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Cell Trapping in Activated Micropores for Functional Analysis

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Abstract— **This paper presents a novel device which provides the opportunity to perform high-throughput biochemical assays on different individual cells. In particular, the proposed device is suited to screen the rare cells in biological samples for early stage cancer diagnosis and explore their biochemical functionality. In the process, single cells are precisely positioned and captured in activated micropores. To show the performance of the proposed device, cultured yeast cells and human epithelial circulating tumor cells are successfully captured.**

I. Introduction

Breast cancer is the most common cancer and the second leading cause of death from cancer in American women. Greater than 40,000 women will lose their lives this year alone to the deadly disease [1]. A variety of treatment options have improved survival rates in recent years. However, once the disease has metastasized, or spread to organs in other parts of the body, survival rates decline precipitously. A woman diagnosed with stage I breast cancer where the tumor is 2cm or smaller and has not spread has a ten year survival rate of 93%, while a woman with stage IV cancer, where the tumor has spread to a distant site such as the bone, liver, lung, or brain, has only a 13% chance of surviving 10 years [1]. Once metastatic disease has been detected, current treatment is generally ineffective. For this reason, understanding the metastatic process may lead to greater diagnostic and treatment options and may lead to increased survival for these women.

Recently, new technology has allowed researchers to find breast cancer cells in peripheral blood [2][3]. It is believed that these circulating tumor cells (CTCs) play a key role in metastasis (reviewed by Smerage and Hayes [4]). Studies have shown that some women with metastatic breast cancer have cancer cells in their blood, while women without breast cancer do not [5]. It is not known if these cells originate from the primary tumor and are the cells responsible for producing metastases, or if these cells are being shed by metastatic foci and are a marker indicating that metastasis has already occurred. Because so little is currently known about these cells and their role in disease, understanding their molecular characteristics could add valuable information to the fight against cancer.

Cells that make up breast cancer are of epithelial origin. Normally, there should be no epithelial cells in the blood. By utilizing markers that specifically target epithelial cells, the CTCs can be labeled and isolated from blood. However, CTCs are rare and isolating them is difficult: in one milliliter of blood there may be only one cancer cell among greater than 5 billion blood cells. Current technology only counts the number of CTCs in blood. Commonly 5-10ml of blood is drawn from a patient and the cells are fixed to help them remain intact for the isolation protocol. There are several published isolation protocols, but all these methods kill the cancer cells and leave them contaminated with some red and white blood cells. Thus, being able to isolate, purify, and count single rare cells while keeping them alive for further functional analyses are key requirements for future technologies.

Studies have shown that in breast cancer patients with measurable metastatic disease, those with five or more CTCs have faster disease progression and lower survival rates [5][6]. This indicates that not only does the presence of CTCs act as a predictive measure of survival, it can also indicate which patients may benefit from more aggressive treatment. Moreover, molecular and biological analyses of these cells may provide information to guide therapy. Targeted therapies are an emerging field in breast cancer. However, the targets are defined by the molecular characteristics of the primary tumor. It is not known if tumor cells in metastases and blood have different molecular features than the primary tumor, and would therefore respond to an alternative therapy.

Our research hopes to produce a device that will isolate CTCs and maintain their viability while at the same time, remove all contaminating blood cells. By obtaining live cells, we will be able to use multiple techniques to determine their metastatic potential, their genetic relationship to the primary tumor or to specific metastatic sites, and finally, to determine their response to different chemotherapy treatments. This technology should provide better predictive indicators for disease outcome, improve treatment options, and ultimately increase survival. Additionally, this technology should be useful in improving treatment for other

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types of cancer such as prostate, lung, colon, kidney and more.

In this paper, we describe a protocol for using activated micropores for trapping single cells on a microarray platform. The process flow for making the device is explained in Section II. In Section III, we mention the experimental results of arraying single cells on the proposed device and show the performance of the device on capturing cultured yeast cells and circulating tumor cells. Finally in Section IV, we discuss about the advantage of the proposed device with other reported systems and the future directions.

