Creation of a 3D construct to aid cell migration and promote cell capture

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CREATION OF A 3D CONSTRUCT TO AID CELL MIGRATION AND PROMOTE CELL CAPTURE

By

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Joseph M. Sanders

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ABSTRACT

Most cancer-related deaths are attributed to metastasis. The tumor microenvironment is a complex environment which is not fully understood. The Nano Intravital Device (NANIVID) is a versatile, biocompatible device that allows for the manipulation of the tumor microenvironment in vitro and in vivo, providing a platform to study various aspects of tumor progression. The purpose of this study is to modify the NANIVID to resemble the tumor microenvironment in order to allow for a seamless transition from the in vivo environment into the engineered environment within the NANIVID. This engineered microenvironment will promote cell migration and cell capture. It has been shown that metastatic cells respond to a chemotactic gradient created by Epidermal Growth Factor (EGF)[1]. Thus far, attempts at actual capture of these metastatic cells through the use of the NANIVID have been sporadic and not reproducible. By utilizing electrostatically spun microfibers, which mimic the size of fibers within the natural tumor microenvironment, as well as incorporating collagen, which is the most prevalent extracellular matrix (ECM) proteins in this environment, the cells will be able to follow the gradient released by the NANIVID in a seamless manner. Cells will be collected to validate this platform in an in vitro system and demonstrate the potential use of this platform in vivo.
Chapter 1. Introduction

1.1 Overview of Cancer and Metastasis

Cells are influenced by their surrounding microenvironment. Both mechanical and chemical cues affect the formation and subsequent migration of tumorigenic cells [2]. Cancer is characterized by uncontrolled cell growth and proliferation. Cancer cells exhibit many properties that make them unique compared to their non-cancerous counterparts. Tumor cells are able to proliferate indefinitely due to their active telomerase activity [3]. Normal cell growth processes become inhibited when two non-cancerous cells encounter one another. Cancerous cells do not exhibit contact inhibition, which allows them to continue to grow uncontrollably, eventually aggregating and forming tumors [4]. As the tumor continues to grow, its need for sustenance will outpace what is available and the tumor will be forced either to recruit blood vessels and metastasize, or to arrest growth [5]. The migration of tumorigenic cells to new sites around the body, or metastasis, is responsible for 90% of cancer-related deaths [6]. The study of metastatic cancer cells is hoped to shed light on the changes that are occurring on the cellular level, which may lead to a better understanding of the metastatic process.

Metastasis consists of a series of sequential steps, all of which must be successfully completed in order for a tumor to form in a new location. These include intravasation from a primary tumor into the blood stream, circulation through the body, survival of cells in the circulation, arrest in a new organ, extravasation into the surrounding tissue, and finally, growth and vascularization of the secondary tumors. Metastasis is a widely studied subject, though knowledge is still very limited [7]. It is believed that through the study of metastatic cells a better understanding of metastasis will be achieved, leading to therapeutic methods that can target
susceptible mechanisms within the metastatic process. To accomplish this end, it is important to study metastatic cells directly and in their natural environment.

1.2 The Tumor Microenvironment

The tumor microenvironment is comprised of many growth factors, proteins, cell types, and other chemical and physical entities which influence the natural environment of the tumor [8]. In order to study the metastatic cells within the tumor, it is crucial to recreate this microenvironment and mimic the natural environment experienced by metastatic cells *in vivo*. The extracellular matrix (ECM) is a collection of extracellular molecules secreted by cells that provides structural and biochemical support to the surrounding cells. This ECM is laid down by cells, allowing them a 3-dimensional structure to adhere to and move within. It consists of many components including collagen, laminin and fibronectin, which can influence tumor cell behavior. It is also a rich source of growth factors that can be released upon proteolytic degradation, which has been shown to be able to increase metastasis [8]. Growth factors are cytokines that affect the growth of target cells and regulate cell movement [9]. Figure 1 shows the multitude of cell types and structures that can be found within the tumor microenvironment. Although the depiction is not exhaustive, it is useful to demonstrate that many pieces come together to form the intricate environment.
The ECM is not merely a passive scaffold for tissues, but also via the cell-ECM interactions influence a number of biological activities such as cell proliferation, differentiation, biosynthetic ability, polarity and locomotion through many different receptors [10]. The ECM has many functions, including acting as a physical scaffold, facilitating interactions between different cell types and providing survival and differentiation signals. In breast tissue, a large amount of the ECM is comprised of collagen. In order to mimic the physical scaffold, Poly (lactic-co-glycolic acid) (PLGA) microfibers were created and embedded in a collagen matrix. The collagen was shown to adsorb to the microfibers as well.
1.3 The Extracellular Matrix and Collagen

