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# Fluorescent Detection of Nucleosomes using Functionalized Magnetic Beads on a Digital Microfluidic Device

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## ABSTRACT

Epigenetics, the study of inheritable mechanisms that regulate gene expression, has clinical ramifications from cancer to autoimmune disorders to psychiatric pathologies. The main tool to study epigenetics is chromatin immunoprecipitation (ChIP), which probes the relationship between DNA and its structural nucleosome-forming histone proteins. Standard benchtop ChIP has three major drawbacks: (1) it requires a large input volume of cells, (2) it is very time consuming and work intensive, and (3) it is low throughput. Digital microfluidic biochips (DMFB) have proven to be successful at utilizing small volumes of reagents and samples to perform high throughput bioanalyses and assays of macromolecules. Their ease of configurability, automation, and high sensitivity make them an ideal platform for ChIP adaptation, addressing the three biggest issues facing epigenetic study and workflow. Herein, we demonstrate the first step towards ChIP implementation on a DMFB by detecting specifically modified nucleosomes, the building blocks of chromatin, in a nucleosome immunoprecipitation assay. Using magnetic beads to capture the nucleosomes with magnetic fields generated by embedded current wires and fluorescent conjugated antibodies for detection, this DMFB system allows complete on-chip isolation and detection without the need for external magnets or specialized fluoroscopy equipment. This assay design can be adapted to probe for multiple specific nucleosome modifications, thus establishing a rapid screening method for antibody specificity and sensitivity. Most importantly, this novel confirmatory checkpoint, currently unavailable when running ChIP, ensures that the target analyte has been isolated prior to intensive downstream analyses such as PCR and sequencing.

**Keywords:** Digital Microfluidic Biochip (DMFB), Electrowetting-on-Dielectric (EWD), Chromatin Immunoprecipitation (ChIP), Nucleosome Immunoprecipitation (NuIP), functionalized magnetic beads, Lab-on-a-Chip (LoC), ChIP-on-chip, epigenetics

## 1. INTRODUCTION

Human cells all share the same genotype, or DNA sequence. However, the differences in phenotype, or cell type and function, manifest from the expression or suppression of particular genes[1]. Epigenetics is the study of the inheritable mechanisms and environmental factors that control gene expression in both healthy and diseased states without altering the underlying nucleotide sequence. Epigenetic studies contribute to our understanding of biological systems from the molecular level to the tissue level, within an organism, across a population, and over generations[2-4]. Epigenetic behavior is inheritable but also highly variable. Furthermore, since external environmental factors can alter an individual's epigenome even within a single lifetime, it is important to probe the epigenome at great granularity. Epigenetics has broad clinical ramifications from searching for cell to cell variability in cancer analysis, to looking at the broader effects of the vast medications, products, and therapies currently available, to studying the underlying causes of autoimmune disorders and psychiatric pathologies[1,5-9] .

The main tool to study epigenetics is chromatin immunoprecipitation (ChIP), which probes the relationship between DNA and its structural nucleosome-forming histone proteins, highlighted by the square in Figure 1. However, standard benchtop ChIP has three major drawbacks: (1) it requires a large input volume of cells, (2) it is very time consuming and work intensive, and (3) it is low throughput.

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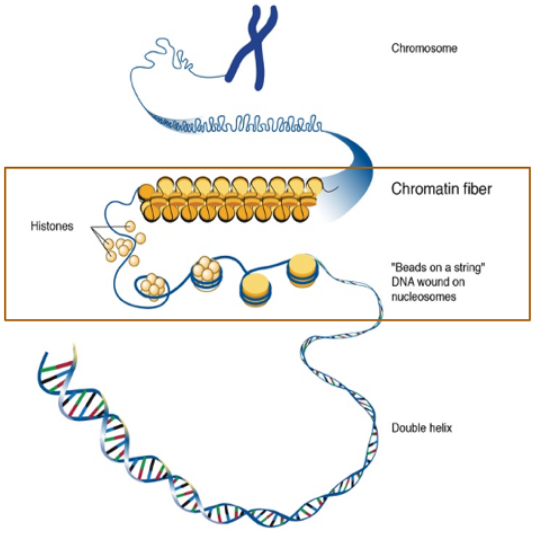


Figure 1. Chromatin structural hierarchy. Nucleosomes and their histone components are indicated in the box[10].