II. Device Design and Fabrication

The proposed device is designed to capture single cells in a regular platform which enables screening all of the cells individually and simultaneously. Patterning adhesiveness [7], microfluidic patterning [7], dielectrophoretic registration of cells to microelectrodes [8] and magnetic absorbance of the cells on patterned microscale magnets [9] are recently developed techniques for making regular array of cells. However, making single-cell microarrays are still under development.

This device consists of arrays of chemically-activated micropores embedded in a silicon nitride membrane. Individual cells are precisely positioned and captured in activated micropores, which are functionalized pores in synthetic membranes. Arraying the cells provides the basic platform for high-density, parallel and simultaneous biochemical assays over individual cells. That will allow us a better understanding of physiological properties of various cell types involved in disease processes and will greatly facilitate our ability to develop better and more efficient clinical protocols for the treatment of complex diseases.

Silicon micromachining processes have been used to fabricate array of 10,000 cylindrical micropores with a diameter of 2µm, which is smaller than a cell size, in low-stress silicon nitride membrane. This device has the capability to trap up to 10,000 individual cells, which can be easily scaled up by increasing the pore density in membrane or making bigger devices. To increase the mechanical strength of the device, the silicon nitride membrane is made up of one hundred compartments (Fig. 1a). Each compartment contains 100 cylindrical holes (Fig. 1b). The fabricated device possesses adequate mechanical strength.

TABLE I
Device Characteristics

Chip Size	2cm x 2cm
Total Number of pores	10,000
Number of pores per compartment	100
Pore diameter	2µm
Pore pitch	100µm

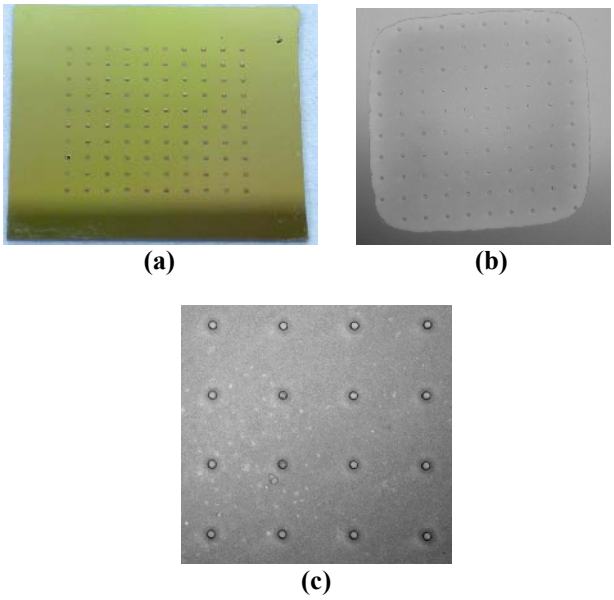


Fig.1. Microarray cell trapping device. Array of silicon nitride membranes and the micropores within are shown.

Although silicon nitride membranes are strong, silicon nitride is practically inert for biomolecule attachment, while on gold, further surface chemistry modifications can be performed through forming self-assembled monolayers (SAMs) of alkanethiols. Therefore, a thin film of Cr/Au is sputtered on top of the membrane.

The process flow for fabrication of the device is shown in Fig. 2)

