable toll on healthcare worldwide. The far-reaching and multi-faceted causes of cancer makes the disease difficult to detect and even more difficult to monitor. Melanoma, a particularly devastating form of cancer, proves to be increasingly difficult due to rapid spread and difficult detection. Current methods require ample time and resources, an increasingly effective method is needed. Additionally, with every patient producing a unique response to treatment, an improved patient-tailored diagnosis and treatment protocol is needed.

Photoacoustic Flow Cytometry (PAFC) poses a viable answer to real time melanoma detection. Described in detail throughout this paper, PAFC utilizes photoacoustics to detect abnormal cells based off their response to laser excitation. Specific detection of melanoma cells combined with quantitative results offers helpful analysis for physicians and oncologists to determine the disease state of a patient. Additionally, the collection of patient blood samples, preparation, and use of the PAFC system can be completed in less than an hour and only requires a blood draw from the patient. This improved detection system provides a quick and effective method to melanoma detection, reducing the burden on both patient and physician.

Further developing the notion of Photoacoustic Flow Cytometry as a diagnostic, we were able to correlate the detection of CMCs and metastasis in late-stage melanoma patients. This knowledge, paired with increased detections exhibited by Stage IV patients, is helpful in disease- state determination by physicians. Increased CMCs could be the sign of poor patient response to chemotherapy and a need for more rounds of treatment. This information will become increasingly valuable in improving treatment methods in the future.

Moving forward the Photoacoustic Flow Cytometry system poses great revolution to medical diagnostics. Cutting down on both time and money while providing accurate results, the system provides improvements on all fronts. With potential to be adapted to various forms of cancer or even bacterial or viral infections, the applications of the system are limitless.

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