Honours Project Report

Epigenetic Profiling on an Array of Nanochannels

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Acknowledgment

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Last but not least, I am very grateful for the constant encouragement and support that my family and girlfriend have provided me throughout the three years of my undergraduate studies. Their support has allowed me to climb to a greater height.

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List of Abbreviations

DNA - Deoxyribonucleic acid

BSC-seq – Bisulphite conversion followed by sequencing

ChIP-seq – Chromatin Immunoprecipitation followed by sequencing

PDMS – Polydimethylsiloxane

AFM – Atomic Force Microscopy

SEM – Scanning Electron Microscopy

DWL – Direct Writing Laser

PIPES - Piperazine-N,N′-bis(2-ethanesulfonic acid)

KCl - Potassium Chloride

NP40 - Nonidet P-40

Dd H20 - Deionized (DI) water

DTT – Dithiothreitol

PMSF – Phenylmethanesulfonylfluoride

SDS - Sodium Dodecyl Sulfate

EDTA - Ethylenediaminetetraacetic acid

Tris - Tris(hydroxymethyl)aminomethane

HCl - Hydrogen Chloride

NaCl - Sodium Chloride

PBS - Phosphate Buffered Saline

Summary

DNA sequence alone cannot explain why some genes are displayed while others are not. Epigenetics information gives the extra insight on gene expression. Hence, epigenetics modification has been increasingly studied[1]. The current methods to extract epigenetics information from living organism are known as BSC-seq[2] and ChIP-seq[3]. These mentioned methods require a large quantity of cells and time-efficient computation method to sieve out the essential epigenetics information. Hence, in this thesis, a proposed method that uses lesser quantity of cells was tested to extract epigenetics information from a living organism.

The method was split into two parts to investigate the possibility of using an integrated micro & nanofluidic device to map an epigenetic profile of a living organism. In the first part, extraction of chromatin from a single cell was investigated and tested while in the second part, linearization of chromatin in a nanofluidic device was investigated before combining both the microfluidic & nanofluidic device into the integrated device.

In the first part, the chromatin can be successfully extracted from a single cell and pushed to the other end of the microfluidic device. However, linearization of chromatin proved to be difficult as will be explained in the latter part of the thesis - development of a new chip design was done to provide a better platform for the linearization of chromatin. Optimization of the certain procedures such as the amount of cell, chromatin and chemical was done to improve the process.

Following the project, the new nanofluidic device will be tested and it is highly expected that the linearization of the chromatin can be achieved. This will provide us a bigger step forward such that in the near future, the marking of living organism's epigenetic profile in the integrated micro & nanofluidic device can be done.

1 Introduction

The answer to how living organisms are formed lies in the extraordinary molecules called deoxyribonucleic acid (DNA). DNA was recognized in the twentieth century[4] as the fundamental heredity material in all living organisms that holds large information that are related to various biological traits. Since the discovery, different technologies such as gel electrophoresis, recombinant DNA technology and polymerase chain reaction (PCR) have been developed to extract the genetic information from the DNA so that living organisms can be better understood.

In recent times, there is a limitation to the information that pure DNA sequence can convey to us. Hence additional information is required for us to understand more about living organisms. The additional information is those encoded "on top" of the DNA which do not include the modification to the underlying sequence such as translocation, insertion or deletion. The additional information includes complex and dynamic physical and chemical conditions of chromosomes and structure that encloses the DNA and are significant in the regulation of cellular processes from transcription to translation. All these information are known as epigenetics modification[5] and they might provide us the right information to have a better understanding of living organisms.

In this thesis, the layers of epigenome will be discussed first, followed by the motivation and objectives of the project. After which theories related to the experiment will be discussed followed by the result and discussion on the experiment of using array of nanochannel also known as integrated micro & nanofluidic device to identify epigenetic modification profile on mice cell.

1.1 Layers of Epigenome

Genomes of living organisms are packed into a small nucleus through the series of hierarchical layers. It can be seen from Figure 1.1.1 on how human DNA length of about two metre long[6] is packed into a nucleus of diameter about 6μm[7]. The structural and chemical changes of these hierarchical packing layers influence gene activity and numerous cellular activities.

The first layer of compaction comes about when roughly 146 base pairs of DNA are wound around a protein octamer to form nucleosomes. The protein octamer consists of eight histones

(two of each histone type H2A, H2B, H3 and H4). The underlying DNA and histones can be subjected to modification which will change the DNA accessibility for transcription also known as gene expression. One such modification is DNA cytosine methylation, in which a methyl group is added to the fifth carbon residue of cytosine and this has an effect of reducing gene expression; typically occurring at Cytosine Guanine Site. Another example of first layer epigenetic modification is the exchange of histone with variant type such as H2A.X, H2A.Z and H3.3. These epigenetic modifications coupled with its location and timing will define the structure of the chromatin and epigenetic state which will decide the accessibility of the gene that leads to whether or not a gene is expressed.[8]

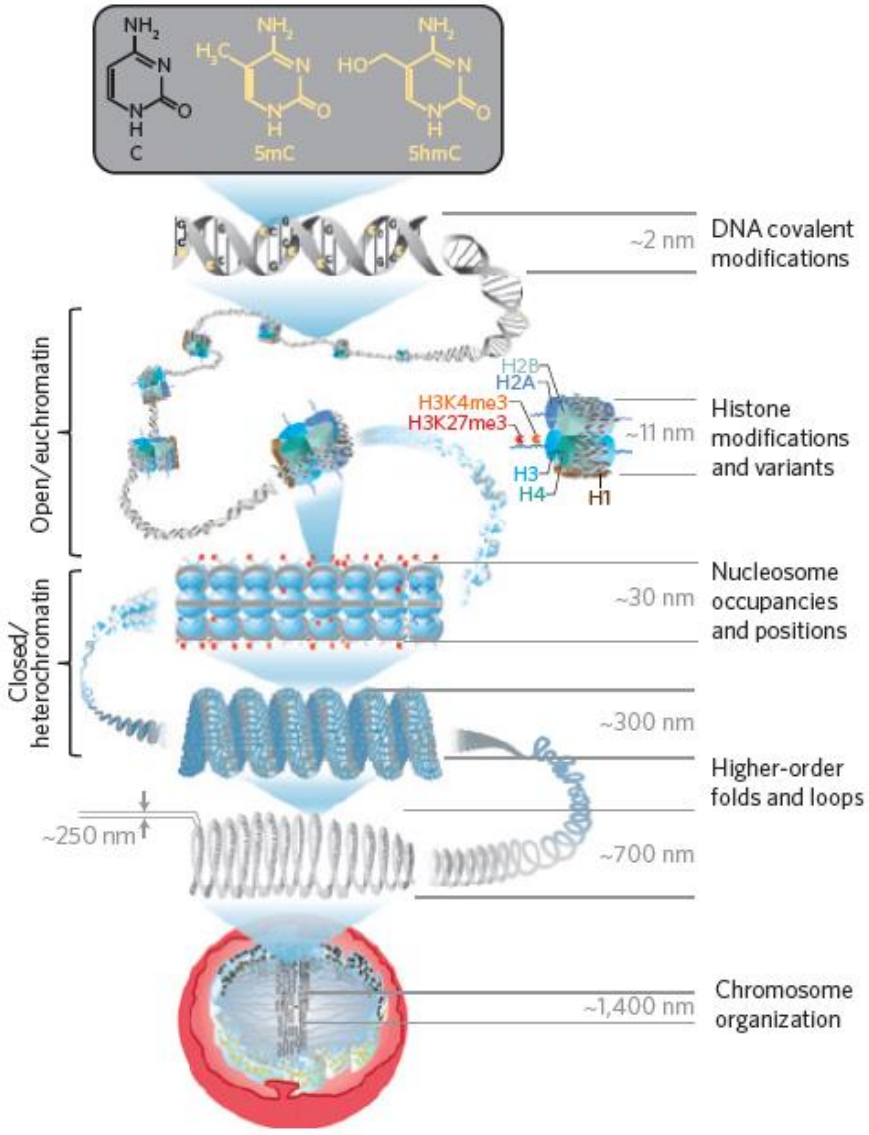


Figure 1.1.1 Outline of layers of epigenomes that highlights the different types of epigenetics modification that could happen in a living organism and also its corresponding size scale.[8]

The accessibility of the chromatin is determined by its form (open/euchromatin or closed/heterochromatin) which can be seen in Figure 1.1.1. In the open form, the chromatin is much more accessible for DNA-binding proteins and polymerase and naturally gene rich areas tend to be packaged in euchromatin. In the other form, the heterochromatin form, it usually contains chromatin that has sequences that are non-coding and repetitive sequence. It contains fewer genes than euchromatin form. Heterochromatin is usually packed into condensed structure as they are not as useful as euchromatin and it will save lots of space in the nucleus.[8]

Epigenetics state can be described by the chromatin packaging structure and transcriptional activities affected by the modification. Chromatin is dynamic as it will adjust itself according to environmental influences such as stress, nicotine, infection and carcinogen.[9] Chromatin structure has higher order of folding and loops which will influence how it is packaged in a nucleus. In this thesis, the epigenetics modification of concern will be those from the first layer which is the DNA methylation and histone modification.

