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Probing the tumor microenvironment: collection and induction

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ABSTRACT

The Nano Intravital Device, or NANIVID, is under development as an optically transparent, implantable tool to study the tumor microenvironment. Two etched glass substrates are sealed using a thin polymer membrane to create a reservoir with a single outlet. This reservoir is loaded with a hydrogel blend that contains growth factors or other chemicals to be delivered to the tumor microenvironment. When the device is implanted in the tumor, the hydrogel will swell and release these entrapped molecules, forming a gradient. Validation of the device has been performed in vitro using epidermal growth factor (EGF) and Mena INV, a highly invasive, rat mammary adenocarcinoma cell line. In both 2-D and 3-D environments, cells migrated toward the gradient of EGF released from the device. The chorioallantoic membrane (CAM) of White Leghorn chicken eggs is being utilized to grow xenograft tumors that will be used for ex vivo cell collection. Device optimization is being performed for in vivo use as a tool to collect the invasive cell population. Preliminary cell collection experiments in vivo were performed using a mouse model of breast cancer. As a second application, the device is being explored as a delivery vehicle for chemicals that induce controlled changes in the tumor microenvironment. H2O2 was loaded in the device and generated intracellular reactive oxygen species (ROS) in cells near the device outlet. In the future, other induction targets will be explored, including hypoglycemia and the manipulation of extracellular matrix stiffness.

Keywords: Microfabrication, BioMEMS, Metastasis, Chemotaxis, Cell Collection

INTRODUCTION

Metastasis is a complex, multiple step process in which cancer cells depart from the primary tumor and proliferate in a distant organ. Chemotaxis, or the directed movement of cells in response to an extracellular gradient, has been implicated in many steps of metastasis. An EGF-CSF1 macrophage-tumor cell paracrine loop has been identified in breast cancer and linked to intravasation and invasion. The migration of tumor cells toward EGF can be used as a means to isolate the invasive cell population. The current technique used to collect these cells in vivo utilizes a needle based assay. In this assay, a needle containing EGF and matrigel is inserted into the tumor and invasive cells will migrate toward the growth factor gradient and be collected. After collection, gene expression profiles of the invasive cells were compared to those of the primary tumor cells to determine the invasive signature. The NANIVID is being developed as an improved tool for in vivo cell collection. The device is made of biocompatible materials and offers transparency to enable imaging of the cell migration and collection process. A hydrogel is loaded in the device which will swell upon hydration and release the entrapped EGF molecules over many hours. Released EGF molecules will form an exponentially decaying concentration gradient originating from the device outlet in the tumor microenvironment. The small device size and sustained release of the chemoattractant will allow for use in vivo in long term migration studies.

In this work, the CAM was chosen as a means to grow xenograft tumors for device insertion due to its low cost and ease of use. The CAM is a membrane found attached to the inside of the eggshell of a developing chick embryo. This membrane is involved with regulating nutrient and gas exchange and is therefore highly vascular. Studies of cancer cell metastasis have been performed using the CAM system. A human metastatic mammary adenocarcinoma cell line was grown on the CAM and the NANIVID was used in experiments for cell collection in the resulting tumor. Additionally,
in vivo cell collection was performed using a mouse model of breast cancer metastasis. The cells responded to the EGF gradient produced by the device and were collected, consistent with the results of the needle based assay.

An additional application is being explored in which the induction-NANIVID is loaded with chemicals that induce controlled changes in the tumor microenvironment. Induction targets have been chosen that have been related to tumor progression and invasion. Hypoxia was the first induction target, studied in previous work with the delivery of CoCl2 from the device\textsuperscript{6}. Currently, the induction-NANIVID is being used to generate intracellular ROS by releasing H\textsubscript{2}O\textsubscript{2}. Increases in intracellular levels of ROS have been implicated in promoting tumor progression and metastasis. Increased ROS within cancer cells have been shown to promote oxidative inactivation of tumor suppressing phosphatases like PTEN\textsuperscript{7} as well as promote the activation of transcription factors leading to enhanced proliferation and invasiveness\textsuperscript{8,9}. Additionally, elevated levels of ROS in neighboring cells can also influence the metastatic potential of proximal cancer cells through the production of matrix degrading enzymes\textsuperscript{10}. Due to the link between ROS levels and cancer metastasis, a device capable of producing an oxidant gradient may provide a novel approach to collecting particularly aggressive cancer cells.

