Method for Overcoming Bandwidth Limitations of Standard Fluorescence Microscopy

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KEYWORDS. CD45, fluorophore, microfluidic, photobleaching, relabeling

BRIEF. A procedure for measuring fluorescence from multiple sources in microfluidic devices

ABSTRACT. During physical injury, pain and swelling are common symptoms of that injury. Likewise, we believe that at the microscopic level, our cells respond to the body's physical and chemical injuries by releasing chemicals and producing proteins. However, in order to detect these microscopic changes, standard fluorescent microscopy, which is the viewing of cells under a microscope with the aid of fluorescent labels, is required to measure the expression of surface proteins on cells. Unfortunately, it is limited in the number of colors that can be viewed simultaneously without causing blending and color overlap, resulting in a blurry and inaccurate image. This effectively limits the number of labels that can be used at one time to three or four per cell. Therefore, the purpose of this project was to develop a protocol that would bypass those limitations by recycling the bandwidth in the color spectrum through a color removal and replacement process referred to as photobleaching and relabeling, respectively. In practice, this should allow for the labeling of a single sample of cells many times over, which will be very useful for indentifying human cell markers which range in the hundreds.

INTRODUCTION.

Trauma, defined as any physical damage to the body, is the leading cause of death for Americans aged 1 to 44, with an estimated economic impact of over $120, 000 per injury [1]. Many of these individuals have served in the military and have suffered physical injuries due to war, while others are civilians, many of whom experienced bodily damages due to motor vehicle accidents. Historically, research funds have been used to contribute to heart, cancer, and mental disorders research, which are not directly related to physical injury [2]. This has been the case for decades now and consequently, full understanding of cellular reactions to trauma has not been attained because of inadequate funds to perform research. The reason that this research is necessary is that in many cases, similarly treated trauma patients will experience varying results for reasons currently unknown to doctors. Although new theoretical and *in vitro* discoveries are being made to identify possible factors which account for the differing responses of similar therapies, there is still not enough information to understand the actual interactions that may be occurring in the cellular level [3].

Researchers use microfluidic devices (Figure S1), or transparent platforms visible under the microscope in which cell assays can be performed, to capture cells using the U-shaped traps in the devices to monitor cellular activities and gather new information about existing disorders. For example stem cell research has been enhanced through the use of microfluidic devices to study Chronic Myeloid Leukemia and Hematopoietic stem cell disorder, which has allowed for the expansion of our knowledge on chemoresistance—specific resistance acquired by cells to chemicals [4]. This technology has been further used to study the mobility of cells such as marine ciliates [5]. However, both of these studies have focused primarily on direct observations of organisms on the scale of a few micrometers, instead of fluorescent labeling of membrane and intracellular proteins.

The major limitation of standard fluorescent microscopy is the overlapping of the spectrum of ordinary fluorescent dyes when three or more are used simultaneously. This effectively limits the information bandwidth of a fluorescent microscopy experiment. Additional technologies have been developed to enhance the degree to which information can be gathered from a sample, including Multispectral Fluorescence Imaging, which is similar to standard fluorescent microscopy except that it may include infrared imaging and has the potential to improve the accuracy of disease detection and monitoring of treatment responses [6]. However, it is a very costly process and requires using new samples each time. Cheaper methods such as the use of Quantum dots (semiconductors whose size is closely linked to the amount of energy needed to excite the crystals) are used to develop dyes [7]. Unfortunately, quantum dots are not photobleachable and so their reusability is limited. Because of these many limitations of traditional techniques, it is difficult to fully study and understand the cellular interactions in trauma patients; thus novel techniques are needed to effectively enhance patient side care.

In order to deal with the limitations of current measurement technologies, new methods need to be created to extract more data from a single sample, reducing reagent and cell amounts needed. Using multi-trap nanophysiometers (MTNPs), data can be gathered from cells of various origins, and identified using surface markers [6]. However, there are limitations with this technology that only allow for the measurement of about three to five different colors from the visible color spectrum. Therefore, novel clusters of differentiation (CD), or group of proteins having a unique structure used for identification, membrane marker labeling techniques are needed to allow for the distinction between cell types and can be done before or after cell fixation and multi-color fluorescent staining. After each test, the fluorophore, or fluorescent molecule that attaches to a membrane marker, may be removed through photo bleaching. This allows for the ability to attach other membrane CD markers to the same sample. Thus, it would allow for the ability to measure the dynamic responses of white blood cells (WBCs), including the expression of new membrane markers in response to trauma, and interrogate WBC subsets and their sub-classes via CD membrane markers. The dynamic responses to microenvironment challenges of subclasses of WBCs in trauma patients may increase our understanding of how the body's cells respond to injury and thus allow us to better diagnose and treat trauma patients.