1.2 Motivation and Objectives

From the layer of epigenomes above, it can be seen that epigenetic modification influences transient activation and repression of gene expression and various cellular activities. DNA cytosine methylation and several histone modifications are some of the examples of epigenetics modification that regulates the development of living organisms. These epigenetics modification change the gene accessibility in the chromatin.

These stated epigenetic patterns also control drimental cellular activities such as cancer. One such example is the pattern of hypermethylation of cytosine and guanine dense region in different types of cancer cell such as breast cancer, colon cancer and glioma cancer. Cytosine and Guanine dense sequence was largely unmethylated but hypermethylated was found in nearly every tumour type. Differences between a normal and cancer cell can also be seen in the hostone modification as many tumour suppressor gene in a cancer cell undergoes chromatin remodelling due to modification in histone which lead to peculiar gene expression and tumorigenesis. It is of no surprise that epigenetic plays a huge role in cancer suppressor or activation and characterizations of these important epigenetic marks have been developed.[8]

The two technologies that are currently used to generate epigenetics profile are bisulphite conversion followed by sequencing (BSC-seq)[1] and chromatin immunoprecipitation followed by sequencing (ChIP-seq).[2] These two technologies provide detailed profile of epigenetics modifications but large amount of cells are required to determine statistically significant result from the background. In addition, computational limitation is associated with the mapping as time efficient computational method are required.

Therefore, the first objective for this thesis is to develop a relatively inexpensive and simple single cell analysis method i.e. integrated micro & nanofluidic device to profile the epigenetic modification that can reduce the required number of cells to one while achieving a high precision of epigenetic profile. Optimization of the procedure will help to achieve a simple and efficient methodology. The schematic result to achieve is shown in Figure 1.2.1.

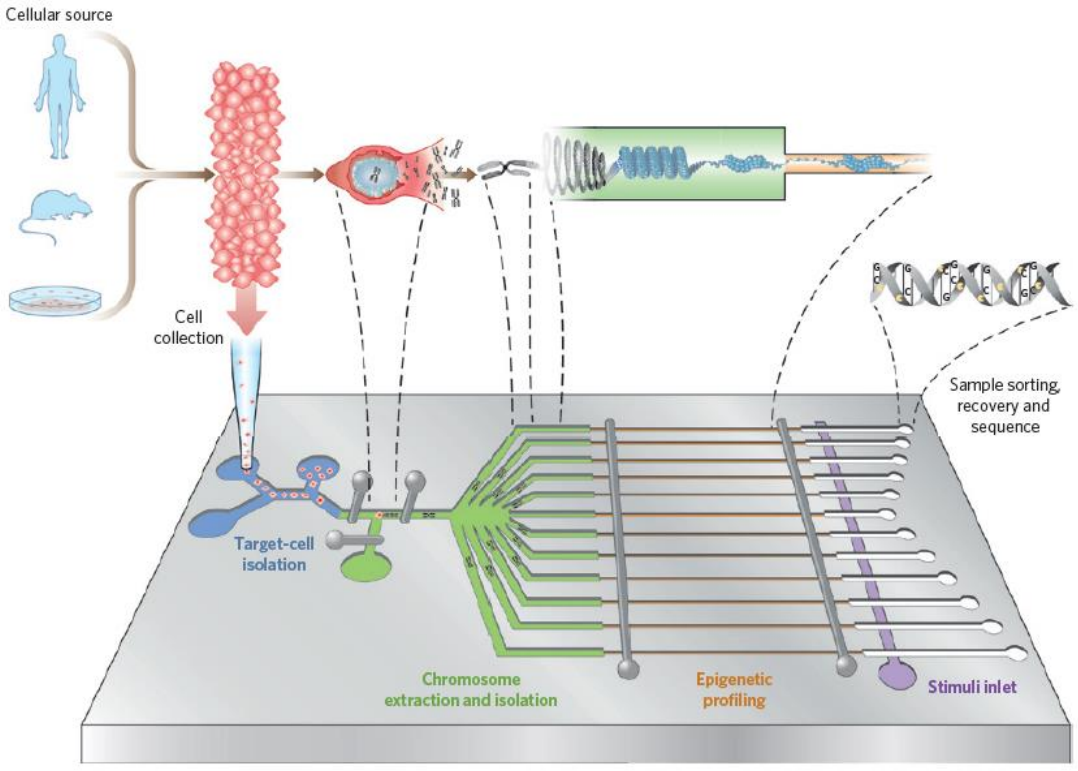


Figure 1.2.1 Idealised integrated micro & nanofluidic device to linearize the chromatin extracted and isolated from a single cell before treatment such that its epigenetic profile can be visualize.[8]

The proposed method is to use lithography fabricated integrated micro & nanofluidic device to manipulate the cell and extract the chromatin following by linearizing the chromatin in a nanochannel for optical detection. Pure DNA could be linearized in nanochannel and for the case of chromatin, it can also be done.[8,11,12,13,14,15] Details on how the proposed method was

carried out will be covered in Chapter 2. The theoretical references such as linearization of polymer and effect of salt concentration on effective diameter of polymer are shown in the following section.

1.3 Theory

The theory is adapted and summarised from Introduction to biopolymer physics (Maarel, J. R. C. v. d. ,2008), reference 10. Refer to Introduction to biopolymer physics (Maarel, J. R. C. v. d. ,2008) for the detailed derivation.

1.3.1 Polymer Conformation

Polymer such as DNA, proteins and polysaccharides can be described by a long thin elastic strand that obeys Hooke's elasticity law under small deformation. This model is known as worm like chain model. This model assumed the polymer to be smooth and continuous by giving the step length, *l* a value very close to zero while the numbers of segment, N approach infinity.

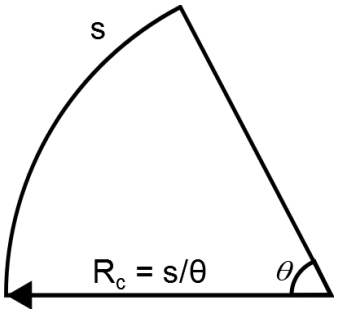


Figure 1.3.1 An elastic filament of length *s* and curvature *θ/ s*

For the elastic filament as shown in Figure 1.3.1 above and with Hooke's law, the elastic bending energy can be derived as shown in the equation below.

$$\Delta U = \frac{1}{2} s k_b \left(\frac{\theta}{s}\right)^2$$

With *s* – length of the filament, k_b – bending rigidity constant and θ – bending angle.

The directional correlation of the worm like chain can be expressed in terms of an exponential of the distance from the current location and persistence length as shown in the equation next page. It can be observed from the equation that the directional correlation will be lost when the length scale exceed the persistence length.

$$\langle cos\theta(s)\rangle = \exp\left(-\frac{s}{L_p}\right)$$

For small s, the LHS and RHS of the above equation can be expanded to the second term as shown below.

$$\langle cos\theta(s)\rangle = 1 - \frac{1}{2}\langle\theta^2(s)\rangle + \cdots$$

$$\exp\left(-\frac{s}{L_p}\right) = 1 - \frac{s}{L_p} + \cdots$$

By taking the thermal average of bending angle, the second term from the directional correlation expansion can be found as follow. The factor of 2 justified the bending of the filament in both directions.

$$\langle\theta^2\rangle = 2\frac{\int \exp\left(-\frac{\Delta U}{kT}\right)\theta^2 d\theta}{\int \exp\left(-\frac{\Delta U}{kT}\right)d\theta} = 2\frac{s}{k_b}kT$$

From the three equations before, persistence length is set to be,

$$L_p = \frac{k_b}{kT}$$

Hence for the case of DNA, the persistence length is around 50nm.