MATERIALS AND METHODS

1.1 Device Design

The device consists of two etched glass substrates sealed together with a thin polymer membrane. It has been modified from previous versions to be more suitable for in vivo insertion. The overall dimensions of the device are 5mm x 1.25mm with a front angle of 45 degrees, as seen in Figure 1. The top cover has etched wells in which fluorescent polystyrene beads are loaded. These beads serve as a reference point for locating the device opening in vivo. The bottom reservoir can be divided into two parts: a rear chamber where the hydrogel is loaded and a cell collection chamber near the device outlet. Transparent materials are chosen to permit imaging of the device during in vivo experiments.

![Device Diagram]

Figure 1. Fully assembled in vivo NANIVID Device. Scale bar = 500 µm.

1.2 Device Fabrication

Microfabrication techniques are used to produce the device, as described elsewhere\textsuperscript{11}. Briefly, the substrates are patterned using photolithography and the features are etched into the glass using hydrofluoric acid. Dicing is performed on the resulting cover and reservoir pieces to obtain the desired shape. The reservoir piece is treated in an oxygen plasma prior to loading. The device is designed to carry a variety of materials optimized for particular applications. For example, a hydrogel solution containing epidermal growth factor (EGF, Sigma Aldrich) is loaded into the back of the reservoir and polymerized using UV light for the cell collection experiments. This hydrogel blend consists of 20% polyethylene glycol diacrylate (PEGMA, Glycosan), 7% methoxy polyethylene glycol monoacrylate (PEGMA, Sartomer), and Irgacure 2959 (BASF) as a photoinitiator. The hydrogel solution can also be loaded with a variety of different chemicals for additional applications. Alternatively, a 2mM H\textsubscript{2}O\textsubscript{2} liquid solution, without hydrogel, was placed in the reservoir for the reactive oxygen species experiments. The wells in the cover are filled with fluorescent polystyrene beads and polydimethylsiloxane (PDMS, Dow Corning) is spun on to create a thin membrane. Oxygen plasma treatment is performed to activate the PDMS surface and facilitate bonding to the reservoir piece. Finally, the two pieces are sealed together to form the completed device.
1.3 Cell Culture

MDA MB 231 cells, a human mammary gland adenocarcinoma cell line, were cultured in Dulbecco’s Modified Eagle Medium with high glucose and L-glutamine (Invitrogen), supplemented with 10% Fetal Bovine Serum (Atlanta Biologicals) and 1% penicillin-streptomycin (Invitrogen). The cells were kept under humid conditions with 5% CO₂ at 37°C.

1.4 Chorioallantoic Membrane Cell Collection

Fertilized White Leghorn chicken eggs (Charles River Laboratories) were incubated for 10 days at 37.4°C. The eggshell above the CAM and the air sac were gently punctured. Light suction was applied to the hole on the air sac end to lower the CAM from the eggshell. A square opening was cut into the eggshell to provide access to the lowered CAM. MDA MB 231 cells expressing GFP were centrifuged, and resuspended in a 1 mM MgCl₂, 0.5 mM CaCl₂, and 1% penicillin-streptomycin solution. Then 2 x 10⁶ MDA MB 231 cells were inoculated in each egg. Scotch tape was placed over the window to enclose the eggshell and the eggs were incubated for 7 more days. The tumor was then removed from the CAM with a scalpel and placed in warm culture media. Devices containing either 0 nM or 500 nM EGF were degassed by low vacuum treatment and inspected for the removal of bubbles. The devices were inserted into the tumor for 24 hours and then removed and imaged using a Leica SP5 scanning confocal microscope.