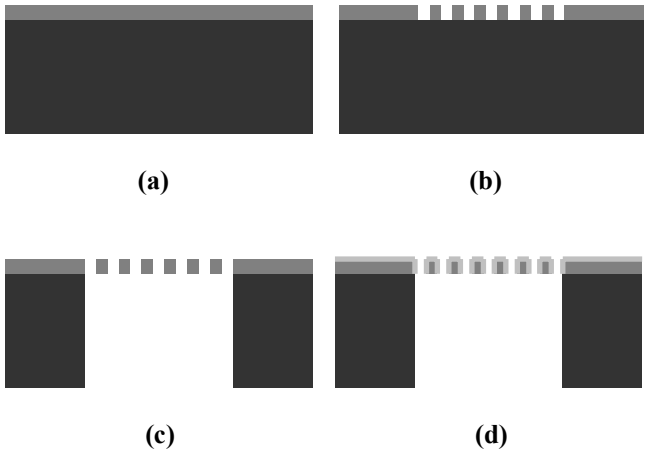


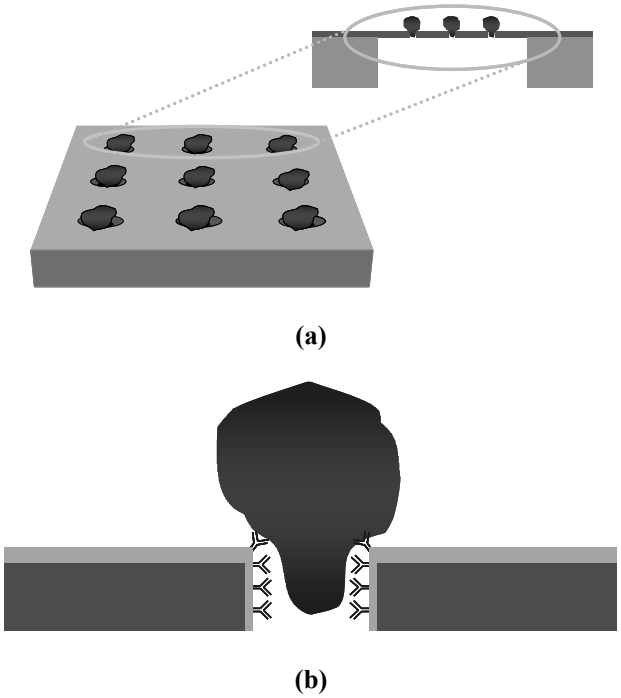
Fig. 1. Process flow of microarray fabrication (a) 1µm of low-stress LPCVD silicon nitride is deposited on silicon wafer. (b) Micropores are patterned in the silicon nitride. (c) Backside of wafer is etched by DRIE to form silicon nitride membranes. The wafer was annealed in 800C to remove the residual hydrophobic polymer formed during DRIE. (d) 50nm of Cr/Au is sputtered on the front side of wafer.

The fabricated devices are cleaned by RCA washing and stored in ethanol to wet the pores. Then carboxylate-terminated self-assembled monolayers (SAM), ordered molecular assemblies formed by the adsorption of an active surfactant on a solid surface, is formed on gold. Addition of NHS and the water soluble carbodiimide EDC to the SAMs

results in the formation of an NHS ester which can react with protein side-chain lysine residues and result in the formation of an amide bond [10]. Consequently micropores are activated by covalent binding of probe molecules to the monolayer. The probe molecules are biological macromolecules such as antibodies or membrane markers, which have selective binding affinity to the cells.

To position the cells precisely to the micropores, the probe molecules should be only attached to the pores. To achieve this requirement, the probes are inserted gently from the backside of the device leaving the front side of the device intact. After incubation, the excess molecules are washed from the backside of the device. This process leaves the top surface of the chip almost free of the probe molecules. The accuracy of positioning the probe molecules through this method is much better than the accuracy of spotting the molecules on the front side of the device, since aligning the spotting point to the micropore is challenging and also making spot sizes comparable to the pore diameter is hard.

After micropores activation is accomplished, the biological solution containing cells is spotted to the front side of the device on the membrane. The single cells are captured and immobilized by employing gentle suction from the backside of the device and affinity binding to the probe molecules in the micropore. Finally further interrogations can be done on the arrayed cells



(a)

(b)

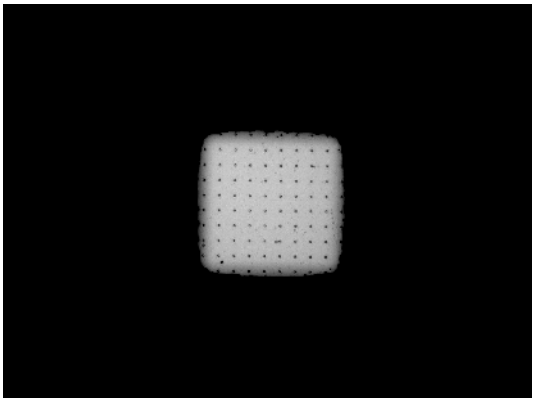
Fig. 3. (a) Schematic of trapped cells in microarray. Single cells are trapped in the micropores by backside suction and affinity binding to the adhesive molecules immobilized on the micropore. (b) Schematic of a single cell trapped in micropore. Adhesive biomolecules are covalently immobilized to the micropore while the front side surface is free of adhesive molecules. Then the biological solution containing cells are spotted on the front side of the device.