The persistence length and directional correlation can be used to calculate the mean square end to end distance of a worm like chain.

$$\langle h^2\rangle = \int_0^L ds \int_0^L ds' \langle\vec{l}(s)\cdot\vec{l}(s')\rangle = 2\int_0^L ds \int_0^{L-s} dt \langle cos\theta(t)\rangle = 2L_p^2\left[\frac{L}{L_p} - 1 + \exp\left(-\frac{L}{L_p}\right)\right]$$

Where t=s'-s

By limiting the mean square end to end distance to the two special cases of total length, $L<<L_p$ and $L>>L_P$, it can be observed that the worm like chain behaves like a rigid rod when $L<<L_p$ and as a Gaussian coil when $L>>L_p$.

$$\langle h^2\rangle = L^2\ for\ L \ll L_p\ (rod)$$

$$\langle h^2\rangle = 2LL_p\ for\ L \gg L_p\ (coil)$$

1.3.2 Confinement of Polymer in a Tube

This section is of the most interest for this project as the size and length of a confined polymer in a tube is being derived. There will be two types of tube being considered here, first a tube with its diameter, D larger than the persistence length of the polymer while the second case is the case in which the persistence length is larger than the diameter of the tube.

For the first case where $D>>L_p$, the monomers inside the length scale of the diameter, D are not affected by the confinement such that they behave as if the tube is not there. With this, the diameter that is not affected by the tube is D and it can be defined as below,

$$D \simeq \lg^{\left(\frac{3}{5}\right)}$$

Where g is the number of link in the blob contained by the tube with diameter D and 3/5 is taken such that the chain can be swollen due to excluded volume interaction.

With the above equation, the extension can be found using the total number of links, number of links in the blob and the diameter of the tube as follow,

$$R = \frac{N}{g} D \simeq Nl\left(\frac{D}{l}\right)^{-\frac{2}{3}}$$

The polymer in the second case of $D<<L_p$ will bend whenever it reaches a wall as shown in Figure 1.3.2. For small deflection angle, θ, it is related to the deflection length and diameter of the tube through the equation below,

$$\theta \simeq \frac{D}{\lambda}$$

Following the next two equations, where the deflection length, λ=s, the extension of the chain with contour length L can be derived as shown in the third equation on the next page.

$$\langle \theta^2 \rangle = \frac{2s}{L_p} = \frac{2\lambda}{L_p} \simeq \left(\frac{D}{\lambda}\right)^2$$

$$\lambda \simeq D^{\frac{2}{3}} L_p^{\frac{1}{3}}$$

$$R = L\langle cos\theta\rangle \simeq L\left(1 - \frac{1}{2}\langle\theta^2\rangle\right) \simeq L\left(1 - \left(\frac{D}{L_p}\right)^{\frac{2}{3}}\right)$$

1.3.3 Effective diameter of polymer with total excluded volume

Interaction energy of two segments can be split into two parts as follow,

$$U = U_0 + U_e$$

Where U_0 is the hard core interaction energy and U_e is the electrostatic interaction energy. Consequently, the excluded volume can also be split into two parts,

$$\beta = \beta_0 + \beta_e$$

The two parts can be derived as follow,

$$\beta_0 = \int_{Vol\ Overlap} d\vec{r} = 2l_k^2 D_0 \int_0^{\frac{\pi}{2}} d\phi (sin\phi)^2 = \frac{\pi}{2} l_k^2 D_0$$

$$\beta_e = \int_{Vol\ Non-Overlap} d\vec{r}\,\langle 1 - \exp\left[-\frac{U_e}{kT}\right]\rangle = 2l_k^2 \int_0^{\frac{\pi}{2}} d\phi (sin\phi)^2 \int_{D_0}^{\infty} dR\left(1 - \exp\left[-\frac{U_e}{kT}\right]\right)$$

$$= \frac{\pi}{2} l_k^2 \kappa^{-1}\left(ln w' + \gamma - \frac{1}{2} + ln2\right)$$

Where,

$$w' = 2\pi v_{eff}^2 l_B \kappa^{-1}$$

$l_k - kuhn\ segment$
$D_0 - bare\ diameter$
$\kappa^{-1} - screening\ length$
$\gamma - Euler's\ constant, 0.57721$
$l_B - Bjerrum\ length$
$v_{eff} - effective\ number\ of\ charge\ per\ unit\ length$

Total excluded volume can be written as a function of an effective diameter as follow,

$$\beta = \frac{\pi}{2} l_k^2 D_{eff}$$

And the effective diameter will be,

$$D_{eff} = D_0 + \kappa^{-1}\left(ln\varpi' + \gamma - \frac{1}{2} + ln2\right)$$

It can be seen from the equation above that the effective diameter is dependable on the screening length and the effective number of charge per unit length through the parameter ϖ'. This tells us that the diameter will depend on the salt concentration as the screening length and effective number of charge per unit length is dependable on the salt concentration. For a quick verification, the effective diameter will reduce to its bare diameter if it is in a high salt concentration which is true as in the following statement.

$$D_{eff} = D_0 + \frac{1}{\sqrt{8\pi l_B \rho_s}}\left(\ln \varpi' + \gamma - \frac{1}{2} + ln2\right)$$

$$D_{eff} \approx D_0 \; for \; \rho_s \gg 1$$

The effect of salt concentration will help us to estimate the concentration of the buffer such that the chromatin can get into the nanochannel.

2 Experimental Methodology

The project aim is to come up with the integrated micro & nanofluidic device for single cell analysis that replicates the one shown in Figure 1.2.1 in which cells extracted from a living organism are inserted into the integrated micro & nanofluidic device, made from Polydimethylsiloxane (PDMS) casting. After which the chromatin will be extracted from the cells for further treatment before it is linearized and the profile of its epigenetics marks are taken. Therefore this project was split into three components so that each individual component can be monitored closely and altered easily when changes are needed before all the components are merged to produce the integrated system.

The three components are as follows, 1) Manipulation of cells in microfluidic device, 2) Linerization of chromatin in nanofluidic device and 3) Optimization of procedure for a simple and yet efficient process. In this experiment, the visualization device used is the Nikon microscope attached with an ANDOR scientific camera with a 100x oil immersion objective lens and a set of filters such that the visualization of cell, chromatin and fluorescence particle can be done.

2.1 Manipulation of cells in microfluidic device

Firstly, the sample used in this experiment was prepared cultured mice cells. The mice cells were stained with YOYO-1 fluorescence dye and incubated for ten minutes before they were injected into the microfluidic device shown in the Figure 2.1.1. (The microscope image of the microfluidic device and some of its dimension can be seen in Appendix A). The development and preparation of microfluidic device is shown in Appendix B. After the cells were injected into the device, the micro injector was utilised to provide pressure to push the cells from the reservoir to the preferred location which is labelled as location 1 in Figure 2.1.1. After which, the cells were drawn into the side channel by applying a low pressure at the other end of the side channel's reservoir.

When the cell was situated at location 2 as shown in Figure 2.1.1, lysis solution from SIGMA-ALDRICH was introduced through the buffer reservoir so that the genetic material, chromatin, can be extracted for the other steps. Evident of high elasticity of chromatin was observed and this is partially due to the presence of actin in the chromatin. Hence, demecolcine was added into the device to depolymerise the actin fibre so to increase the

mobility of the chromatin such that chromatin can be easily manipulated. At the same time, antibodies with fluorescence dyes was added into the chip so that the specific binding site of epigenetics modification will bind with the antibodies and it can be visualized with the fluorescence dye. The antibody used in this experiment is Histone H3 Antibody with Alexa Fluor Conjugate.