Digital microfluidic biochips (DMFBs) have proven to be successful at utilizing small volumes of reagents and samples to perform high throughput bioanalyses of macromolecules using various assaying techniques. Their ease of configurability, automation, and high sensitivity make them an ideal platform for ChIP adaptation. Droplet manipulation using electrowetting-on-dielectric (EWD) has been demonstrated extensively for moving, merging, splitting, and mixing of droplets. These capabilities, in conjunction with magnetic bead control using magnetic field gradients generated by a current running through a wire on the device, provide all the necessary functionality to successfully run ChIP more efficiently on a DMFB[11-14]. Translation of the benchtop ChIP protocol onto a DMFB will address the three biggest issues facing epigenetic study and workflow. Smaller volumes will reduce reaction time and reagent and sample use, while providing greater granularity and higher resolution when probing a sample. Automation will decrease turnaround and hands-on time for users. Parallel operations and multiplexing will increase throughput. Finally, streamlining ChIP onto one device greatly reduces sample loss, thereby expanding the types of possible studies to include low-yield and rare tissue types. Herein we demonstrate the first step towards ChIP implementation on a DMFB by detecting specifically modified nucleosomes, the building blocks of chromatin, in a nucleosome immunoprecipitation (NuIP) assay.

## 2. EXPERIMENTAL SETUP

### 2.1 Device design and fabrication

As shown in Figure 2, a thin layer of titanium (for adhesion), followed by a thicker layer of copper is sputtered onto a silicon wafer. The device design is transferred onto the wafer using photolithography and the electrodes and current wires are created using wet etching with copper etchant and BOE. Then, using vacuum deposition, Parylene C (the dielectric material) is deposited onto the devices. Finally, a hydrophobic CYTOP® layer is spun and baked on. The top plates are made by coating CYTOP® onto a layer of sputtered ITO (Indium Tin Oxide) on an acrylic substrate. The two plates are joined using a double sided adhesive SecureSeal™ gasket layer. This forms the channel in which droplet and bead manipulation will occur. This channel is filled with silicone oil during operation to prevent droplet evaporation and biofouling of the device surface. Figure 3 shows an image of the current wires and electrodes used for magnetic bead control[14]. Figure 4 is a representative photomask of the DMFB used in this study with labelled components.

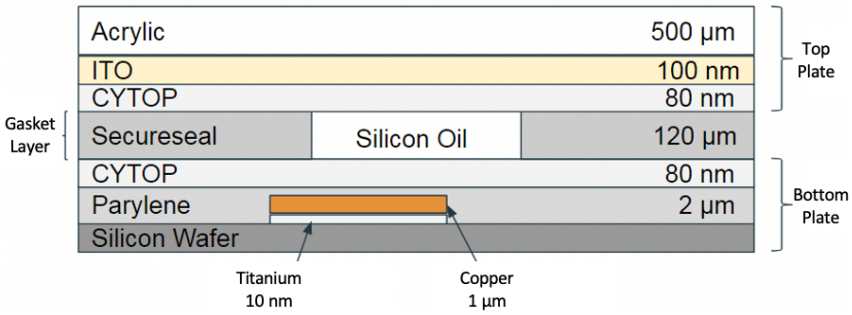


Figure 2. Side view of DMFB.

