Towards a Fast, Efficient Assay for Isolating Circulating Tumor Cells

PI: Professor David Eddington
Grad Student: Cari Launiere
Author: Joseph M. Labuz
jlabuz2@uic.edu

July 31, 2009

Abstract
CTCs offer and intriguing option for cancer diagnosis. By assaying for their presence in blood, rather than through biopsy or imaging technology such as mammography, cancer screening can be made cheaper, more efficient, more comprehensive, and more effective. However, an adequate method to elucidate the presence of CTCs in whole blood has yet to be determined. We purpose a microfluidic separation device based on three proteins (anti-EpCAM, anti-CAV-1, and E-selectin) to increase capture efficiency, purity, and versatility. A passive, grooved PDMS mixer is also incorporated into the design to increase frequency of contact between the cells in suspension and the protein-functionalized surface of the bed of the channel. Current microfluidic capture devices rely solely on anti-EpCAM to bind to CTCs in flow. By incorporating E-selectin, anti-CAV-1, and a passive mixer into our chip, we are able to increase capture efficiency, purity, and versatility to yield a more robust detection assay.
Keywords: *CTCs, microfluidics, cancer screening, EpCAM, E-selectin*

Introduction
In 2008, over half of cancer-related deaths were caused by prostate, lung, breast or colon cancer.[1] The four diseases all share the threat of metastasis – the ability to spread from the original tumor site throughout the body via circulating tumor cells (CTCs). These cells detach from the tumor site and are transported throughout the body by either the circulatory system or bone marrow.

In blood, these cells are extremely hard to find (between one in ten million and one in a billion)[2] since they are mixed in with red blood cells, leukocytes, platelets, etc. Yet, they offer an extremely attractive method for cancer screening and diagnosis since they are often detectable before the symptoms of the tumor itself become apparent. As an added benefit, such a screening method would only require a small blood sample as opposed to a traditional biopsy which necessitates invasive surgery and the removal of a tissue mass. Moreover, such a method could test for the presence of metastatic cancers throughout the whole body, rather than just a particular site as current medical imaging methods (like mammography) do.

One thing many carcinoma cells, and by extension CTCs, have in common is that they express epithelial cell adhesion molecule (EpCAM) at a very high rate,[3, 4] while the protein is only negligibly present in normal blood cells. Previous attempts to isolate CTCs using a variety of methods all proved to have severe deficiencies. Reverse transcriptase polymerase chain reaction procedures commonly yielded sensitivity rates below 35 percent[5] and immunomagnetic bead based technologies managed to achieved high throughput and purity, but low efficiency.[6]

In 2007, the Toner group built a microfluidic device that takes advantages of the overexpression of EpCAM in carcinoma cells.[7] By fabricating 100 μm posts in a Si wafer via deep reactive ion etching (DRIE), then coating them with anti-EpCAM, they were able to isolate CTCs by simply flowing whole blood through the chip. The CTCs adhered to the anti-EpCAM-coated posts and the rest of the blood was flushed out of the device (Fig.1).

This leveraging of microfluidic technologies for the isolation of CTCs proved much more effective than previous methods. The device was able to process whole blood with no prior sample preparation giving such a method the potential for rapid, high-throughput screening.

Moreover, the device was very sensitive, detecting CTCs in 115 out of 116 cancer patients. Finally, this method isolated a purer sample than previous methods.[7]

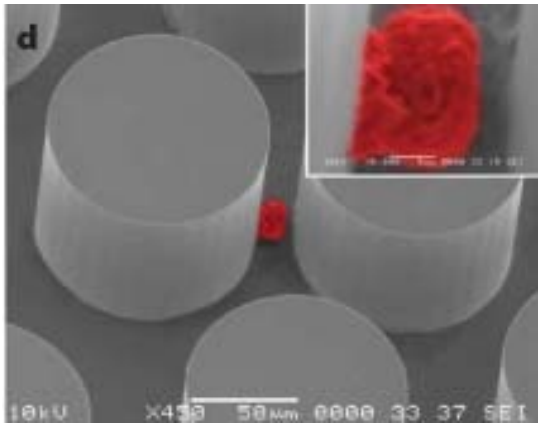

Fig.1 – micrograph of 100 μm x 100 μm Si posts from Toner's device, with captured CTC, colored red for visibility. The posts are fabricated by DRIE and are coated with anti-EpCAM and readily bind to CTCs which overexpress the protein.