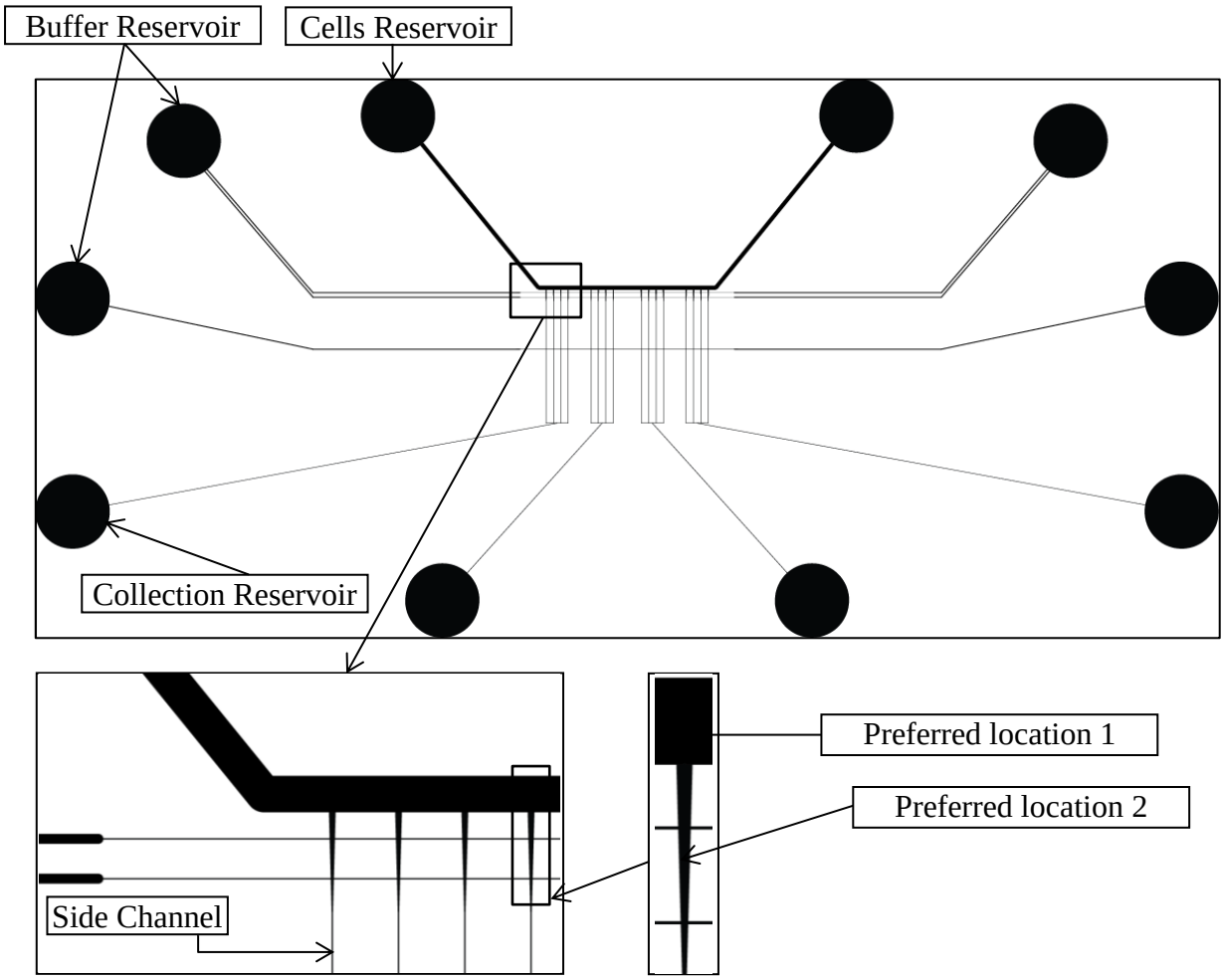


Figure 2.1.1 Microfluidic device used in experiment with its design enlargement and the details of preferred cell locations.

Mice cell chromatin is very long as there is roughly 3 billion base pair in its heredity material.[11] Consequently, enzyme is be needed to cut the chromatin into smaller pieces before the fragmented chromatin can be relocated to the nanochannel. The two types of enzymes used are fastdigest EcoRI and NruI restriction enzyme.

2.2 Linearization of chromatin in nanofluidic device

Two methods of extracting chromatin from cells before it was injected into the nanofludic device were attempted. The first method was to lyse the mice cell in test tube and introduce the two fast digest restriction enzymes mentioned in the previous section to nick the chromatin to smaller fragment. The second method was to prepare the chromatin according to the procedure shown in Appendix C and this was done by Dr Abdollah. The first method will introduce other material beside the chromatin i.e. the broken cell membrane while the second method will eliminate other material except the chromatin before the chromatin is introduced to the nanofluidic device.

After the mice chromatin was prepared with either method, it will be stained with YOYO-1 fluorescence dye and incubated for ten minutes. In addition, it was also treated with demecolcine to depolymerise the actin fibre.

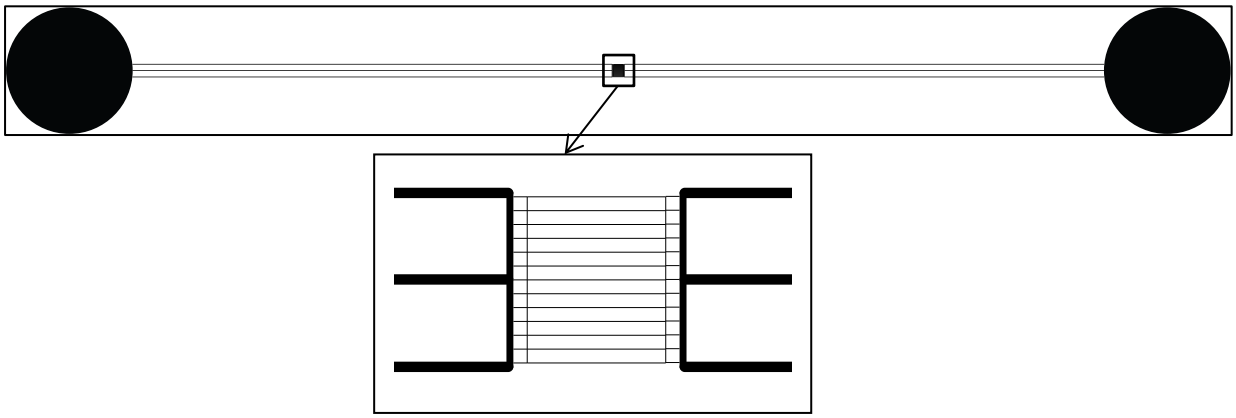


Figure 2.2.1 Nanofluidic device used in experiment with its design enlargement.

After the treatment, the chromatin was injected into the nanofluidic device as shown in Figure 2.2.1 above (The microscope image of the nanofluidic device and some of its dimension can be seen in Appendix A). By applying the right concentration and right pressure or electric field to the nanofluidic device, the chromatin will be able to get into the smaller channel. Two types of nanofluidic device were used in this section, a 500nm by 500nm nanochannel and a 250nm by 250nm nanochannel. Different dimensions of nanofluidic device were used to linearize the chromatin as the dimension in which the chromatin is linearized into the suitable length for epigenetic profiling was uncertain.

2.3 Optimizing of procedure for a simple and yet efficient process

Several optimizations were done in this experiment such that the whole process can be improved and simplified. The following list presents the optimizations done in this experiment which will be discussed more in Section 3.3

- Designing of new chips to allow a better manipulation of cells
- Optimization of the amount of YOYO-1 fluorescence dye
- Optimization of the concentration of chromatin and cells to provide a good control
- Optimization of the amount of demecolcine used for chromatin and cells