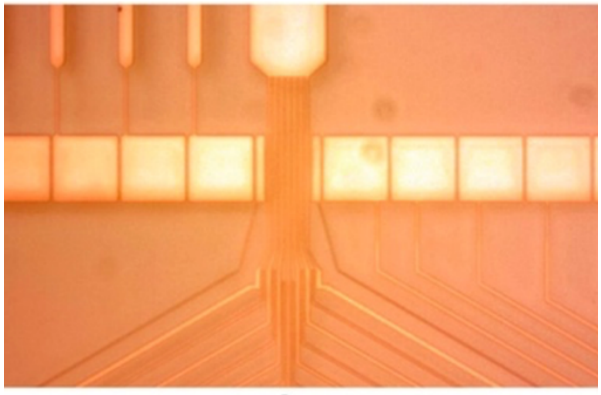

Figure 3. Top view of DMFB. Current wires and electrodes are shown[14].

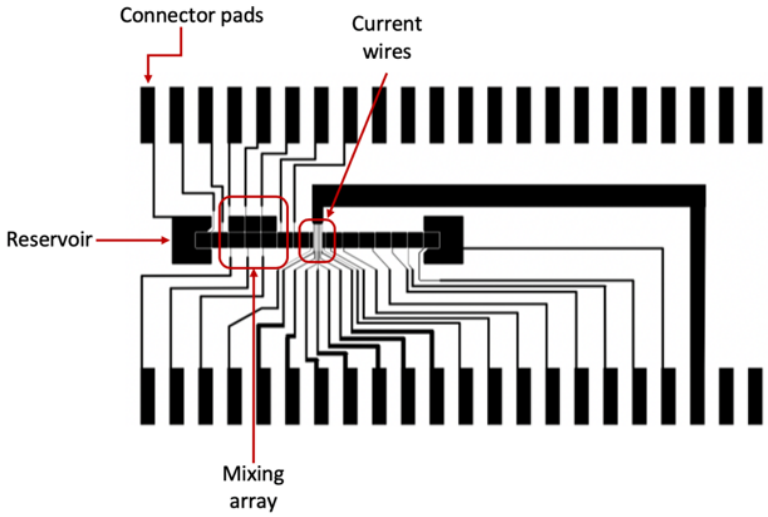


Figure 4. Photomask of DMFB with labelled components.

### 2.2 Electrical control and fluorescence imaging setup

There are two control systems, one for the voltage across the electrodes and another to regulate the current supply to the wires. A waveform generator coupled with an amplifier provide the necessary voltage to a switch array. Meanwhile, a multichannel optical relay passes current through the wires to generate a magnetic field. These controls are connected to the device via two clips attached to a metal stage. This stage acts as both a point of connection and as a heat sink for the device[14]. Figure 5A shows the microscope used to view droplet movement, the metal stage (shown also in 5B holding a device), and the connectors to the voltage and current supplies. Figure 5C shows the PCB (printed circuit board) at the top, which controls EWD actuation, and the optical relay circuit that provides the current for magnetic field generation.

For fluorescence detection, a mounted LED fitted with a bandpass filter of the appropriate wavelength for the fluorescent antibody is set up at an empirically determined incident angle to the metal stage to excite the sample. A long pass emission filter is placed at the end of the microscope just before the CCD camera, which is used to collect images and videos. Figure 6 shows the fluorescence imaging and detection setup. For the current fluorophore used, a 405 nm LED fitted with a 395 nm bandpass excitation filter and focusing lens is used to excite the detection antibody. The Alexa Fluor 405 conjugated antibody has an emission wavelength of 421 nm. This signal is collected using the microscope shown in Figure 5A fitted with a 420 nm longpass filter and coupled with a CCD camera.

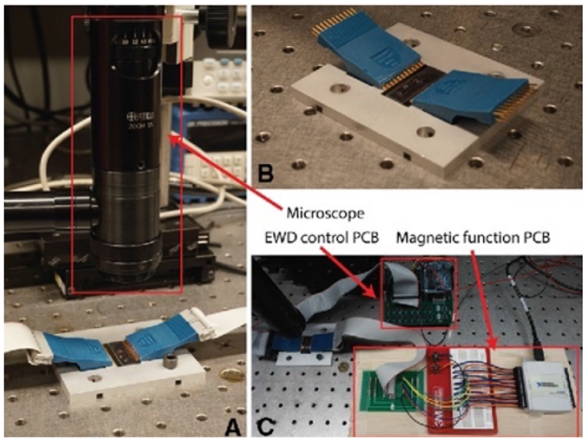


Figure 5. Electrical control setup[14]. (a) Imaging microscope and connected metal stage. (b) Metal stage loaded with device. (c) EWD control PCB and magnetic function PCB.