Collagen is the major insoluble fibrous protein in the ECM. This family of proteins is composed of at least 28 genetically distinct species [10]. In fact, it is the single most abundant protein in the animal kingdom [11]. Collagen is distinct from other proteins in that it is made up from three polypeptide chains (α chains). These three α chains are able to wind around each other and form a triple helix due to the amino acid glycine being a major constituent of the α chains. This small amino acids allows for the tight winding of the collagen fibrils [11]. Among the isotypes of collagen, type I is the principal structural and functional protein and is composed of two α1 chains and one α2 chain. The specific complement of α subunits present within the fibril defines the material properties of the polymer [12]. Type I collagen is found throughout the body and is synthesized in response to injury [11]. It can also be deposited by fibroblasts in response to specific chemical cascades [13].

Since the beginning of the 80s, researchers have utilized collagen as a culture substrate for breast cancer cells, in order to evaluate the effects that the collagen matrix/film has on attached cells. Collagen has been found to be important in regulating proliferation as well as in enabling the mammary epithelial cell to respond to trophic factors [14]. It is clear that the matrix provides different signals to cells and that cell-ECM interactions are important for cells to behave as though they would in their natural environment.

The interaction between cancer cells and the ECM is especially apparent in breast carcinomas. Breast carcinoma progression characteristically changes the ECM by manipulation of its organization and composition and by depositing greater amounts of ECM than are normally found in mammary glands. These changes result in stiffer matrices, leading to the universally
known “lumps”. The ECM remodeling and increased stiffness have been a top area of research due to their clear correlation with tumor progression [15]. The Nano Intravital Device (NANIVID) has been shown to be able to mimic a signaling cascade that results in ECM deposition. In these studies, the NANIVID released various chemicals to cause cells to manipulate their ECM in a desired manner. The collagen matrix was manipulated indirectly through the influence of the NANIVID on cells occupying the ECM [13].

For the purposes of these experiments, it was necessary to recreate the collagen microfibers as closely as possible. Collagen microfibers in PyMT mice were studied utilizing multiphoton second harmonic signals as visualized in Figure 2 [16]. As can be seen in Figure 3, the most frequently observed diameters were 1.5-2.0 µm. 2.0 µm was thus chosen as the desired diameter of microfibers to be incorporated into the NANIVID.

![Figure 2. Z stack of collagen fibers taken *in vivo* using multiphoton second harmonic generation. Courtesy of Edison Leung at Albert Einstein College of Medicine.](image)
Figure 3. Diameters of *in vivo* collagen microfibers were determined from analysis of Z-stacks obtained using multiphoton second harmonic generation. The most frequently observed diameter, 2.0 µm, was chosen for the microfibers. Courtesy of Edison Leung at Albert Einstein College of Medicine.