3 Experimental Results and Discussion

3.1 Manipulation of cell in microfluidic device

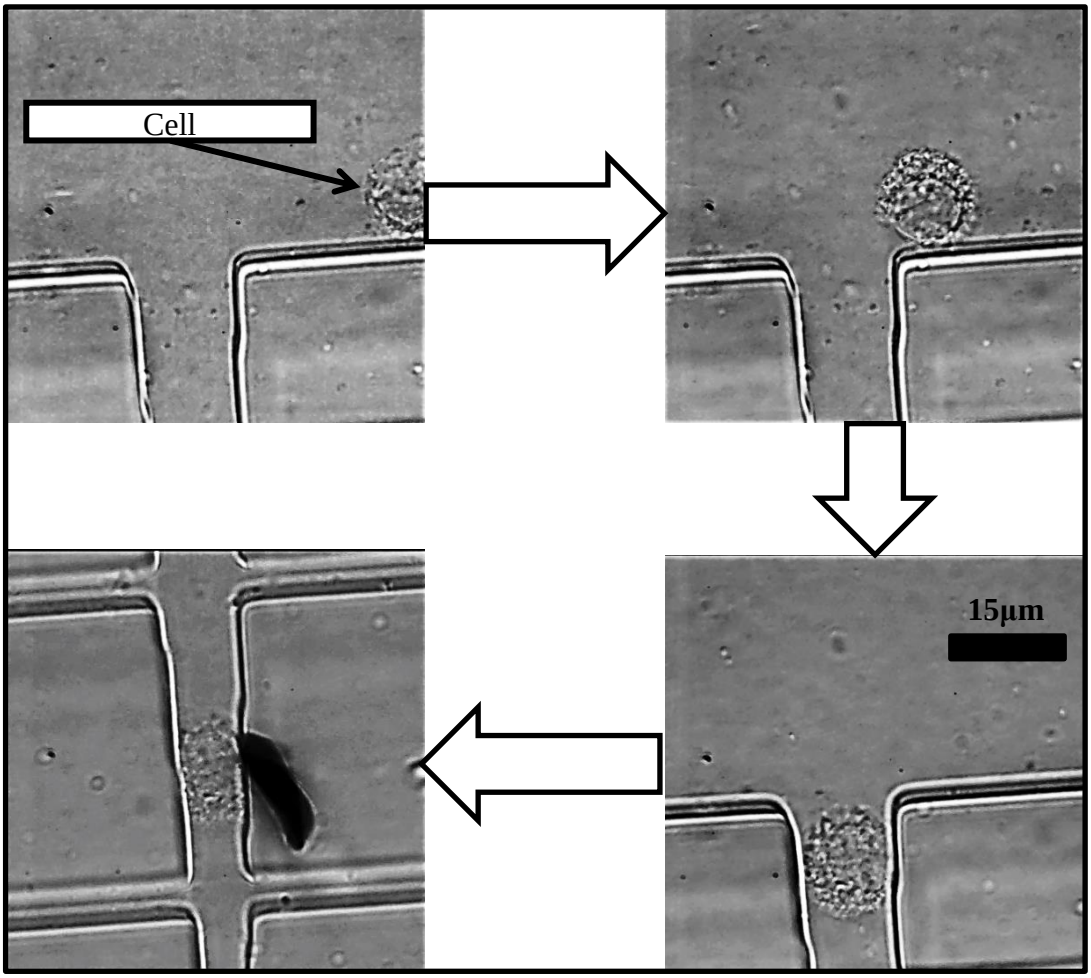


Figure 3.1.1 Process of getting a cell into the side channel

From Figure 3.1.1 above, it can be seen that the cells can be effortlessly pushed to the preferred location 1 and 2 shown in Figure 2.1.1 for the planned procedure such as cells lysing, antibodies binding and other treatment for further procedure before they are pushed into the smaller nanochannel.

The pressure applied to push or pull the cells into the side channel was not constant as there were a lot of variables that affect the mobility of the cell. The possible reasons are the quality of cells, the duration that the cells are incubated with YOYO-1, and the quality of microfluidic device as cell might interact with the PDMS glass or device.

From the video taken as illustrated with the images in Figure 3.1.2, the cell would slowly slide from the side channel towards the main channel when no pressure is applied due to the

elasticity of the cell and its natural preference to be in a larger volume instead of a confined space. The optimization of this process to ensure that the cell can be kept in a confined space without the applied pressure will be discussed in Section 3.3

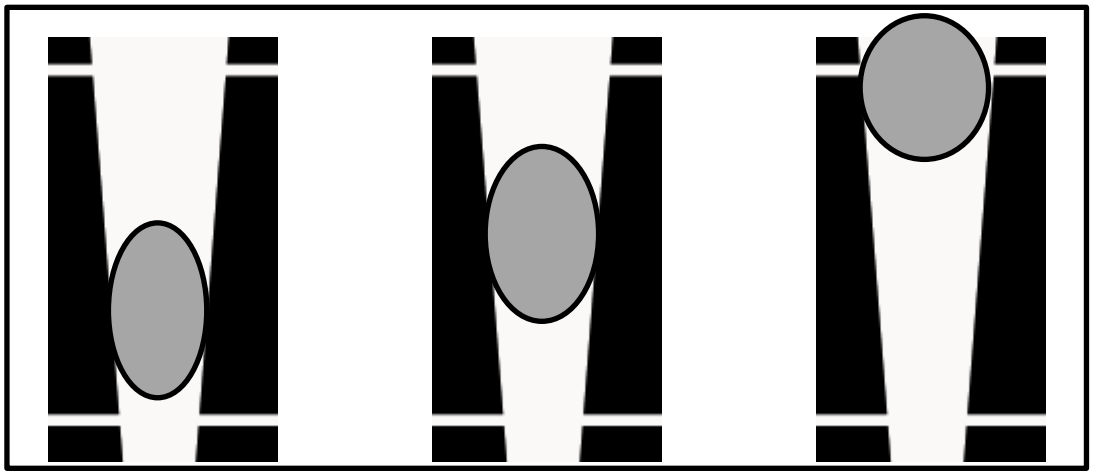


Figure 3.1.2 Illustration of cell moving out under no applied pressure

After the cells were situated in the preferred location, the treatment of the cell was done at preferred location 2 as shown in Figure 2.1.1 where the various solutions i.e. lysis solution, demecolcine and H3 antibodies with Alexa Fluor were injected in at the buffer reservoir. The two images in Figure 3.1.3 (the white line is to help visualize the channel) highlight the lysing process in fluorescence. It can be observed that the right image has its fluorescence material spread out and it is less dense as the cell membrane is being lysed. The image in Figure 3.1.4 shows the binding of H3 antibodies to the chromatin which is visible due to the Alexa Fluor.