However, since it is a micro-scale device processing meso-scale samples of blood, the tests can be rather time consuming, often negating any benefits reaped by the elimination of pre-processing the sample. Also, the complexity and cost of DRIE is prohibitive to any near-term, laboratory reproduction of the cancer screening chip. Though the purity of the captured cells sample is markedly improved from past methods to 50 percent,[7] still higher purity yields are necessary for effective cancer screening.

Subsequent projects have made use of similar phenomena,[8, 9] but significant drawbacks nevertheless remain for each. Through the fabrication and characterization of a novel microfluidic device, we will address and improve upon the range of cells that can be isolated by microfluidic techniques as well as the capture purity and efficiency of the device.

Furthermore, previous CTC capture microfluidic devices have focused exclusively on EpCAM-based capture. It is not uncommon, however, for some CTCs to have comparatively low levels of EpCAM expression. It has generally been found that as EpCAM expression decreases, CAV1 expression increases[10]. For this reason, we hope that by incorporating anti-CAV1 into the protein coat, we will be able to capture a broader range of CTCs than previously possible, making our device a more powerful tool for cancer diagnosis.

Another protein, E-selectin is common on the endothelial surface of the vasculature and it readily interacts with blood cells as well as CTCs. E-selectin exhibits very fast binding kinetics (via a catch-bond mechanism) which makes it an ideal protein for pulling CTCs out of flow, in the process increasing the amount of time they are available for capture. Its incorporation will allow for greater CTC exposure to the protein-functionalized surface, leading to higher purity and capture rates, as well as allowing for flow rates at least six times higher than have previously been achieved. Blood cells will roll along the E-selectin surfaces until they reach an anti-EpCAM or anti-CAV1 patch, become unbound, and are perfused out of the device.

We present a device consisting of a multi-channel polydimethylsiloxane (PDMS) mold bonded to a glass slide coated with a protein cocktail engineered to isolate CTCs from whole blood as it is perfused through the device. In order to improve capture efficiency, as well as isolate CTCs with comparatively low EpCAM expression, the protein coating is composed of E-selectin and anti-CAV1, as well as anti-EpCAM, whereas previous devices focused on capture based exclusively on EpCAM adhesion.[7-9] In order to increase incidents of contact between suspended cells and the protein coat, a passive mixer was also incorporated into the PDMS mold.

Method

The PDMS mold consists of 41 parallel, independent channels with grooved ceilings designed to induce transverse flow, forcing the cells to the bottom of the device. PDMS molds were made via soft lithography from Si masters fabricated by photolithography.[11, 12] SU-8 2100 was spun at 2600 RPM to coat a 100 mm diameter Si water with a layer of photoresist 100 µm thick. After soft baking, the photoresist was masked and exposed to UV light and post baked. A second, 160 µm layer was spun and 1100 RPM and the process repeated. To complete the master, the device was developed and then silanized for 2 hours to allow for easy removal of PDMS molds. PDMS was mixed in a 10:1 ratio (by weight, polymer to curing agent), poured over the Si mold, degassed and cured at 75 °C for 90 minutes. Both lithography processes are illustrated in Fig.2.[13]