1.4 MTLn-3 Cells and EGF Response

Differences are observed when studying migration of cancer cells *in vitro* versus *in vivo*. Thus, it is critical to develop an *in vivo* model to accurately study the biology of metastasis. One key difference between *in vivo* studies and *in vitro* studies is the high degree of persistent linear motion of carcinoma cells in tumors compared with the random walking of equivalent cells in culture. It is believed that three major factors allow cells to undergo persistent linear movement over long distances and at high speeds *in vivo*. These are: the ECM, amoeboid movement of the
cells and linearity of the ECM microfibers. These factors contribute to the efficiency of invasion by allowing cells to traverse long distances within the tumor, and can enhance intravasation, as ECM microfibers often converge on blood vessels in mammary tumors. It has been found that MTLn3 cells have no motility in areas where there are no vessels or collagen microfibers. This could be due to a lack of EGF signaling in these areas [17]. Thus, to induce cell capture, EGF will be released from the NANIVID and the path towards the gradient will be lined with collagen to attempt to mimic what the most motile cells experience \textit{in vivo}. The microfibers will be directed linearly to force the cell’s path to be linear.

\subsection*{1.5 Scope of this Thesis}

The goal of this thesis is to expand the NANIVID from a platform of controlled manipulations to the tumor microenvironment to a platform for cell capture. This will enhance the use of the NANIVID in better understanding the relationship between the tumor microenvironment and the progression of metastatic disease. This work will validate this platform \textit{in vitro} to expand upon the current features of the NANIVID by adding a microenvironment within the device that is similar to the natural tumor microenvironment experienced by the metastatic cells of interest. A breast cancer model was chosen for validation. Components specific to the ECM of breast tissue will be mimicked to allow for the capture of these metastatic cells.

The enhancement of the NANIVID will be achieved through three objectives. In objective one, electrostatically spun microfibers will be deposited within the NANIVID in an aligned manner. These microfibers have been tailored to mimic the natural collagen microfibers found in breast tissue, along which the metastatic cells travel as described in Chapter 3 [17]. In
objective two, collagen will be introduced into the NANIVID to create a gel for the cells to migrate through. In objective three, the device will be inserted into an in vitro system to collect cells through the chemotactic migration towards an EGF gradient. The in vitro validation of the NANIVID as a way to collect cells using the mechanisms outlined in these objectives previously described will allow for the transition to in vivo collection studies utilizing the NANIVID. Use of this platform in vivo to study the biology of metastatic pathways will help to continue developing knowledge of metastasis and may eventually lead to therapeutic strategies that target vulnerable processes within metastasis.

Chapter 2. The Nano IntraVital Device

2.1 Device, Concept, Designs and Applications

The NANIVID is a versatile, biocompatible device that allows for the manipulation of the tumor microenvironment in vitro and in vivo, providing a platform to study various aspects of tumor progression. It was designed to manipulate the tumor microenvironment through the controlled release of various molecules from the device. Existing chemotaxis assays and microfluidic devices do not have the flexibility for use in both 2-dimensional as well as 3-dimensional systems, nor for use in both in vitro and in vivo systems. Several designs of the NANIVID have been created and validated for in vitro and in vivo models [18].

The NANIVID combines microphotolithography techniques, polymer chemistry and microfluidics to function as a chemotaxis device that has the flexibility to be used beyond the capabilities of other chemotaxis assays. This system provides a unique method of isolating the response of subpopulations of metastatic tumor cells to changes within the tumor microenvironment. Previous work has focused on manipulating the tumor microenvironment by
introducing various chemicals to elicit responses such as migration, hypoxia, and collagen deposition[19][13][20]. That work has chemically induced physical changes in the tumor microenvironment to allow the study of these effects in a localized manner and at the single cell level [21]. Quantification of these changes and elucidation of the underlying molecular mechanisms represents a significant and ongoing challenge in this emerging field but also has the potential to uncover novel insights into how cells invade and metastasize and to identify new therapeutic targets. The manipulation of the interior environment of the NANIVID will allow even further work and diversify the experiments which can be done.

2.2 Device Fabrication and Assembly

The device design is achieved through use of L-edit software to map the device features and alignment marks. The resulting map is turned into a transparency mask to be used during the fabrication process. Fabrication of NANIVIDs begins by depositing a 50 Å chromium adhesive layer followed by a layer of gold which is 1000 Å thick onto a glass substrate via electron beam evaporation. The substrates are spin-coated with positive photoresist, and the desired reservoir shape is exposed using a transparency mask designed with L-edit software. The resist is developed and the features are etched through the metal layers and into the glass using gold and chromium etchants and hydrofluoric acid respectively. Once the etching is completed, the remaining photoresist and metal layers are removed and the glass is cleaned in piranha etch (three parts sulfuric acid to one part hydrogen peroxide). The general process flow is outlined in Figure 4 below. Dicing is performed on the etched glass substrates to produce the desired NANIVID shape and size.