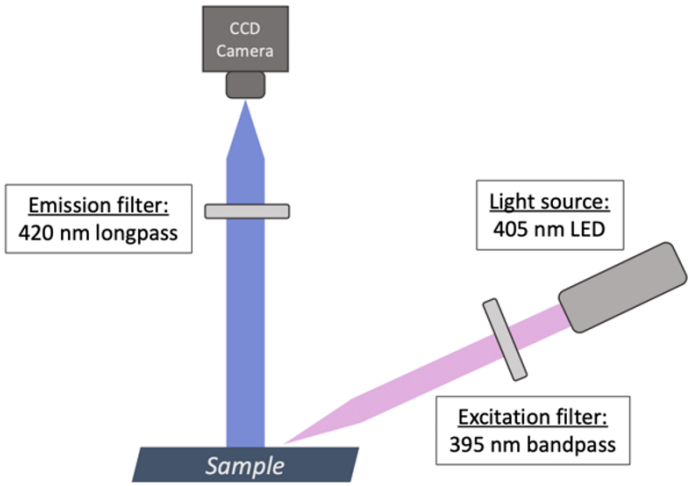


Figure 6. Fluorescence imaging and detection setup.

### 2.3 Nucleosome Immunoprecipitation (NuIP) Assay

A preliminary benchtop assay was developed to detect the presence of biotinylated nucleosomes. A purified, biotinylated, human nucleosome sample of H3K4me2 (dimethylated 4$^{th}$ lysine on Histone 3) was mixed with streptavidin coated magnetic beads and incubated for 6 hours with agitation at 4°C. Simultaneously and under the same conditions, but separately, anti-H3K4me2 (an antibody raised in rabbit against the nucleosome) was incubated with Alexa Fluor 405 Goat Anti-Rabbit secondary antibody. After washing the beads, the detection complex was added and incubated again. After a final wash, the beads were re-suspended, and a drop was placed atop a device and imaged. Figure 7 shows the workflow for this assay protocol.

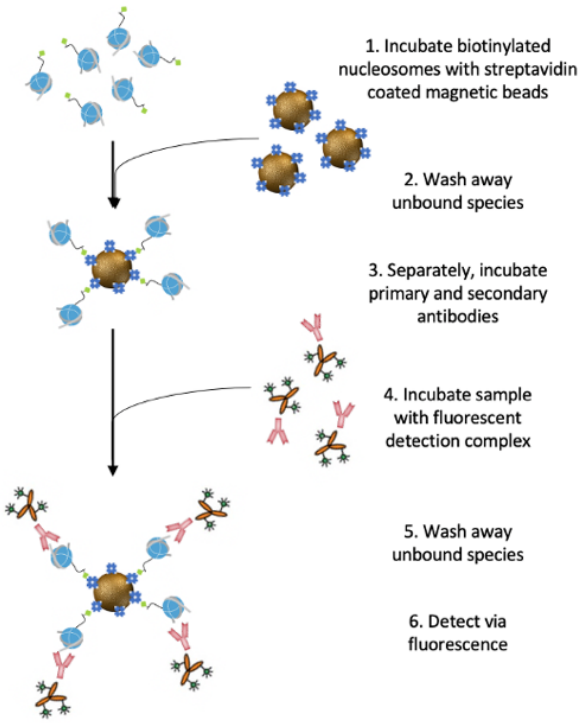


Figure 7. Nucleosome Immunoprecipitation assay schematic.