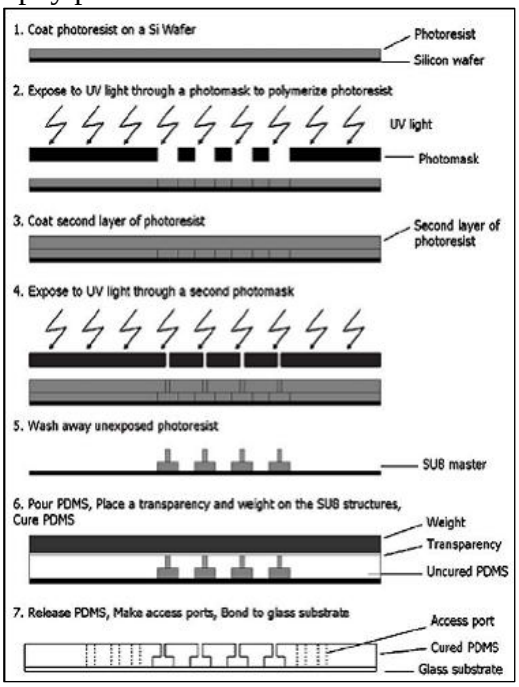

Fig.2 – photo and soft lightography are often coupled. A Si master is made using photolithography, and a PDMS mold taken during soft lithography.

To coat the surface of the slide, the PDMS device is first bonded to the glass with the aid of a corona treater and the channels filled with positive pressure from a syringe with an anti-EpCAM/anti-CAV1/E-selectin cocktail and allowed to settle for 4 hours. The device is flushed with phosphate buffered saline (PBS) then vacuum filled[14] with bovine serum albumin (BSA) to cover any of the glass surface not yet coated and prevent non-specific binding. After another PBS rinse, the device is ready to be used. Until the sample is infused, the device remains filled with PBS to prevent the protein coat from becoming dehydrated.

MDA-MB-231 breast cancer cells were chosen because the cell line binds well to E-selectin but does not overexpress EpCAM,[10] making it undetectable in EpCAM-exclusive assays. Since our device incorporates a three-protein capture scheme, we hope to isolate MDA-MB-231 cells, as well as other lines overexpressing EpCAM. MCF-7 type cells were also considered, but were not chosen because though they bind well to anti-EpCAM, they exhibit poor E-selectin affinity.[15]

Finally, CTC capture is quantified using a microscope. For sixty seconds, whole blood is perfused at 0.1 mL min^{-1} through the device by a syringe pump and pictures taken at one second intervals. From these pictures, the number of cells captured can be determined, however it is necessary to develop a way to filter out particles, cells that have already attached to the protein coating, and cells that are not attached and just flowing through the frame.

To analyze the images, the mixer pattern was first subtracted from the frame. This is done using imageJ's 'Subtract' function. By taking a 'control' picture, before the device is filled with cells,

we can remove the mixer pattern (and associated glare) from subsequent images where cells are present.

To quantify cell capture, two separate programs are used. The series of timelapse images is analyzed using ImageJ software.[16] A custom-built macro sums images to filter out cells that are flowing through the frame, applies a threshold to the resulting image, and then counts the cells, excluding things like debris and scratches based on size and circularity restrictions. The data is exported to a text file to be analyzed by MatLab.

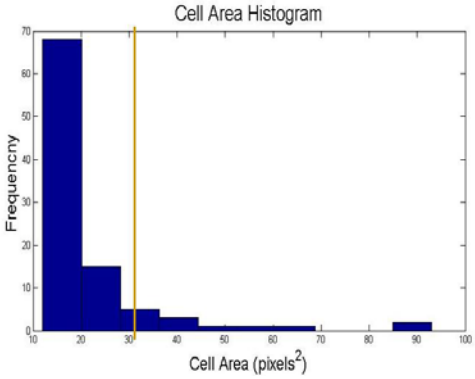

Fig.3 – Cell size histogram. Cells larger than the mean plus standard deviation (those to the right of the gold line) were considered clumps.

In MatLab, a program receives the ImageJ output and searches for particles larger than the standard deviation plus the average size (Fig.3). When one is found, the area is divided by the average cell area and the total cell count amended accordingly.