The device consists of two Pyrex (glass) substrates processed using the previously mentioned photolithography processing techniques and bonded together using a thin film of
PDMS (Polydimethylsiloxane) with oxygen plasma treatment. The synthetic hydrogel is made up of 20% polyethylene glycol diacrylate (PEGDA, Glysocan), 7% methoxy polyethylene glycol monoacrylate (PEGMA, Sartomer) and Irgacure 2959 (BASF) as a photoinitiator. This synthetic hydrogel is cross-linked through exposure to UV light for 2 minutes. Inside the device, the source chamber is loaded with the aqueous, solvent-based hydrogel blend (PEGDA/PEGMA) containing growth factors, which is UV cross-linked with a short curing time. As stated above, EGF is the growth factor of interest in our studies.

All of the materials used in this system, including PDMS, glass and hydrogel are commonly used materials in the biomedical field [23]. Biocompatibility is a primary concern in design changes to the NANIVID, as the end result is a device that can be utilized in vivo. Other important considerations include transparency, for the purposes of imaging and retrievability so the device can be collected after either delivering a payload or collecting desired cells or materials of interest in the specific situation. Previous designs can be viewed in Figure 5 below.
Figure 5. Multiple designs of the NANIVID which are created utilizing the process flow above. The device design has been tuned to the specific needs of the experimental model to be used and can continue to be modified as the needs of the device continue to evolve.

The *in vitro* design is being modified for these experiments as it has the largest interior volume and thus will allow for the most modification. The *in vitro* NANIVID is ideal for optimizing molecule concentrations, release kinetics and in this case, incorporation of the ECM within the device. This design allows for faster throughput than would be possible in an *in vivo* only system.
Chapter 3. Create a Modified NANIVID Containing Electrospun Microfibers

3.1 Electrostatic Spinning Background

3-dimensional constructs which mimic the natural environment of cells of interest are required to bioengineer cells to form tissues. Electrostatic spinning has been an indispensable tool in recent years for creating scaffolds for tissue engineering. This technology has led to the recreation of the native ECM of cells due to its ability to generate microfibers similar to the fibrous structures of native ECMs of varying tissues throughout the body. A wide variety of materials can be used in the electrospinning process, allowing for careful selection of biologically compatible materials. This process is relatively straightforward and allows for great manipulation and control over various features of the microfibers, including diameter, modulus, density, alignment and porosity. The large surface area of electrospun microfibers, as well as their porous structure, makes them favorable for cell adhesion, proliferation, migration, and differentiation [23].

The electrospinning process is used widely in tissue engineering and requires four main components. These components are a metallic needle attached to a syringe, a syringe pump, a high-voltage power supply, and a grounded collector. Figure 6 depicts the generic set up for the electrospinning process with the grounded collector plate set up to collect aligned microfibers. The syringe pump allows for a constant flow of solution out of the metallic needle. A charged liquid jet is formed as the electric field created by the power supply overcomes the surface tension of the droplet. The jet is then elongated and whipped by electrostatic repulsion on its way to the grounded collectors. During its travel from the needle tip to the grounded collectors, the solvent evaporates and the jet solidifies to form a nonwoven microfiber [23].
Figure 6. The Basic components of the electrostatic spinning technique are pictured here. The grounded collector plate has a gap which allows for microfibers to align along the length of the gap.