1.5 Mouse Cell Collection

Devices containing hydrogel loaded with either 50nM or 100nM EGF were inserted into the breast tumors of Dendra 2 color mice. These EGF concentrations were chosen so that the concentrations at the cellular level are physiologically relevant. The mice were anesthetized using isofluorane and the chest wall was opened. Degassed devices were inserted just under the surface of the tumor and left in place for approximately one hour followed by removal and imaging. Images of the cell collection chamber from the bottom to the top of the device were taken in 10µm steps using a multiphoton microscope.

1.6 Reactive Oxygen Species Induction

A device loaded with 2mM H₂O₂ liquid without the hydrogel was placed in a glass bottom dish with MDA MB 231 cells, in culture media. A rectangular in vitro device design that has been described previously was used in this experiment. The device was incubated with the cells for 15 minutes, which allowed the H₂O₂ to diffuse out of the device. Then, 1 µL of 10 mg/mL 2’,7’-dichlorodihydrofluorescein diacetate (H₂DCFDA, Invitrogen) was added as an indicator of reactive oxygen species generation. After 15 minutes, imaging of the area in front of the device outlet was performed using a Leica SP5 scanning confocal microscope. Three images were merged to create a large field of view outside of the device.

RESULTS AND DISCUSSION

1.7 Cell Collection from Excised CAM Tumor

The NANIVID was previously validated as an in vitro chemotaxis chamber. Rectangular shaped NANIVID devices were used to form a chemotactic gradient of EGF that attracted metastatic breast cancer cells in a culture dish. While robust, the rectangular design was not suitable for tumor insertion in vivo. A new pointed or needle shaped design was implemented for the in vivo application. This design allows for easy device insertion and less damage to the tumor. The outlet is located on the side of the device to prevent clogging during insertion. Finally, the elongated back end of the device provides an area to grasp the device during insertion and removal.

Collection of MDA MB 231 cells was performed using the needle shaped NANIVID loaded with EGF. A device was inserted into an excised tumor grown on the CAM and incubated in culture media for 24 hours. Figure 2A shows a merged image of the entire 500 nM EGF device after removal from the tumor. Both cancer and CAM cells were seen in the opening of the device after its removal, as shown in Figure 2B. This was the first demonstration of cell collection using the NANIVID. Cells were also collected in the opening of a 1 µM device, but in a lower number (data not shown). This may be due to the higher concentration device creating an EGF concentration at the outlet that is too high to attract cells inside the device. Control devices containing hydrogel without EGF also collected both cell types, as shown in Figure 2C, although the majority collected resemble CAM cells, as indicated by size and lack of GFP fluorescence.
few cancer cells that were collected were much smaller in number than in the 500 nM EGF device. Additional optimization of the experiment is on going.

Figure 2A) Merged overlay image showing the entire 500 nM EGF device after removal from an excised MDA MB 231 tumor grown on the CAM. Scale bar = 500 µm. B) Expanded view of the device outlet and cell collection chamber with MDA MB 231 GFP cells collected. Scale bar = 100 µm. C) Expanded view of cell collection chamber of a control, 0 nM device after removal from tumor. Scale bar = 100 µm.

1.8 Cell Collection in vivo from a Mouse Model of Metastasis

A 50 nM EGF loaded needle shaped NANIVID was inserted into the tumor of a Dendra2 mouse. These mice have macrophages which express cyan fluorescent protein and were injected with Dendra2 green fluorescent metastatic mammary adenocarcinoma cells. The device was removed from the mouse after approximately 1 hour in the tumor. Images of the removed device were taken using multiphoton microscopy, as shown in Figure 3. Many cancer cells (green) and a few macrophages (blue) can be seen inside the device. The co-collection of cancer cells and macrophages is consistent with what has been shown with the needle based assay. Faint fluorescence from the out of plane polystyrene beads can be seen in red. A second experiment using a device with a higher EGF concentration, 100 nM, was also performed under the same protocol. Both cancer cells and macrophages were also collected in this device, but fewer in number (data not shown). Continuing work is focused on device optimization, utilizing lower EGF concentrations, and controls.
Figure 3) Cell collection chamber of 50 nM device inserted into tumor from Dendra2 mouse. Cancer cells are shown in green, macrophages in blue, and fluorescent polystyrene beads in red. The edge of the device and cell collection chamber are shown outlined in white. Scale bar = 100 µm.

