**Abstract**

a

**Main**

Fluorescence microscopy has proved to be invaluable for biological research. However, fluorophores intrinsically have broad excitation and emission spectral bandwidths. This limits the number of unique markers that can be distinguished reliably from each other. Currently that limit is 4 and can be 5 on special higher bandwidth systems. This poses significant limitations on biological studies as biological pathways are highly intertwined and yet for any sample, we can only label 4 of those components at once. A typical strategy to increase marker count is to use different markers on different samples and aggregate their results together. However, this is far from ideal given noise in biological system are high and many results are subject to local properties and conditions of the system. A strategy to further dampen this impact is to use adjacent slices in a tissue sample with different markers. Still at best this is a Riemann sum getting closer to the integral result. Having all markers on the exact same tissue slice would reduce the biological noise to a minimum and thus be a more ideal way to study many biological systems.

Strategies to increase the marker count are referred to as multiplex techniques and can be broken down into three broad categories: mathematical, fluorophore unbinding and fluorophore destruction. The mathematical approach is referred to as compensation and is widely used in FACs in which spectral information is used in conjunction with a mathematical theory to further separate fluorophores from each other (ref 1). This has a cap at around 6-7 unique markers and is thus a light multiplexing technique. Fluorophore unbinding is where in between successive images, fluorophores are unbound and then the tissue is restained with the same fluorophores, but new markers. The leader in this technique is the Codex system (ref 2). That system utilizes a DNA barcode system to bind fluorophores to antibodies. This allows it to specifically cleave certain sequences and add on just a few fluorophores to label known antibodies with the complementary strand bound to themselves per cycle. A second type in this style is to use microwaves to break the bond and effectively strip the antibody complexes off the binding site (ref 3). The last type is where fluorophores are bleached, which permanently puts them into a dark state. This enables one to make successive images with a sample and after destroying fluorescence in between images, they can restain with same fluorophores, but with different markers. The most notable in this style is CyCIF. First discovered by Gerdes (ref 4), it was later refined and expanded by the Sorger lab (ref 5). In this technique, a high pH solution of H2O2 is used to chemically bleach cyanide-based fluorophores. While this limits fluorophore choices, it still leaves enough to generate a full spectrum of dyes.

CyCIF is a very attractive multiplexing strategy due to it being an affordable, well documented protocol that uses easily obtainable reagents. We identified 3 primary areas that CyCIF pose significant barriers and issues to using it. The first is the bleaching solution cannot bleach any widely available and excellent performing dyes in the red range and while the solution effectively bleaches Alexa-488, it takes an hour to accomplish it. Next is due to needing to stain and bleach tissues, the coverslip must taken on and off of the slide. Lastly, the overall labor is high and mostly in the form of numerous small tasks such as solution removals, washes and finding specific fields of view each cycle.

In this paper we present a new microscope that utilizes robotics to drastically reduce the labor burden that the CyCIF technique originally presented and eliminates the need to use coverslips. In addition, we introduce a new bleaching solution that drops the bleaching time from 60 minutes to 5 minutes and expands out the list of dyes that are compatible with it, including better options in the red emitting range.

**AutoCIF Microscope and the Invisi-Slip System**

The coverlsip’s usefulness in CyCIF is to prevent the tissue from drying out. To circumvent the need for a coverslip we developed a water dipping upright microscope which has the tissue always submerged while imaging and boosts the resolution of the system beyond that of air systems. To automate the washes, bleaches and stains that the sample undergoes, we developed a microfluidic system that not only isolates and confines microfluidic volumes directly on top of the tissue, but also enables water dipping imaging to be done on the tissue. We dubbed this microfluidic device, the invisi-slip system as it employs a coverslip as an imaging window which allows the microfluidic volume to be constricted to just over the slide and at the same time is ‘invisible’ when water is both above and below it. To achieve the invisibility aspect of the imaging window, we chose to make it out of FEP film. The invisi-slip device needed to be reversible and still have a good seal on a slide so that it could be reused. Normally, PDMS has a weak Vander Waal based bond with glass. To enhance this bond, we built most of the device out of PDMS mixed with iron oxide dust and built neodymium magnets directly underneath the slide as this tactic has been shown to triple the bond PDMS has with glass (ref). To control the microfluidic system, we used an open-source stepper motor based peristaltic pump (ref), a pinch value system from xxx, and developed a custom Arduino based controller which can control DC and stepper motors and well as interface with the pinch value controller to modulate what valves are pinched or open. We operated the entire system in python and micromanager 2.0 with pycromanager being used as the bridge program between python and micromanager (ref 6).