Methods for manipulating these electrospun microfibers have been a large area of research and have resulted in multiple different methods of collections to manipulate alignment, density, layering and other features to make them more amenable to the desires of tissue engineering. Some parameters of the electrospinning process have been studied widely including solution viscosity, conductivity, applied voltage, spinneret tip-to-collector distance and humidity, which all impact the final microfiber structure [24]. Methods for aligning microfibers are of great interest in this project, as they allow microfibers to be oriented from the outlet of the device to the hydrogel within the back of the device. This alignment will allow the cell a directed path towards the source of the growth factor. The trends in changes to other parameters of the electrospinning process which are thoroughly discussed in a variety of literature allowed microfibers to be tuned to mimic the characteristics of the natural microfibers found in the ECM of breast cancer cells.
The simple method of introducing a gap into the conventional collector is able to achieve uniaxially aligned microfibers over large areas. As assisted by electrostatic interactions, the microfibers are stretched across the gap to form a parallel array. Figure 7 demonstrates fibers which have been spun onto a planar grounded collector while Figure 8 depicts fibers that have been aligned between the parallel collector plates. This demonstrates that a wide range of diameters can be achieved through manipulation of the specific electrospinning parameters. These microfibers can be transferred onto other substrates with relative ease, as they are suspended between the parallel collector plates. In this case, the fact that ungrounded substrates within the electric field have essentially no influence on the quality of the resultant microfibers was utilized. The NANIVID was placed on a glass slide within the electric field and the size and alignment of the microfibers resulted as expected.

![Image](image.png)

Figure 7. SEM image of unaligned PLGA fibers collected on a planar surface. Scale bar =300nm
Figure 8. SEM image of fibers that have been aligned utilizing the parallel plate method. Scale bar=1µm

3.2 Tuning the Microfibers

An important aspect of the microfibers to be incorporated into the NANIVID is their biocompatibility. A key design factor of the NANIVID is its ability to be used both in vitro as well as in vivo. PLGA was chosen for its recognized biodegradability and biocompatible properties [25]. PLGA is one of the most commonly used biodegradable polymers in tissue engineering [26]. Its wide use in the electrospinning process makes it a great choice due to the vast literature on manipulation of its various features including mechanical properties and biological compatibility. Materials chosen for in vivo work should not elicit short nor long term immune response. In the case of PLGA, neither the polymer itself, nor its degradation products are toxic [27].
Surface biocompatibility is associated with the surface chemistry of the material. It was important to choose a polymer that would not only be compatible with our choice of cells, but also with our choice of ECM protein. In this case the surfaces of the polymer were coated in collagen. The chemical characteristics of a material surface will mediate the adsorption of biologic molecules that regulate cell activities, such as adhesion and migration [28]. It has been demonstrated that ECM proteins physically adsorb to microfibers. Figure 9 below shows that collagen I immunostaining demonstrated coating of the microfibers after an incubation of 12 hours. The microfiber aspect ratio was unchanged after the protein coating, as seen in Figure 10.

Figure 9. Collagen I immunostaining demonstrates adsorption of collagen I by the microfibers. The height was slightly increased by adsorption of the collagen. There was little change to the width and aspect ratio. Courtesy of Ved Sharma at Albert Einstein College of Medicine.
Figure 10. The height and width measurements demonstrate little change in either measurement. There is also little change in the aspect ratio.

The mechanical properties of an engineered scaffold are dominated by intrinsic factors, such as the chemistry of the material and by extrinsic factors, such as the dimension or architectural arrangement of the building blocks. Mechanical force measurements were taken via atomic force microscopy (AFM) on the microfibers. All analysis was conducted using a Bioscope Catalyst II AFM (Veeco digital instruments). Mechanical measurements were made using sharpened silicon nitride tips (Bruker). These tips have a resonant frequency of 150 kHz, a spring constant of 0.7 N/m, a nominal length of 70 µm and a nominal width of 10 µm. Peak force quantitative nanomechanical property mapping (PF-QNM) was used to measure the modulus while imaging the sample. This mode allows us to generate high resolution data in much less
time than other AFM modes, such as force volume mapping. This mode oscillates the cantilever at sub-resonant frequency, monitoring interaction to ensure that peak force remains constant[29]. For PF-QNM measurements, the deflection sensitivity was measured on glass and the spring constant of the cantilever was calibrated using the thermal tune method. The Poisson ratio of the cells was taken to be 0.5, as is typical for soft biological material [30]. The Derjaguin-Muller-Toporov (DMT) model was used in conjunction with the PF-QNM method as follows:

\[ F_{\text{tip}} - F_{\text{adh}} = \frac{4}{3} E \sqrt{R d^3} \]  
\[ E = \left( \frac{1 - \nu_s^2}{E_s} + \frac{1 - \nu_{\text{tip}}^2}{E_{\text{tip}}} \right) \]  

where \( R \) is the tip radius, \( d \) is the deformation of the sample, \( F_{\text{tip}} - F_{\text{adh}} \) is the force on the cantilever relative to the adhesion force, \( E \) is the modulus, and \( \nu \) is the Poisson’s ratio.

The AFM was used to determine relative Young’s Modulus. The force was measured as a function of height. Since the fiber is 2 µm in diameter, the maximum force experienced is when the tip scans across the area that is 2 µm tall. Here the measured Young’s Modulus was found to be higher than the stiffness of the ECM normally experienced by cells [15]. The increase in Young’s modulus has not had a negative impact in the cell’s ability to move on the fibers and thus is not considered to be as important of a factor as others for this experiment.

Scanning electron microscopy (SEM) was performed using a Leo1550 scanning electron microscope. This technique was used to measure microfibers to ensure that they were of the desired diameter. As can be seen in Figure 11, the desired microfiber diameter of 2.0 µm was achieved.
Figure 11. SEM image of the general dimension of the microfibers created by the electrospinning process.

Cell migration has been studied in previous work and Figure 12 demonstrates that cells are able to move on fibers. Cells are able to migrate persistently and linearly due to the confined dimensions of the fiber [16].
Figure 12. The cells on the top left fiber have a noticeable movement between panels A and B. Image adapted from Williams et al. 2014; Used with permission [22].

3.3 Electrospinning Microfibers in the NANIVID

Since the most frequently observed diameter of collagen microfibers in vivo was 1.5-2.0 μm, this relative size was desired for incorporation within the NANIVID. To achieve aligned
microfibers, the standard electrospinning set up was utilized with grounded parallel plate collectors. A 21% PLGA solution was found to produce microfibers with the desired diameter of 2.0 µm, when electrospun under the constraints as described below.

### 3.4 Materials and Methods

One-dimensional aligned microfibers were created from a 21% (w/w) PLGA (LACTEL) solution dissolved in hexafluoroisopropanol (HFIP, Krackeler) with 1% NaCl (Sigma Aldrich) and stirred overnight. The solution was mixed with Sulphorhodamine B (SRB) dye to allow fluorescent imaging of the microfibers and cells simultaneously. The solution was fed into a 3 mL syringe, the flow rate was controlled by a syringe pump, and the high-voltage was applied using a high voltage power supply. The aligned microfibers were attracted to two grounded parallel collector plates consisting of cleaved silicon. A glass coverslip was placed in between the parallel collector plates and the loaded NANIVID reservoir was placed on the glass coverslip. The outlet was perpendicular to the parallel plates in order to align the microfibers from the front of the device to the back of the device. The collector plates were 15cm from the needle tip. The flow rate was set to 16 µL/min and the voltage was set to 12 kV. The NANIVID collected fibers for 30 seconds each time.

### 3.5 Results and Discussion

A uniform layer of electrospun microfibers was deposited on the NANIVID, as can be seen in Figure 13 below. The microfibers are aligned as expected and flow from the outlet towards the back of the device as anticipated. The process is easily repeatable when the conditions described are adhered to. Microfibers were collected on up to six devices at a time, with no noticeable difference in amount of deposited microfibers or alignment.
The ability to electrospin these fibers in repeatable way allow the opportunity to expand on this platform and add in the collagen matrix. These fibers will allow directed migration with great speed from the outlet towards the EGF loaded hydrogel. This platform is expected to enhance cell capture.