Results/Discussion

The ceiling grooves were optimized according to previously conducted research to induce maximum transverse flow.[17] To ensure that the calculated channel dimensions of 100 um and 160 um – critical to mixer optimization – were achieved, a series of experiments was performed to determine the correct spin speed for SU-8 coating. The results are summarized in Fig.4.

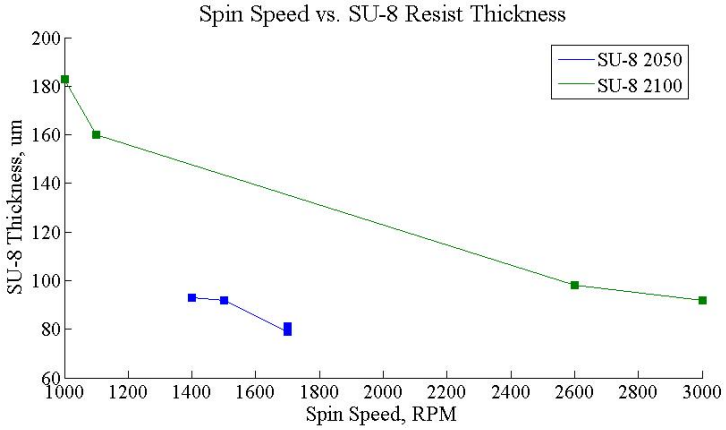

Fig.4 – graph of SU-8 photoresist layer thickness versus spin speed. The 2100 curve is similar to that found in the literature, though shifted left. The same is expected of the 2050 curve had more trials been run.

It was found that a spin speeds of 2600 and 1100 RPM corresponded to thicknesses of 100 um and 160 um respectively. This differed significantly from published data from Microchem,[18, 19] but this error is most likely due to higher than normal laboratory temperatures, which would in turn decrease the viscosity of the photoresist. This is consistent with the data.

In order to validate the use of the 'Subtract' function in ImageJ, a control image of the device was taken before it was filled with cells. An image with capture was then selected (Fig.5-b) and

subtracted from the control (Fig.5-a). The result (Fig.5-c) indicated that such a method would be appropriate for filtering out the effects of the mixer pattern, as well as other scratches or defects on the surface of the slide.

Fig.5-a – 'Control' image of device used to cancel out interference caused by PDMS mixer pattern.

Fig.5-b – Image with captured cells

Fig.5-c – Result of subtracting 5b from 5a. This validates our method for removing noise caused by the mixing pattern.

The counting algorithm was compared to two different hand counted photographs to validate the procedure. To be sure the program could handle a variety of images, one image containing ~100 cells was chosen and one image containing ~1000 cells was chosen. After each image was counted by hand using imageJ, the program was run and the results compared. The numbers were in a good agreement; in each case the error was ~1 percent.

From preliminary results, we were able to elucidate several key points about out assay. First, it was found that a flow rate of ~0.1 mL min^{-1} improved interaction between the cells and the protein surface. This is a lower flow rate than desired, but nevertheless, it is six times faster than those achieved by other devices.

As expected, the cells rolled on E-selectin, however there was not much anti-EpCAM and even less anti-CAV1 capture. This could be due to two reasons. First, it was observed that as rolling cells entered the mixing region, they became detached and were washed away. It could be possible that the mixer is "over-optimized" and the flow is so turbulent that it rips bounds cells off the bottom of the device and washes them away.

Second, it is possible that the protein coating was flawed. We have yet to determine the correct ratio of anit-EpCAM, anti-CAV1, and E-selectin to obtain maximum capture. Furthermore, in preparing the slides, the BSA was left to coat the devices for longer than planned, giving rise to the possibility that it may have displaced some anti-EpCAM or anti-CAV1 due to its higher affinity for PDMS adsorption. Either way, more careful, controlled deposition of the protein coat is necessary and will be attempted in successive trials.