The proposed device can be reused by pumping buffer solution from the backside of the chip which releases the trapped cells in the micropores.

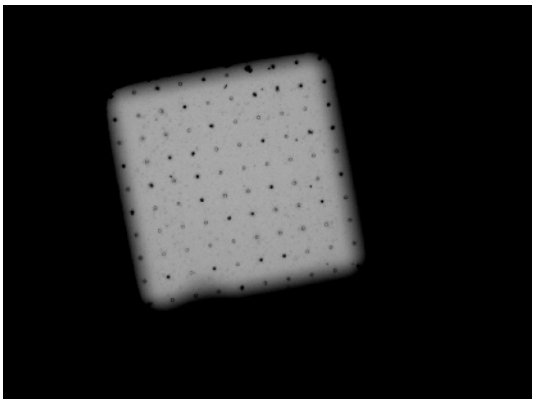
III. Experimental Results

Cultured yeast cells and circulating cancer tumor cells are arrayed by using the described chip. In the first set of experiments, the micropores are not activated and the cells are captured by applying suction from backside of the chip. As shown in Fig 4b, the capturing performance is poor in this case and more than half of the pores are not filled with the cells. In the second set of experiments, the micropores are activated by Concanavilin-A protein and a monoclonal antibody targeting CD-44 to capture yeast cells and CTCs respectively. By applying suction from backside, almost all of the micropores are filled with single cells, as shown in Fig 4c.

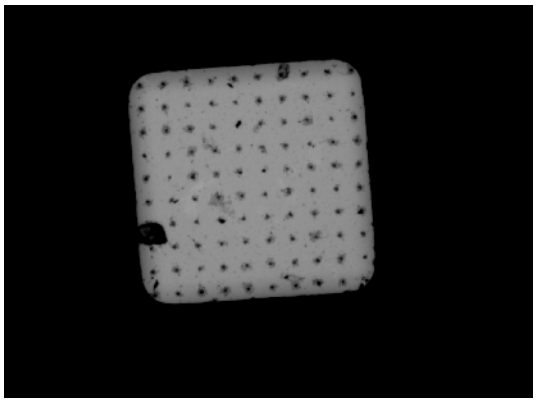
All of the experiments are done with live cells, so any desired biochemical assays can be performed on cells. In the case of detecting rare cancer cells, fluorescent cancer markers can be added to the chip and the cancer cells can be easily detected by fluorescent microscopy, since the cells are separated and positioned in a regular platform. Moreover, individual cells can be treated differently and in parallel.



(a)



(b)



(c)

Fig. 4. (a) RCA washed silicon nitride membrane. This compartment contains of 100 micropores. (b) Trapping the years cells by applying gentle suction from backside of the device. As seen here, less than half of the micropores are occupied with cells. (c) Trapping the yeast cells in activated micropores by applying gentle suction from backside. All of the pores are filled with cells.

Due to the great capturing performance, even though there may be very few cancer cells in a vial of blood, this new platform may allow for even one cancer cell to be identified.

The next step, after arraying single cells, is to identify the cancer cells. We are currently using an epithelial marker called EpCAM. Epithelial cells, but not blood cells, express this protein on their surface. We target this protein with a fluorescent antibody that specifically binds to EpCAM and no other protein.

IV. Discussion

As discussed previously, by activating the micropores, the efficiency of trapping the cells improves significantly. Also by maintaining the viability of the cells, further biochemical analysis becomes possible.

In conclusion, the advantage of the proposed platform is trapping single cells in a regular array and allowing high-throughput and parallel biochemical interrogations on single cells individually. Meanwhile all of the cells or a selected population of the captured cells can be released by applying pressure from backside of the device. Therefore the proposed novel device is well suited for rapid drug screening, detection of chemical and biological warfare agents, and fundamental studies of cell biology. We believe that this technology will allow us to rapidly screen large numbers of cancer patients in a cost and time efficient manner and would be useful in determining the molecular characteristics of CTCs in patients with known metastatic disease and it would also be useful as a screening technique for early detection of cancer.

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