Figure 13. Optical image of aligned microfibers running from the outlet of the device towards the loaded hydrogel in the back of the device.
Figure 14. Optical image of the fibers from Figure 13 zoomed in. These were imaged while taking advantage of the fluorescent SRB dye.

Chapter 4. Create a NANIVID Containing a Collagen Matrix to Mimic the \textit{in vivo} Microenvironment

4.1 Rationale

It has been demonstrated that coating microfibers with collagen improves the ability of cells to adhere and move along microfibers [16]. By incorporating collagen gel, a close mimic of the ECM, the natural tumor microenvironment is imitated, allowing cells to move within the NANIVID. Providing cells a protein constituent—which is regularly encountered in their natural environment—to interact with is expected to allow the cell to continue to move in a directed manner along the released gradient.

4.2 Materials and Methods

Collagen type I from rat tail was purchased from Corning. This specific type of collagen was chosen due to its prevalence in the ECM of breast tumors. The collagen was diluted to 1.3
mg/mL to simulate the tumor microenvironment specific to the breast tissue environment. This concentration has been used in previous work to create a matrix [19]. The solution was put into the NANIVID reservoir on the layer of microfibers and allowed to gel for 4 hours. A subsequent layer of microfibers was then electrospun on the NANIVID in the same manner as previously described. The process can be visualized in Figure 15 below. Figure 16 gives a visualization of the side view of the NANIVID. From this perspective, the outlet can be viewed which gives an idea of how much depth the ECM can fill in the device. Once the artificial ECM was in place, the edges of the reservoirs were mechanically scraped to expose the maximum amount of glass possible. The covers were subsequently plasma treated and the reservoirs and the covers were sealed.

Figure 15. The electrospinning procedure is shown through the addition of the second fiber layer.
Figure 16. SEM image of the outlet of the NANIVID. This shows the relative depth of the reservoir which is being filled with the PLGA fibers and collagen gel. The glass slide begins with a nominal thickness of 0.15 mm.

4.3 Results and Discussion

The collagen and microfibers were incorporated into the NANIVID to be used in the experiments. Figure 17 below demonstrates many microfibers aligned within the NANIVID as well as the collagen gel placed within the NANIVID. This combination is expected to correctly mimic the in vivo ECM closely enough that cells will be able to move into the NANIVID and towards the hydrogel which is the source of the growth factor.

The addition of the matrix did not have a negative impact on the ability to seal the device. This allows for future work in manipulation of the interior of the NANIVID as well as a positive outlook on the incorporation of the ECM and its longevity in the design of the NANIVID.
Figure 17. Optical image of the NANIVID device after microfibers have been electrostatically spun and a collagen gel has been deposited on top, followed by a second layer of microfibers. The device has not been sealed at this point.

Chapter 5. Cell Collection by the Hydrogel and ECM Loaded NANIVID

5.1 Rationale

Thus far, attempts at cell collection have had limited and sporadic success when utilizing the NANIVID. It is proposed that, through the incorporation of ECM components into the NANIVID, the ECM will be recreated within the NANIVID. Cells will not be able to
differentiate between the natural environment normally experienced *in vivo* in this *in vitro* system, thus promoting cell migration towards the EGF gradient and eventually cell capture. The components of the natural ECM, which have been incorporated as previously mentioned, will allow the device to be utilized for cell capture *in vitro*.

### 5.2 Methods and Materials

#### 5.2.1 Cell Culture

MTLn3-GFP cells were maintained in alpha-MEM (Gibco) supplemented with 5% fetal bovine serum (Gibco), 0.2% sodium bicarbonate (Sigma Aldrich) and 1% penicillin/streptomycin (Gibco) in an incubator at 37°C with 5 % CO₂. The starvation media for MTLn3-GFP cells contained Leibovitz L-15 media (Sigma Aldrich) with 0.8% bovine serum albumen (Sigma Aldrich). The imaging media was Leibovitz L-15 media (Sigma Aldrich) with 5% bovine serum albumen (Sigma Aldrich).