Conclusion

A microfluidic device made functional by anti-EpCAM, anti-CAV1, and E-Selectin has been fabricated and will be shown to capture circulating tumor cells in good yield and purity. Through a novel design that couples E-selectin with anti-CAV1 and anti-EpCAM for cell capture, faster flow rates and therefore higher throughput were realized. Furthermore, CTCs expressing normal or low levels of EpCAM (such as MBA-MD-231s) will be captured by this

new device, whereas previous devices based exclusively on EpCAM capture were unable to detect such cells. This device brings us one step closer to a cheap, effective, high-throughput microfluidic device for metastatic cancer screening.

Acknowledgements
Special thanks to Prof. David Eddington, Cari Launiere, and the BML lab for their guidance and support over the course of the project. Additional thanks to the National Science Foundation and Department of Defense for funding this work through EEC-NSF Grant # 0755115.

Works Cited

1. Cancer Facts & Figures 2008. American Cancer Society (2008).
2. Racila, E. et al. Detection and characterization of carcinoma cells in the blood. Proceedings of the National Academy of Sciences of the United States of America 95, 4589-4594 (1998).
3. Went, P. T. et al. Frequent EpCam protein expression in human carcinomas. Human Pathology 35, 122-128 (2004).
4. Balzar, M., Winter, M. J., de Boer, C. J. & Litvinov, S. V. The biology of the 17-1A antigen (Ep-CAM). Journal of Molecular Medicine-Jmm 77, 699-712 (1999).
5. Zieglschmid, V., Hollmann, C. & Bocher, O. Detection of disseminated tumor cells in peripheral blood. Critical Reviews in Clinical Laboratory Sciences 42, 155-196 (2005).
6. Talasaz, A. H. et al. Isolating highly enriched populations of circulating epithelial cells and other rare cells from blood using a magnetic sweeper device. Proceedings of the National Academy of Sciences of the United States of America 106, 3970-3975 (2009).
7. Nagrath, S. et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature 450, 1235-U10 (2007).
8. Adams, A. A. et al. Highly Efficient Circulating Tumor Cell Isolation from Whole Blood and Label-Free Enumeration Using Polymer-Based Microfluidics with an Integrated Conductivity Sensor. Journal American Chemical Society 130, 8633-8641 (2008).
9. Phillips, J. A., Xu, Y., Xia, Z., Fan, Z. H. & Tan, W. H. Enrichment of Cancer Cells Using Aptamers Immobilized on a Microfluidic Channel. Analytical Chemistry 81, 1033-1039 (2009).
10. Sieuwerts, A. M. et al. Anti-Epithelial Cell Adhesion Molecule Antibodies and the Detection of Circulating Normal-Like Breast Tumor Cells. Journal of the National Cancer Institute 101, 61-66 (2009).
11. Duffy, D. C., McDonald, J. C., Schueller, O. J. A. & Whitesides, G. M. Rapid prototyping of microfluidic systems in poly(dimethylsiloxane). Analytical Chemistry 70, 4974-4984 (1998).
12. Xia, Y. N. & Whitesides, G. M. Soft lithography. Abstracts of Papers of the American Chemical Society 214, 348-PMSE (1997).
13. Mohammed, J. S., Caicedo, H. H., Fall, C. P. & Eddington, D. T. Microfluidic add-on for standard electrophysiology chambers. Lab on a Chip 8, 1048-1055 (2008).
14. Monahan, J., Gewirth, A. A. & Nuzzo, R. G. A method for filling complex polymeric microfluidic devices and arrays. Analytical Chemistry 73, 3193-3197 (2001).
15. Zen, K. et al. CD44v4 is a major E-selectin ligand that mediates breast cancer cell transendothelial migration. PLoS One 3, e1826 (2008).
16. Rasband, W. S. ImageJ. National Institute of Health (1997-2004).

17. Lynn, N. S. & Dandy, D. S. Geometrical optimization of helical flow in grooved micromixers. Lab on a Chip 7, 580-587 (2007).
18. Processing Guidelines for SU8 2100 and SU8 2150. Microchem.
19. Processing Guidelines for SU8 2025, SU8 2035, SU8 2050, and SU8 2075. Microchem.