# 3. RESULTS

## 3.1 Nucleosome Immunoprecipitation (NuIP) Detection

After completing the protocol described in section 2.3, two droplets were sequentially placed onto a device and imaged using the setup shown in Figure 6. The negative control droplet that did not contain any nucleosomes is shown in Figure 8a and the nucleosome-containing sample droplet is shown in Figure 8b. The effect of an external magnet to show that the beads, not the supernatant, are fluorescing is shown in Figure 8c. The same control and sample were then deposited into the reservoir of a DMFB. Figures 8d and 8e show that the sample was manipulated and transported on a DMFB using EWD and on-chip current mediated magnetic control. This assay will be used to test functionality, mixing schemes, and binding efficiency on the fabricated devices. Furthermore, this has established a screening protocol for antibody compatibility and sensitivity.

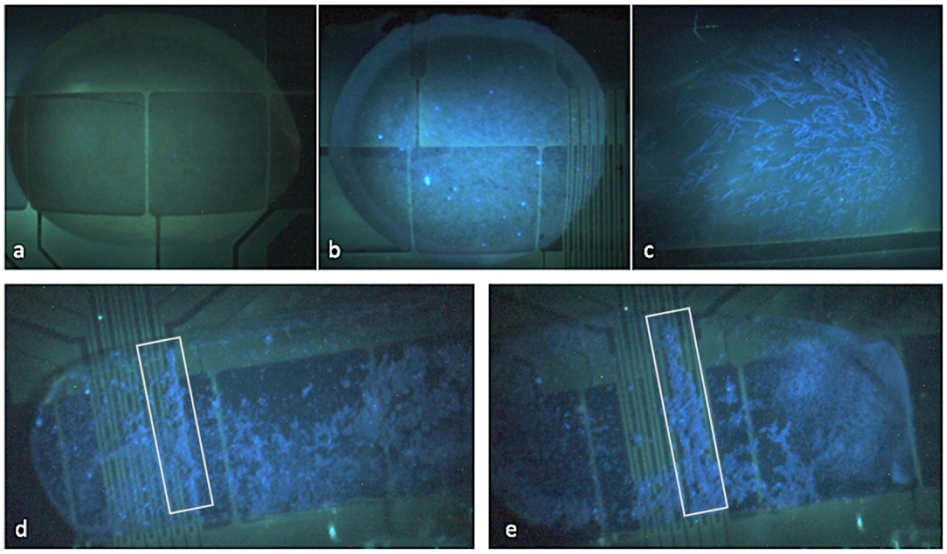


Figure 8. NuIP assay results. (a) Negative control (b) Nucleosome containing sample (c) Sample in presence of external magnet (d,e) Electrowetted sample droplet and current wire magnetic activation. The white rectangles indicate magnetic accumulation of beads on a current wire.

## 3.2 Nucleosome Immunoprecipitation (NuIP) Quantification

Next, the same protocol outlined in 2.3 was used to develop a semiquantitative method of analysis. A series of samples ranging from 0-20 μL of stock H3K4me2 biotinylated nucleosomes were prepared, isolated, and introduced into the DMFB reservoirs. A single droplet was generated and electrowetted to the current wires, where the beads were concentrated onto a single wire. Next, an image was taken and split into its separate color channels using ImageJ. For each concentration, using the blue channel image, equal rectangular areas in the same location were used to analyze the pixel intensity average over that area. These values, normalized using the control droplet, are shown in the graph below. From this preliminary data, we can ascertain that the linear range of this assay is between 0-10 μL of stock H3K4me2 nucleosomes. Figure 9 shows the image analysis protocol. This method was used to collect the data shown in Figure 10.