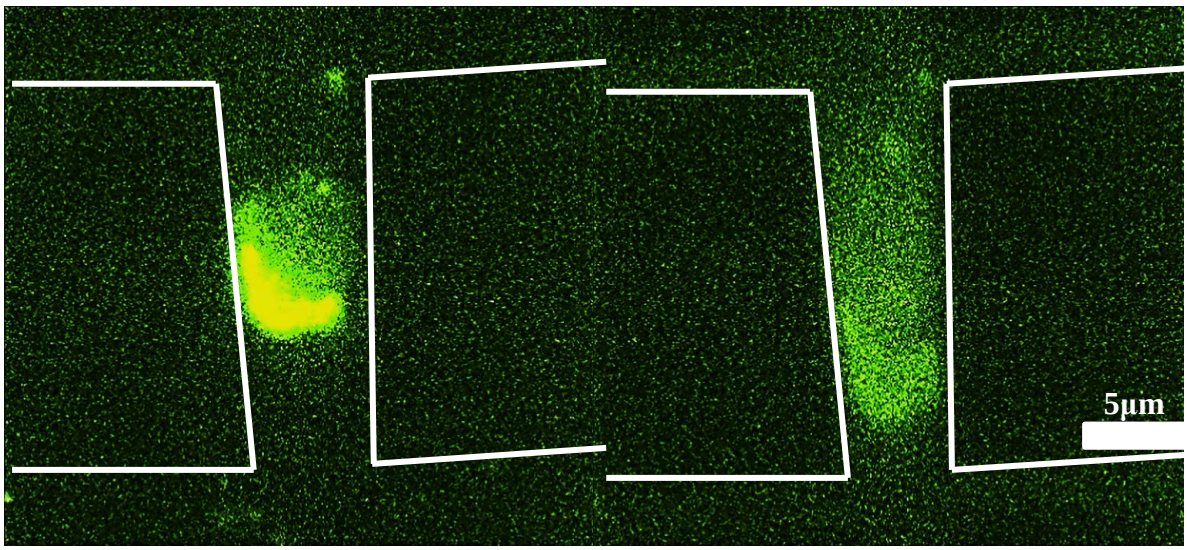


Figure 3.1.3 Lysing process under fluorescence imaging

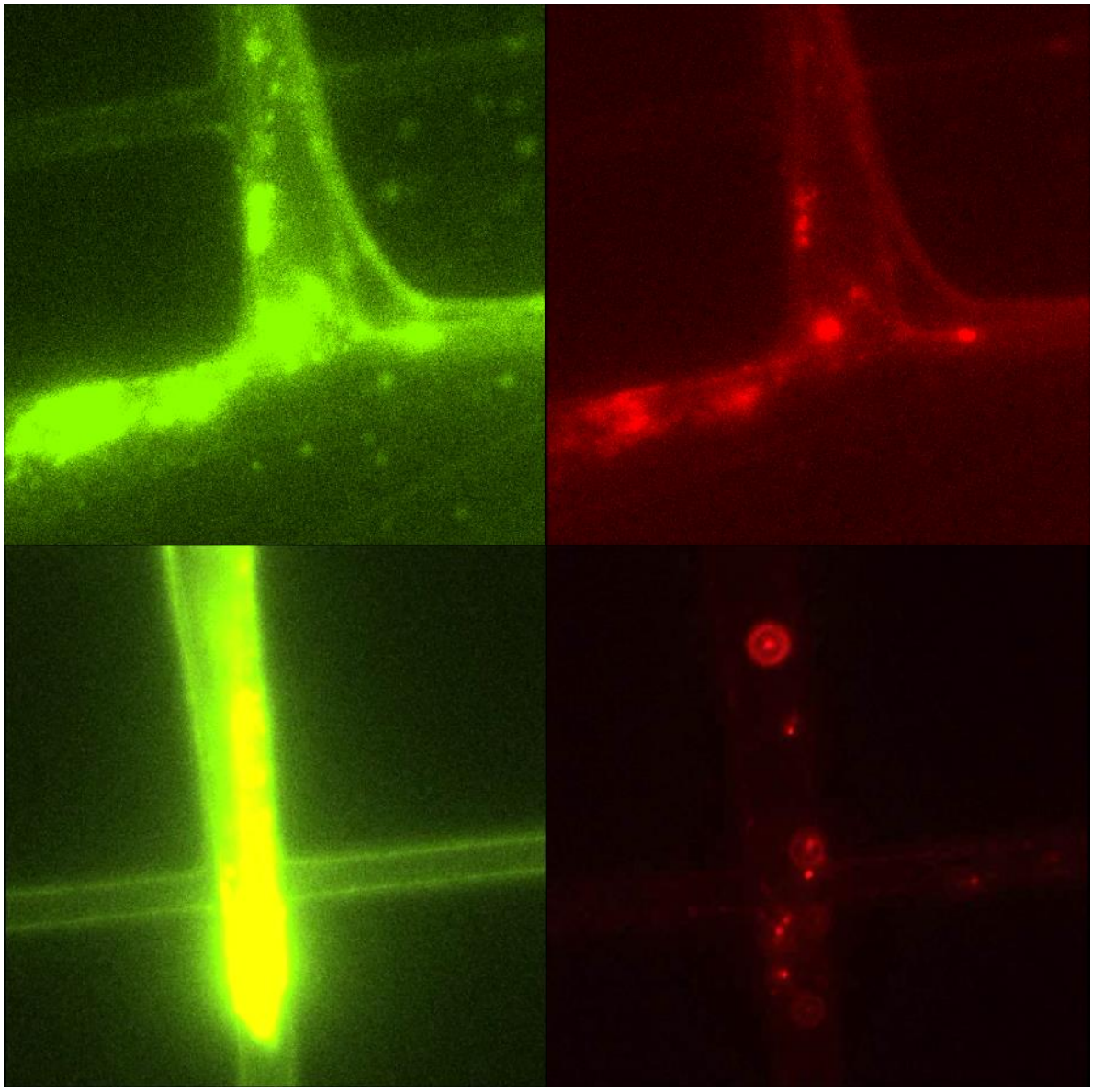


Figure 3.1.4 H3 Antibodies binding to chromatin with Alexa Fluor dye

To cut the chromatin into smaller pieces, EcoRI and NruI were used. After the enzyme was flushed in, the chromatin can be seen broken into a lot of smaller pieces. Some of the fragmented chromatin could not be visualized due to its small size magnitude. To allow for a better and efficient cutting of the chromatin, a heat plate can be used on the microscope so that the enzyme can operate at its optimum temperature of 37°C.

From all the results in this section, it can be observed that the extraction of chromatin from cells looks promising and the priorities will turn towards the movement of chromatin into the nanofluidic device.

3.2 Linearization of chromatin in nanofluidic device

After the chromatin was prepared with the two methods as mentioned in Section 2.2, the chromatin was first injected into the 500nm nanofluidic device as shown in Figure 2.2.1. The chromatin from the first method has difficulty getting into the 500nm nanochannel while the chromatin from the second method does not have any difficulty and this can be seen from Figure 3.2.1.

Therefore the chromatin from the second method of extraction was used for the smaller nanochannel nanofluidic device. The chromatin encountered difficulty in getting into the nanochannel of 250nm. Experiment was carried out with and without demecolcine and with different salt concentration and similar results were obtained.

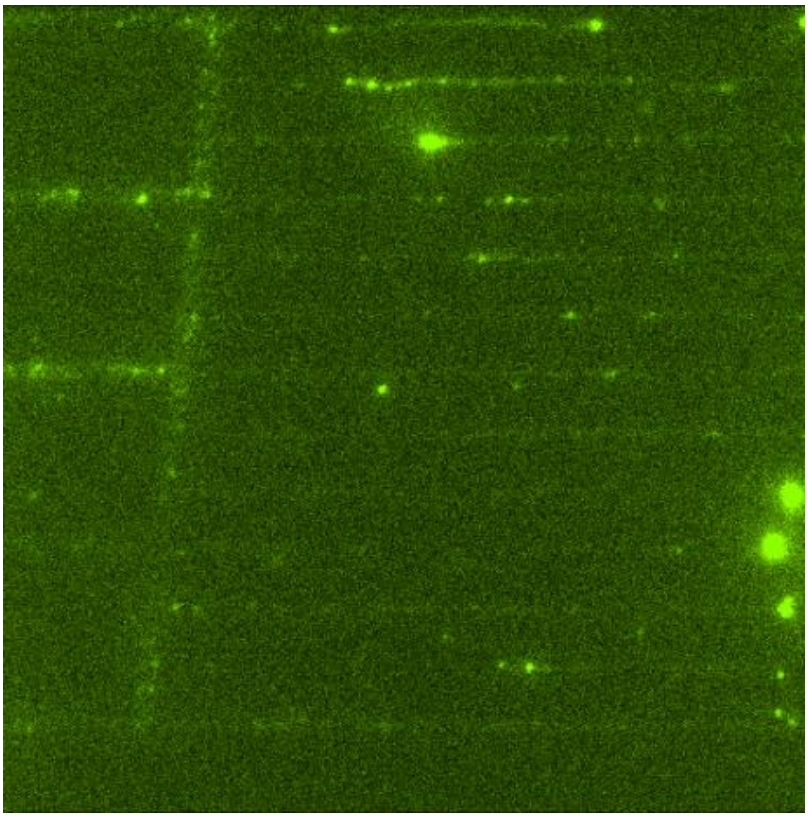


Figure 3.2.1 Chromatin in the 500nm nanofluidic device

Hence a new design of the nanochannel was developed as shown in Figure 3.3.7 with the tapered nanochannel. The tapered nanochannel will allow the chromatin bundle to rest in the start of the funnel first before pressure is applied to the channel to be linearized. It is a similar concept as the improved microfluidic device as explained in Section 3.3.

In pure DNA linearization, electric field is usually applied to get the DNA into the nanochannel before linearization. This is possible due to the fact that DNA is negatively charged while it is not possible for chromatin as it is an almost neutral particle as the proteins such as histone that are attached to the chromatin are positively charged.

3.3 Optimization of procedure for a simple and yet efficient process

The first optimization involves the redesign of the microfluidic device. The previous microfluidic is shown in Figure 3.3.1. It has many side channels and its side channel is of standard size which made it difficult for the cell to get into. There was no need for side channels at both sides of the main channel and it will be more effective to get the cell into the side channel if the number of side channels decreases. In this way, pressure applied at the end of the side channel will not be distributed to more channels.

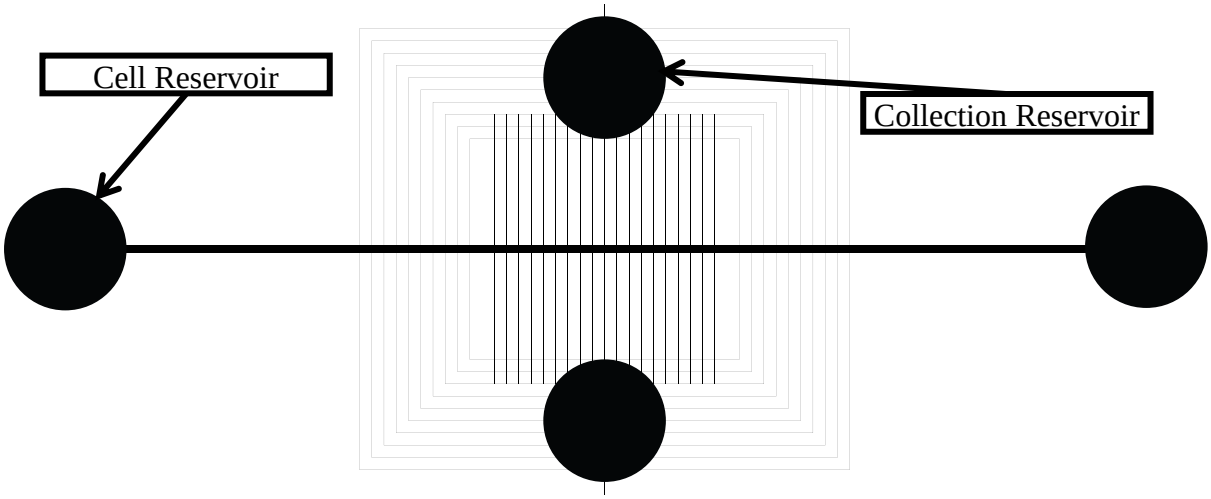


Figure 3.3.1 Old microfluidic device

Hence, a new microfluidic device was designed to allow a more effective way of manipulating the cell in the microfluidic device. In Figure 3.3.2 to 3.3.4, a number of designs are shown and the final design that was developed and used in the experiment is the microfluidic device shown in Figure 3.3.2.It is an improvisation of the other designs. The advantages of the microfluidic device C are also shown in Figure 3.3.4.