MATERIALS AND METHODS.

Photolithography.

In this process, the stencil created through the use of AutoCAD, consisting of three inputs that collect at the center of the device and one output, is then used to create a positive image mask to be used for the fabrication of a microfluidic device (Figure S2). SU-8, an epoxy-based liquid that solidifies when exposed to UV light, is spun on another silicon wafer to achieve the desired height of the device. It is then cured to cause cross-linking between the mixtures and thus harden the device enough to be used with the stencil. The stencil is placed above the newly prepared wafer and UV light is focused from above to harden the SU-8 on the silicon wafer. The excess SU-8 not hardened by the UV light is then washed away with Isopropyl Alcohol (IPA) and a positive image of the stencil remains.

Microfabrication.

In a clean room, the positive image of the stencil was used as a mold for the device, over which a mixture of polydimethylsiloxane (PDMS) and Silicone Elastomer Curing Agent was poured, and baked for at least four hours for the device to solidify completely. Prior to baking, the creation of PDMS involves using standard ratios to mix the various components of the polymer (to attain various degrees of thickness), followed by degassing to remove trapped gases

in the mixture. After the device has become solid, holes are punched for the inputs and outputs using sharpened syringe tips. Following the preparation of the microfluidic device, a Harrick Plasma Cleaner is used to plasma-bond the flat surface of the device containing the channels to the glass in order to seal it.

Cell Culture.

Jurkat T-cells, an immortalized line of lymphocyte cells, are maintained in an incubator at 37°C. Every other day the cells are split under a sterile fume hood, which involves replacement of nutrient media, and reducing the concentration of cells. In order to do so, a hemocytometer is used to count the approximate number of cells so that a dilution yielding between 100, 000 cells to 300, 000 cells per mL can be prepared. The prepared dilution was placed in an incubator. From the remaining cells, approximately 300μL (30,000 to 90,000 total cells) are drawn to be used for the experiments and the rest were disposed.

Standard Fluorescent Microscopy.

Standard procedures for tagging CD membrane markers inside of microfluidic devices involve diffusing fluorescent dyes through the device and allowing binding to the cells to occur. Three to four labels can be used simultaneously without causing much overlap when viewed under the microscope [8]. Other standard but more expensive alternatives include running the sample through a flowcytometer and inputting the conjugated, or artificially attached, markers into the machine while running up to 18 simultaneous fluorescent labels [9].

Modified Fluorescent Microscopy and Image Analysis.

Our modified procedure involved tagging leukocytes with CD membrane labels specific to the different markers contained in the membranes of the white blood cells. Glass syringes, one with labeling media conjugated with Allophycocyanin (APC) and another with nutrient media and cells, were connected to the microfluidic devices. The syringes were placed on nanoliter precision Harvard Micropumps. The device was first primed with nutrient media, and then the cells in the syringe were pumped through the device. The cells then become trapped inside the device.

After loading the device with Jurkat T-cells, we perfused the device at 250 nL/min with a solution supplemented with 150mL/L CD45 Marker. The cells were saturated with marker after 40 minutes (Figure 1). The cell media was then stopped, and the labeling media was allowed to flow through for a period of time specific to the labeling marker. Then, photobleaching, or lose of fluorescence, was measured as the difference in mean intensity at the following intervals: 5, 10, 20, 25, 30, 35, and 40 minutes. Once the time needed for photobleaching and relabeling, or the attaching of fluorophores-conjugated antibodies to membrane markers, is known, one can re-label and photobleach an *n* number of times. ImageJ was used to analyze the intensity of images and numerical data from the images taken using fluorescent microscopy.

RESULTS.