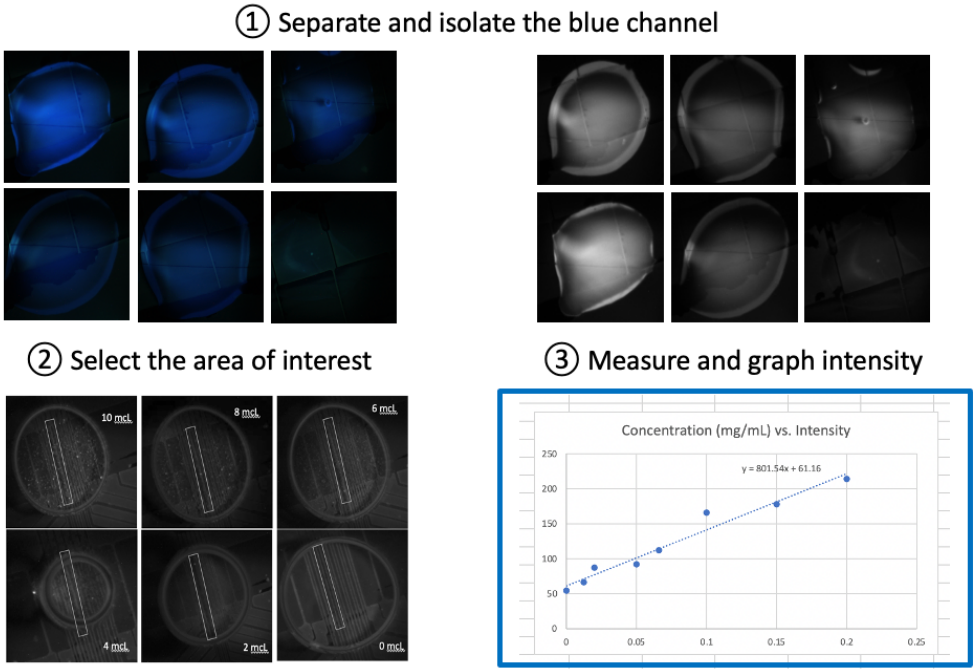


Figure 9. NuIP image and quantitative analysis method.

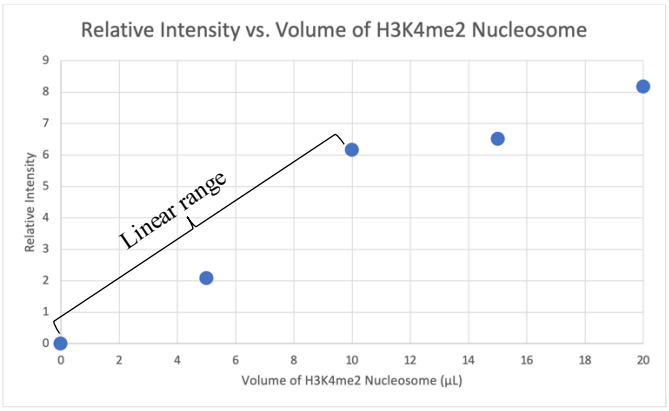


Figure 10. NuIP Assay results. Relative intensity vs. volume of H3K4me2 nucleosomes in sample.

## 4. CONCLUSION

Herein, we demonstrated the first step towards ChIP implementation on a DMFB by detecting specifically modified nucleosomes, the building blocks of chromatin, in a nucleosome immunoprecipitation assay. Using magnetic fields generated by embedded current wires and fluorescent conjugated antibodies for detection, this DMFB system allows complete on-chip isolation and detection without the need for external magnets or specialized fluoroscopy equipment. Furthermore, imaging after amassing the analyte-bound beads onto a single current wire allows for single-channel image analysis on pixel intensity. This is the first step towards developing a semiquantitative method using a standard curve to determine the concentration of analyte in a given sample. This assay design can be adapted to probe for multiple specific nucleosome modifications, thus establishing a rapid screening method for antibody specificity and sensitivity. Most importantly, this novel confirmatory checkpoint, currently unavailable when running ChIP, ensures that the target analyte has been isolated prior to intensive downstream analyses such as PCR, gel electrophoresis and sequencing.

## ACKNOWLEDGEMENTS

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