#### 5.2.2 Confocal Imaging

Devices were removed and cells were imaged using a Leica SP5 Scanning Laser Confocal microscope. The GFP in the MTLn3-GFP was used as a fluorescent tag to locate cancer cells. The SRB dye was used as a fluorescent tag to locate microfibers. A laser with a wavelength of 488 nm was used to excite the fluorescent tag of these cells and a laser with a wavelength of 540 nm was used to excite the fluorescent tag of these microfibers. Images were taken at 40x magnification utilizing oil immersion. Image J software was used to process the images.
5.2.3 Cell Capture Assay

100,000 MTLn3-GFP cells were plated into a collagen gel after it had cured. The NANIVIDs were then degassed and placed into the dish overnight. Control devices were made without the microfibers or collagen, but still containing EGF. The following day the devices were removed and placed into a dish with imaging media to be imaged on the confocal microscope.

5.3 Results and Discussion

The devices were imaged and as shown in Figure 18, cells were found near the outlet of the NANIVID within the device. No cells were found within control devices. Due to the optics of Leica Scanning Laser Confocal microscope, at the magnification used, the Z-axis resolution is limited and is comprised mostly of the glass slide and bottom layer of glass of the NANIVID reservoir. The optics allow limited vision within the NANIVID and do not allow the cover to be imaged. It appears at this point however, that due to the location of cells and fibers on the same Z-plane, that the cells are within the artificial ECM that has been created.

These results closely resemble data from Xue et al[31]. Figure 19 shows MTLn3-GFP cells which have been imaged in vivo on collagen fibers. The collagen fibers are viewed in purple utilizing second harmonic generation [31]. This similarity demonstrates the NANIVID’s ability to capture cells and to be manipulated further. This achievement represents yet another generation of NANIVIDs. As the NANIVID continues to evolve in its design and its use, an ever growing array of studies can be performed to investigate the tumor microenvironment, metastasis, and breast cancer at large.
Figure 18. Confocal image of the NANIVID outlet with MTLn3-GFP cells in green and the SRB dye-labeled microfibers in red. Scale bar=50 µm

Figure 19. An in vivo view of MTLn3-GFP cells on collagen fibers, imaged utilizing second harmonic generation signals. White and red arrows point to GFP labeled cells and orange arrows point to collagen fibers. Scale bar is 20 µm. Image from Xue et al. Used with permission [30]
Chapter 6. Concluding Remarks

6.1 Thesis Summary

It has been demonstrated that aligned microfibers of the size found in the ECM in the breast cancer tumor microenvironment can be electrostatically spun onto the NANIVID reservoir and sealed. These microfibers and the incorporation of a collagen matrix allow the linear path to the EGF gradient normally released in the tumor microenvironment as well as access to one of the major proteins encountered in the natural ECM of the metastatic cells chosen. This recreation of the in vivo tumor microenvironment within the NANIVID has allowed cells to migrate into the device.

Fibers were only located within the device for the duration of the experiment and imaging. It is thus proposed that all cells that were imaged in the Z-plane of these fibers are within the device. It appears that cells are indeed able to utilize the integrated ECM within the device in order to chemotax towards the EGF gradient introduced into the in vitro dish.

Future work will include gene profiling of these captured cells to search for potential markers that impact metastasis. These can be targeted and exploited in further experiments to eventually develop therapeutic techniques.

It is expected that cells within the collagen matrix will exhibit growth inhibition. Currently, it is believed that the reason for the growth arrest is that cells need time to develop the remainder of their ECM when presented with limited amounts of proteins that they naturally experience in vivo [32]. This may be an interesting factor to consider, as, once cells are captured, their growth can be arrested, allowing for longer periods of capture while maintaining the genetic markers of the first generation of metastatic cells collected.
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Most importantly I would like to thank you for reading this. I hope that whoever you are, you escape this place. I hope that the world turns, and that things get better. But what I hope most of all is that you understand what I mean when I tell you that, even though I do not know you, and even though I may never meet you, laugh with you, cry with you, or kiss you, I love you. With all my heart, I love you.
References