To test the viability of a fluorescent bleach protocol, Jurkat T-Cells were cultured so that the concentration would be at approximately 300, 000 cells per mL. The cells were safely flowed into the device, without significant harm, and became trapped in the nanotraps within the device. Most of the cells remained in place throughout the entire experiment, and thus were fixed in place (Figure 1A). Allophycocyanin (APC) fluorophores were conjugated, or artificially attached, to the CD45 and CD3 cell membrane markers.

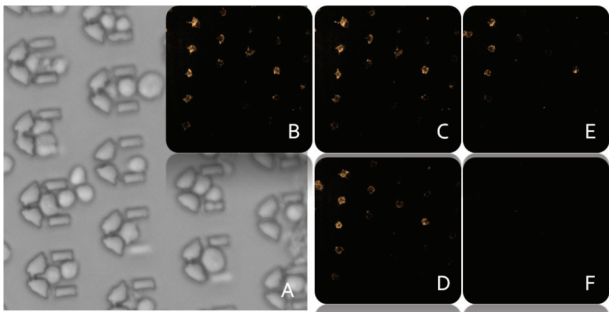

Figure 1. (A) Differential Interference Contrast (DIC) image, which highlights the edges, of Jurkat cells in traps. (B-F) XF102-2 time lapsed image after 0, 5, 10, 20, and 40 minutes respectively of photobleaching the CD45 marker.

The purpose of the first assay was to confirm that fluorescence could be removed from cells labeled with membrane markers. The cells were labeled without the need to incubate in labeling media. After 40 minutes of labeling, the cells were clearly visible through the XF102-2 filter, which only allows orange light to pass and causes the fluorophores to fluoresce. Once fluorescence was confirmed, the process of photobleaching began, and a decrease in fluorescence could be seen over time. The decrease in fluorescence was most noticeable within the first 25 minutes but was noticeable throughout the elapsed time (Figure 2). The removal of fluorescence through photobleaching was confirmed by examining the intensity decrease to approximately zero.

The purpose of the second assay was to determine the ability to track multiple markers and the ability to selectively remove target markers. The CD45 and CD3 conjugated fluorophores were again shown to label cells in a microfluidic device without the need to incubate. For this experiment, the XF102-2 filter was able to detect fluorescence from the CD45 and CD3 conjugates, while the FITC filter, which only allows green light to pass, was only able to detect fluorescence from the CD3 conjugate. There was a visible decrease in the intensity of fluorescence from the Jurkat cells labeled with CD45 and CD3 membrane markers. A change of 3.72 mean intensity was observed through the FITC filter, whereas a change of 5.1 mean intensity was observed through the XF102-2 filter. Therefore, a greater amount of intensity can be seen through the XF102-2 filter than in the FITC filter (Figure S3). The similar decrease of mean intensity through both filters confirms the visibility of CD3 conjugates through the XF102-2 filter, and the ability to target a specific marker without much of an effect to fluorescence of the other fluorophores.

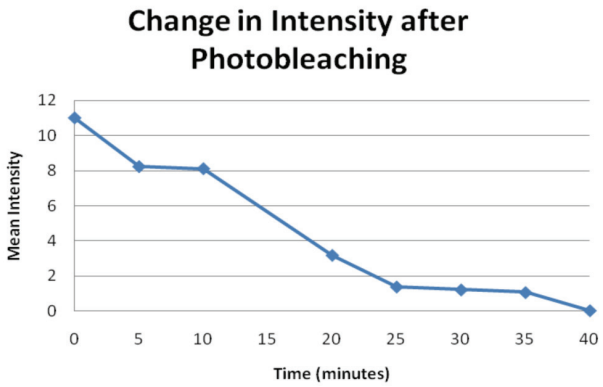

Figure 2. Change in intensity after photobleaching. There is a decrease of intensity as the duration of filtered UV light exposure increases.

DISCUSSION.