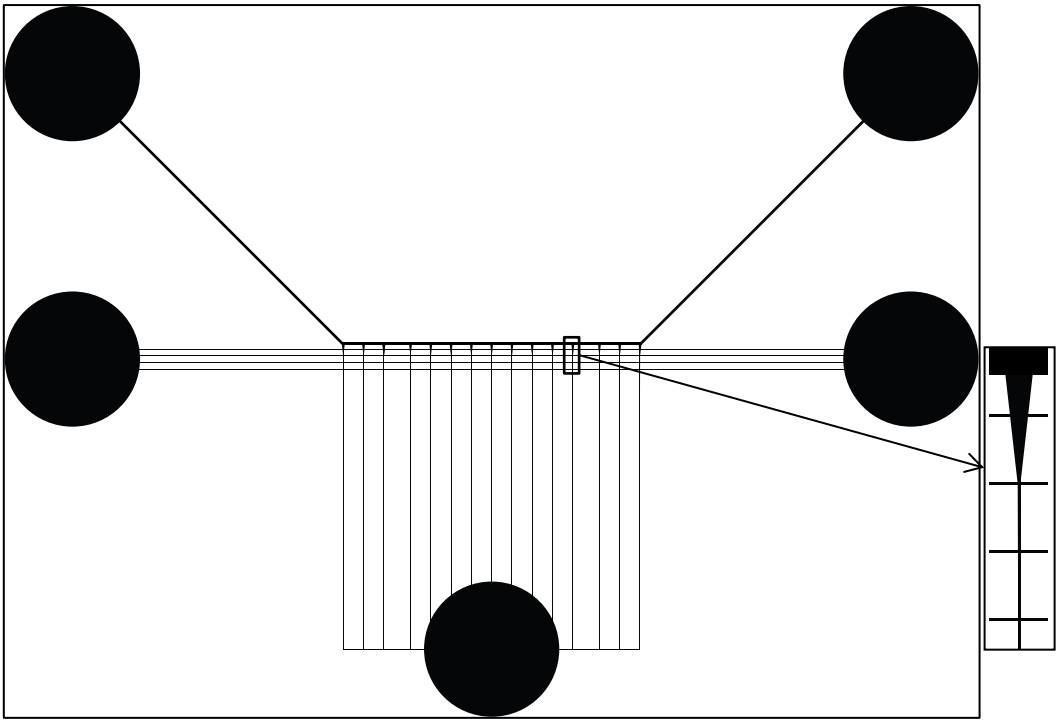


Figure 3.3.2 Microfluidic device design A

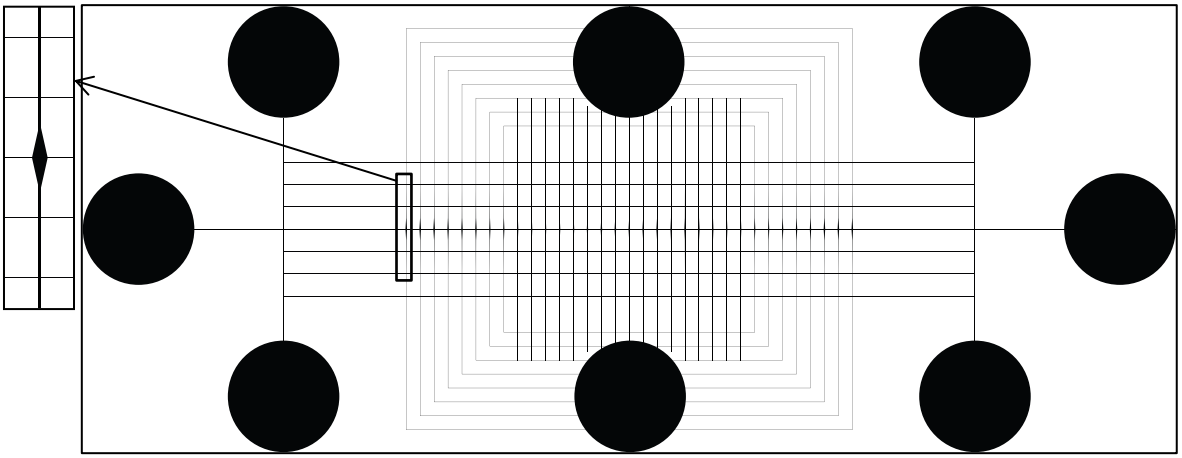
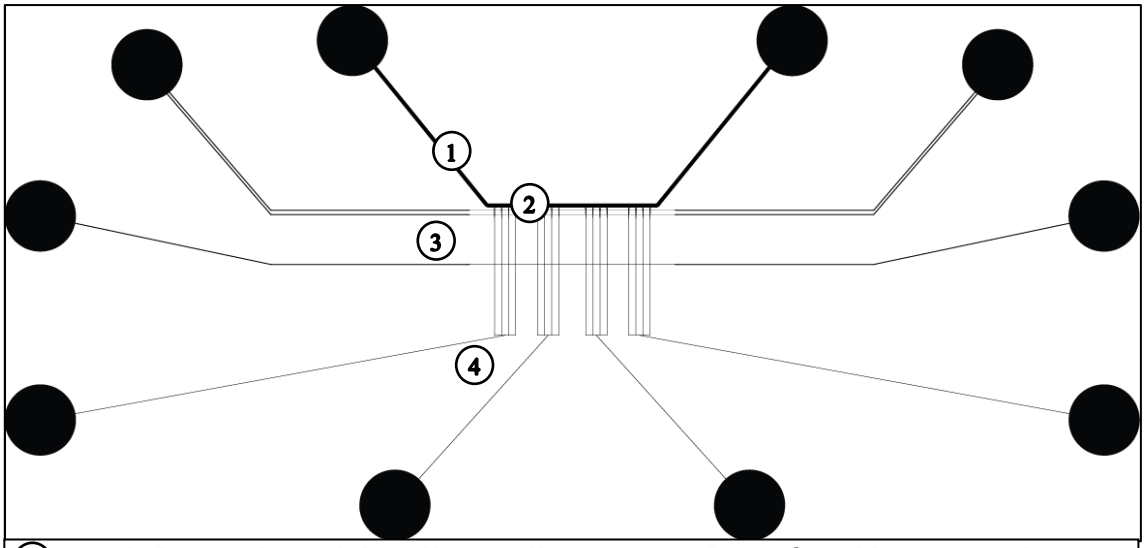


Figure 3.3.3 Microfluidic device design B



① - Angled main channel that allows cell to move to the preferred location at the main channel at a reasonable speed while increasing the chance that the cell get into the side channel

② - Tapered side channel that allow cell to get into the side channel easier

③ - Two buffer channel that can allow the use of different buffers at different locations

④ - Separated collection reservoir if a separated sample is needed

Figure 3.3.4 Microfluidic device design C with its advantages

For future devices, it is recommended that the preferred location 2 as shown in Figure 2.1.1 have a straight wall instead of tapering (illustrated in Figure 3.3.5) so that the cell can maintain its position without the application of external pressure.

Figure 3.3.5 illustration of improved tapered channel

For the nanofluidic device, a new design was drafted according to the discussion in the previous chapter where a tapered channel will be preferred for linearizing the chromatin. The design can be seen in Figure 3.3.6. An illustration of how the tapered channel will help to linearize the chromatin is shown in Figure 3.3.7.