**Methods**

**In Solution Bleaching Kinetics**

Sodium Hydroxide was used to modulate pH of solutions. 30% H2O2 was used for all dilutions. A **blank** spectrofluorometer was used for all emission spectra measurements. We used **X** slit size and **Y** excitation wavelengths. An excel macro book (ref 7) was used to calc approx. NaOH and H2O2 values to achieve noted values. An intensity decay fit was done to interpolate what the half time of intensity decay was.

**Slide Studies**

We ran several slides through the AutoCIF system with the following dye and cycle combos:. We designated the first cycle to always be the one and only primary secondary cycle. We did used a bleach solution of blank for blank minutes. For analysis we used Ashlar to align cycles with each other (ref 8). We also employed deep learning algorithms (ref 9) to segment a group of stains for cell borders. We then used these segmented cells as masks to plot intensity per pixel of different markers that fell within their borders.

**Results**

CyCIF is an approachable and affordable multiplexing method. Figure 1 goes over the basic workflow of the method. Effectively, tissue sections are stained with markers that have certain fluorophores bound to them plus Hoechst, which is used as a fiducial marker channel to align all cycles up with respect to each other. After imaging, these are bleach with a high pH H2O2 solution until the fluorophores are sufficiently dim. This allows for the cycle to repeated until the desired number of markers is reached. While this is a straightforward process, it poses significant labor. We identified the significant labor barriers as: 1. Imaging tissue in the exact same spot each cycle, 2. Taking coverslips on and off.

To combat barrier 1, we chose an approach where the slide stays stationary on a stage. This is ideal because it removes all human error and reduces the inaccuracies down to the intrinsic error of the stage itself. To do that we needed devices that would be able to do washes, stains and bleaches right onto the stage without moving the slide. Figure 2 details diagrams of the devices that we came up with to solve this. The main features of the drain chamber are that it can accept PBS and the bleaching solution as an input and uses another peristaltic pump as a drain pump. It also has a fluid diffuser which creates a wide fluid front to wash over the tissue on a slide and a gutter system funnels the fluid into the drain point. We found that after the pipettor placed a small volume of stain on the tissue that it would rapidly dry out. We installed a lid that can cover the well and expose it. Lastly, it has leveling screws to ensure the tissue is perpendicular to the optic axis.

The pipettor uses a revolver cylinder to hold 8 syringes. It can swing a single syringe underneath its push rod and push out a known amount of stain solution. The wide range stage allows the sample to be both imaged and driven underneath the staining syringe. All of these features allowed everything that was once done by hand to be done automatically and in place on the stage. Taking a coverslip on a off is a different challenge. Typically, a coverslip is attached with a 10% glycerol solution (ref 5) and it falls off after vertically dipping into a container with PBS. This is a very daunting task to do automatically on a stage. The reason a coverslip was ever needed is to prevent the tissue from drying out. We decided to not use coverslips in our system. It wasn’t ever really needed. Tissue cant dry out if its constantly submersed in PBS. What we did was flood the drain chamber with PBS for imaging and image the tissue with a water dipping objective. Not only did this negate any need for a coverslip, it gave us a boosted numerical aperture for the system due to the increased refractive index.

Previous studies (references) only looked at absorbance of dyes. If a dye is unable to absorb a photon then it 100% cannot emit. This misses effects where the dye can still absorb and yet cannot emit. Additionally, previous studies never justified why certain values of pH and H2O2% are used. In fact Gerdes and Sorger use different values. We measured the intensity half time of Alexa 647 as a function of pH and H2O2 % by independently altering those two variables and summarized the findings in Figure 4a. (make some comments about the finds when I know them).

CyCIF is well established for 4 channels (Hoeschst + Alexa 488 + Alexa 555 + Alexa 647). It could be greatly sped up in a 5th channel were available. Figure 4b shows the half-lives of Alexa 750, Dylight 755 and blank. Make some comments about results when I know them.

We applied this machine to a series of tissue slides to investigate (blank hypothesis). The images each cycle are presented in figure 5.

The resulting data showed blank. We are able to confirm that the segmentation algorithm (ref) was able to reasonably segment cellular borders. Some more words about what the results mean.

**Discussion**

We presented here a novel microscope that greatly