**Auto-CyPlex is an open-source platform to automatically execute bleaching based multiplex methods. This platform introduces two novel elements: a reusable microfluidic device with a viewing window made of FEP film which allows for water dipping imaging and a mCPBA bleaching solution which is 4x and 12x faster than Lithium Borohydride and Hydrogen Peroxide respectively. All construction details and plans on the platform are available at (github address).**

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 mathematical theory to further separate fluorophores from each other (ref 1). The last two methods are based around concept of eliminating fluorescence in between successive images and then restain the tissue with the same fluorophores, but new antibodies. Fluorophore unbinding methods accomplish this by releasing the fluorophore from the sample and washing it away. The leader in this technique is the DNA barcode-based Codex system (ref 2). Fluorophore bleaching methods accomplish it with solutions which chemically react with fluorophores in such a way that they lose the ability to fluoresce. The most notable in this style are the Hydrogen Peroxide (H2O2) based CyCIF and Lithium Borohydride (LiBH4) based IBEX methods (ref 3 and 4).

Bleaching based multiplex methods are attractive from an accessibility viewpoint as they use standard immuno-fluorescence protocols, low-cost reagents, and no proprietary systems such as DNA barcoding. The method’s downsides are being labor intensive and time-consuming which has hindered its wide-spread adoption. To address this, we developed an open-source platform which automates all labor steps and significantly sped the process up with a new ultra-fast bleaching solution.

Automation of our multiplex protocol (Fig 1a) required the Auto Cy-Plex platform to have a fluidic system with an ability to change the dispensed liquid, keep the tissue hydrated to eliminate the need for coverslips, automate all functions of a microscope and perform all these actions while the slide is stationary and on the microscope’s stage. That final point is very important as a significant labor hurdle was returning to the previous imaging regions for every cycle.

To merge all the automation tasks together, we created a reusable micro-fluidic device that is placed on slides and over tissue sections (Fig 1b). The device is 2mm thick and has a built-in viewing window made of FEP film which is within the working distance of high NA, water dipping objectives and the FEP is the exact same refractive index as water which allows for aberration free imaging. To enhance the bonding strength of the PDMS glass interface, we doped the PDMS with iron oxide dust and added magnets underneath the slide. This has previously been shown to enhance the bond strength by upwards of 3 fold (ref).

The chemical reaction that fluorophores, primarily cyanine-based ones, undergo with high pH peroxide has never been published. For the first time, we propose a chemical model for this reaction (Supp Info fig 1). This proposed 2 step reaction is as follows, epoxide formation via a Prilezhaev reaction with H2O2 and alkene groups and subsequent vicinal diol formation of the epoxide after a reaction with hydroxide (ref). While high pH peroxide can certainly undergo the Prilezhaev reaction with alkene groups, it benefits greatly from metal complex catalyzers. A logical alternative to this is a commonly used chemical for this reaction in other settings, M-Chloroperbenzoic acid (mCPBA), which requires no catalyzers.

We conducted a series of experiments of fluorophores in 3 bleach solutions (LiBH4, H2O2, mCPBA) to determine what fluorophores in our panel were susceptible to the bleaching solutions and to quantify their relative speeds (Fig 2a). From this, we found that mCPBA has a Ka that is **4x and 12x** higher than that of LiBH4 and H2O2 respectively. We also identified that Alexa-532 is susceptible to mCPBA and can be used in place of the normally used Alexa-555 dye.

Alexa-488 is the bottleneck in the bleaching process as it takes approximately 12x longer to bleach than Alexa-647. In stained tissue on slide bleaching experiments, we found that Alexa-488 was able to be bleached in **60, 10 and 5 minutes** with H2O2, LiBH4 and mCPBA respectively while Hoescht was relatively unbleached in all solutions (Fig 2b,c).

We ran a 16-stain panel on intestinal tissue using the Auto Cy-Plex system with mCPBA bleaching solution (Fig 2d). The tissue showed no issues with accepting new antibodies each cycle, indicating that epitopes were not being blocked via mCPBA.