Microfluidic devices allow for a controlled environment to perform our assays. The traps were shown to effectively hold the cells, and the cells remained alive. We were able to demonstrate that it is possible to label cells with CD markers and detect the fluorescence of individual cells. This is an important step in the development of a cell typing device, or a device that identifies cells based on surface markers. Additionally, the microfluidic devices require very small volumes of reagents and very small numbers of cells. In the case of trauma patients, 10mL are routinely collected by physicians, and only 10mL is collected from a trauma patient, only 20uL or 0.2% of the total blood would be required for this protocol. However, due to the nature of this protocol, it is unlikely that the cells will survive many repetitions. Thus more research would have to be done to determine the exact number of times this can be performed on living cell groups for experiments requiring the typing of live cells. Therefore, it is recommended that assays requiring live cells be performed first or on a separate sample. For extensive typing, it is recommended to fix the cells prior to photobleaching, which should minimize the damage caused by the intense and directed light required for photobleaching the cells, and allow for more assays to be executed. .

Many of the cells died in the experiment, presumably based on increase cell size and loss of rigid membrane, possibly due to the prolonged photobleaching procedure. To reduce the risk of cell death, the procedure can be modified to use with a more photo-bleachable fluorophore to diminish the intensity of the oxidation that occurs. The experiment was repeated with similar conditions except that two markers were used instead, raising the perfusion rate to 500 nL/min. We demonstrated that perfusing for 20 minutes is enough time to adequately label the T-cells, effectively cutting the time required to perform a fluorescence assay in half.

Theoretically, the entire surface of the cell was not coated with labels, and the oxidation caused by photobleaching mainly affected the fluorophores and did not cause a significant additional response from the cell. Theoretically, the cells were not completely labeled, and the oxidation caused by the photobleaching mainly affected the fluorophores more so than the cells. This is supported by a change in intensity after photobleaching with a FITC filter, where there was a similar decrease in the intensity of fluorescence from the Jurkat cells labeled with the CD45 and CD3 membrane makers after selectively photobleaching the CD3 conjugated fluorophore with a FITC filter (Figure 3). This demonstrates the control over the experiment that is achievable within a microfluidic environment. Ideally, future experiments will be able to label the cells in less time than incubation requires, by increasing the rate of perfusion. Additionally, in future tests we expect to decrease time for photobleaching to occur by using fluorophore conjugates of Peridinin chlorophyll protein (PerCP), which is highly photobleachable [10]. Other labs are currently working on typing blood cells by using flow cytometry, but are limited by number of markers that can be used, and high costs of running tests [9]. Our research suggests that through our protocol, it can be cost effective to use whole blood for distinguishing between the subtypes of cells caught in the traps.

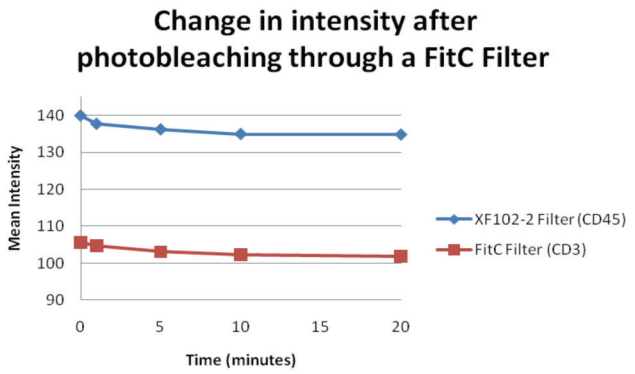

Figure 3. The XF-102-2 Filter (blue) and FITC filter (red) both showed a decrease in intensity overtime.

Our data supports the hypothesis that CD membrane makers, combined with photobleaching, can be used to differentiate between different cell types inside of microfluidic devices. Cells can then be relabeled using the same fluorophore conjugated to a different membrane marker. There is also potential to targeting specific fluorophores to selectively photobleach single types of membrane markers to allow for greater precision in indentifying cell types. These are important advances towards applying these novel technologies to directly observe the dynamic cellular characteristics and responses in trauma patients which would allow us to better diagnose and treat trauma patients.

ACKNOWLEDGMENTS. I would like to thank the VIIBRE and the Searle Systems Biology and Bioengineering Undergraduate Research Experience for providing the equipment and training necessary to conduct my research. I would also like to acknowledge Laura Wertz, graduate student at Columbia University, whose research served as a foundation for the project. I would also like to thank the School for Science and Math at Vanderbilt University and Dr. Vanags for advising me on concerns regarding my research and for providing the necessary preparation for this endeavor.

SUPPORTING INFORMATION.

Figure S1. Microfluidic Device

Figure S2. Device Design.

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