To confirm that the cells collected were inside the collection chamber, rather than on the outside of the device, a Z-stack was taken through the device. 3D image reconstruction was performed using Volocity® 3D Imaging software. A screen capture of Video 1 is shown below with labeling of the approximate locations of the device features. The movie begins with a 3D reconstruction of the device shown in a top down view followed by panning to a side view. Next, the device is rotated and returned to another top view. Given the height of the etched cell collection chamber, approximately 50 µm above the bottom of the reservoir substrate, the cells are clearly inside the device. The fluorescent beads can be seen in a higher Z-plane than the cells, consistent with the location of the etched wells in the device cover.

Video 1) Screen capture from a movie showing a reconstructed Z-stack of the device. Side view image shown with the approximate outlines of the edges of the device reservoir, cover, and the contact plane between the two. Scale bar = 50µm. http://dx.doi.org/10.1117/12.909045.1

1.9 Generation of a Reactive Oxygen Species Gradient

The induction-NANIVID was used to generate intracellular ROS by releasing H$_2$O$_2$. A device containing 2mM H$_2$O$_2$ liquid was incubated with MDA MB 231 cells for 15 minutes, allowing time for the H$_2$O$_2$ to diffuse, followed by the addition of H$_2$DCFDA. H$_2$DCFDA is a non polar, cell permeable compound that is non-fluorescent until the attached acetate groups are removed by esterases inside the cell and oxidation occurs$^{13,14}$. The resulting fluorescence intensity
(Ex./Em. 492-495 nm/ 517-527 nm) is based on the concentration of reactive oxygen intermediates. Figure 4A shows the gradient of H$_2$DCFDA fluorescence formed at 30 minutes after adding the device. The baseline intensity, due to the generation of ROS during normal cellular metabolic processes, can be seen in cells located farther away from the device. Cells located closer to the device outlet showed an increased intensity. A gradient of intracellular ROS has been formed, extending over a millimeter away from the device outlet. The same region was reimaged 15 minutes later, shown in Figure 4B. As expected by the diffusion of H$_2$O$_2$, the intensity of the cells near the outlet has increased and the gradient has extended farther from the device. The H$_2$DCFDA intensity was analyzed using ImageJ software. Starting at the device outlet, 100 μm rectangular sections across the width of the image were analyzed. Mean fluorescence intensity is plotted as a function of distance from the device outlet in Figure 4C. Continued work with reactive oxygen species generation is ongoing. These experiments demonstrate that the NANIVID can be used to initiate controlled changes in cellular oxidation both temporally and spatially.

**CONCLUSIONS**

The NANIVID is fabricated using transparent, biocompatible materials and is currently being developed as an *in vivo* cell collection tool. Sustained release of chemoattractants is achieved using a hydrogel blend which swells upon hydration and releases entrapped molecules, forming a gradient. When loaded with EGF, the device can be used to attract and capture invasive cells from a tumor. Cell collection was performed using two different models, in excised tumors grown on the CAM and in a mouse model *in vivo*. These were the first demonstrations of cell collection using the NANIVID. Ongoing work is under way to improve the device and perform longer duration experiments. The device is also being used a delivery vehicle for chemicals that induce controlled changes in the tumor microenvironment. This induction-NANIVID loaded with H$_2$O$_2$ generated a gradient of intracellular reactive oxygen species. Additional induction targets are being investigated including hypoglycemia and the manipulation of extracellular matrix stiffness.

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