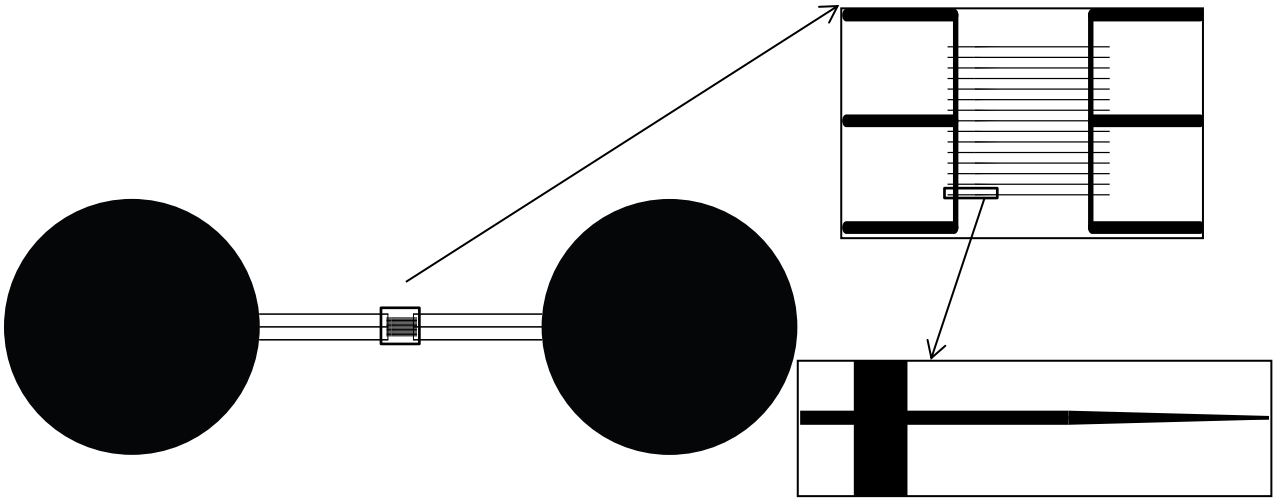


Figure 3.3.6 New nanofluidic device with tapered channel

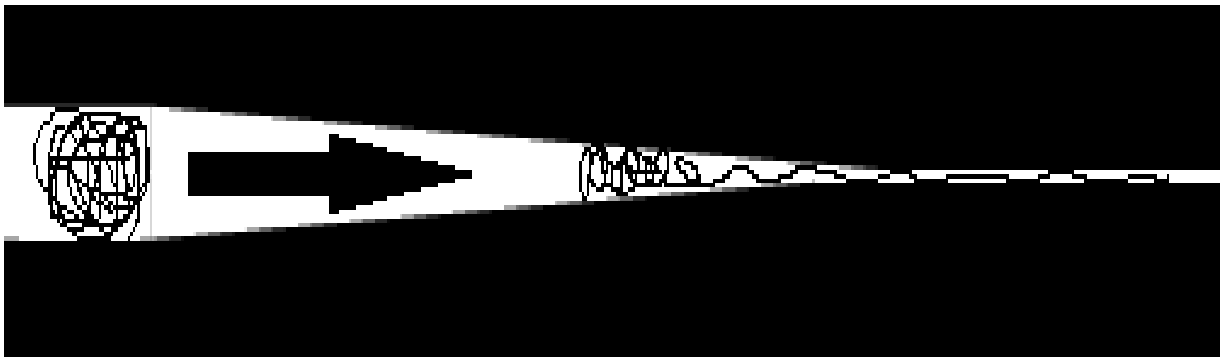


Figure 3.3.7 Illustration on how linearization of chromatin can be done with a tapered nanochannel

Optimization of YOYO-1 fluorescence dye to be used for chromatin and cell was done visually and after a few trials it was determined that the optimized YOYO-1 fluorescence dye to be used was about 2μl/0.06ml. Concentration of cell and chromatin was also tested but it was determined that the concentration of cell and chromatin is not so critical if the quality of cell and chromatin is good.

Caffeine and demecolcine was found to be able to depolymerise the actin filament in chromatin from the reference 18 and 19 respectively. Therefore they were tested in the experiment as the chromatin have actin filament that made them very elastic and hard to control in the microfluidic and nanofluidic devices.

First, caffeine was used and it was established that caffeine could not help to decrease the elasticity of the actin filament in the chromatin as the caffeine will precipitate too fast as it

gets into the microfluidic or nanofluidic device. Hence demecolcine was tested to see if it helps to reduce the elasticity.

Firstly, visual and qualitative analysis on the effect on chromatin was done. Demecolcine was added into the buffer channel via the buffer reservoir in the microfluidic device together with the lysis buffer. It was observed that no effect was seen in the microfluidic device. It was decided that the cell needed to be incubated for a short period of time before the cell can be introduced into the microfluidic device since demecolcine is able to enter the cell membrane and nucleus. After incubating it for one hour, the cell is injected into the microfluidic device and pushed into the side channel for lysing. It can be observed from the video that the chromatin bundle is much more freed up than before.

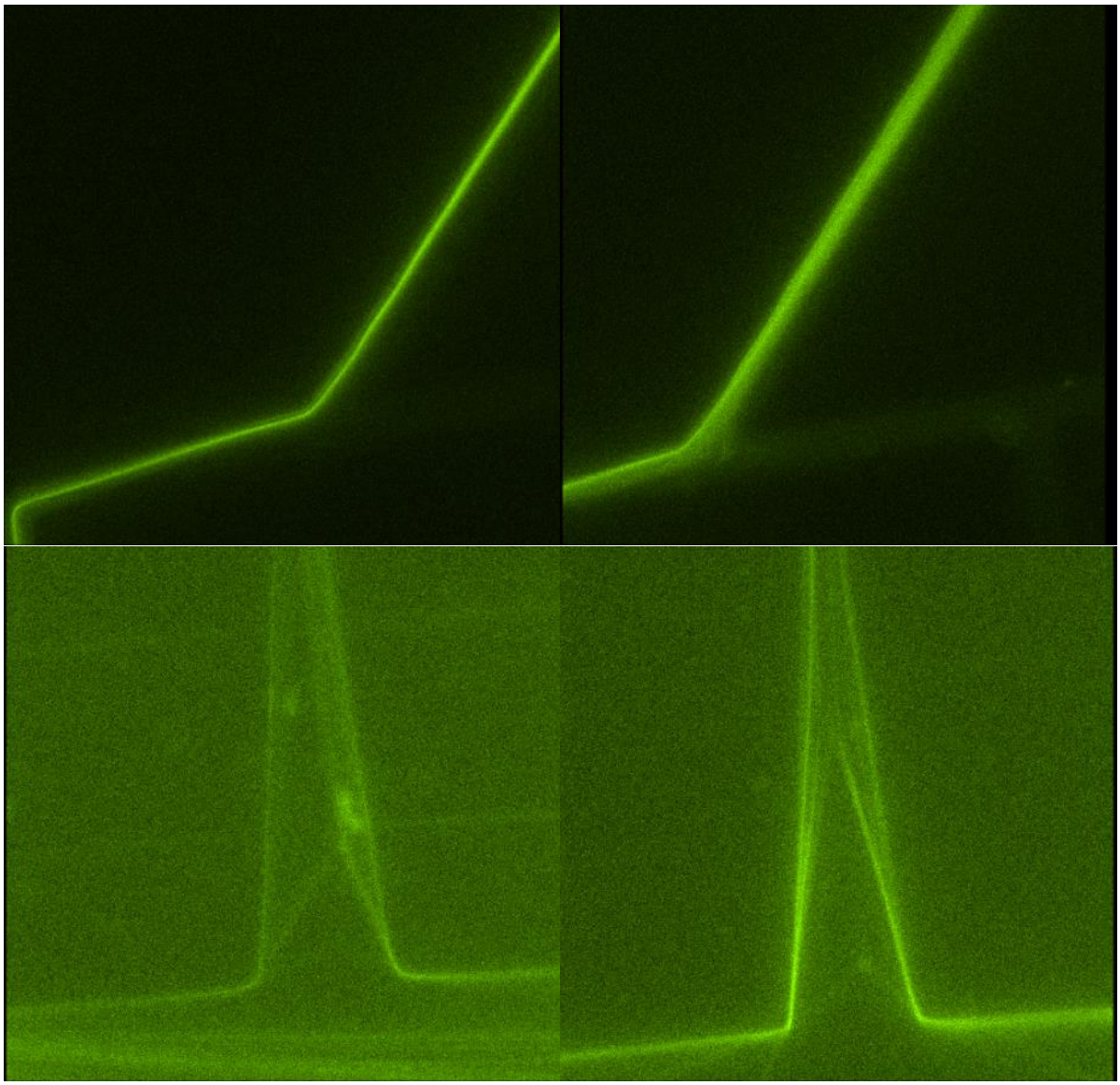


Figure 3.3.8 Images with and withour demecolcine

Figure 3.3.8 proved the effective of demecolcine effectiveness as the top images shows the ability to pull the chromatin to the other end of the microfluidic device while the bottom images shows that the chromatin is too elastic as it is stuck to the opening of the side channel and always return to its original location after being pulled for a few micrometer.

Limited time was left when demecolcine arrived for experiment, therefore justification on how effective demecolcine quantitatively was not done. Two methods can be used to test the effectiveness of demecolcine and to determine the optimized amount of demecolcine and duration of incubation. The first method is to use actin staining dye and to observe under microscope how different concentration of demecolcine and different incubation time affect the outlook of a cell before and after lysing. The second method is to utilise Atomic Force Microscopy (AFM) to observe any changes in the contour length before and after the application of demecolcine.

One of the other experiments done is the effect of demecolcine on cell structure after its incubation time as shown in the Figure 3.3.9 on the next page. Cells can be seen to have deformed for higher concentration of demecolcine but a longer period of incubation, cells do not seem to deform. This could be further looked into such that appropriated amount of demecolcine and duration of incubation time can be determined.

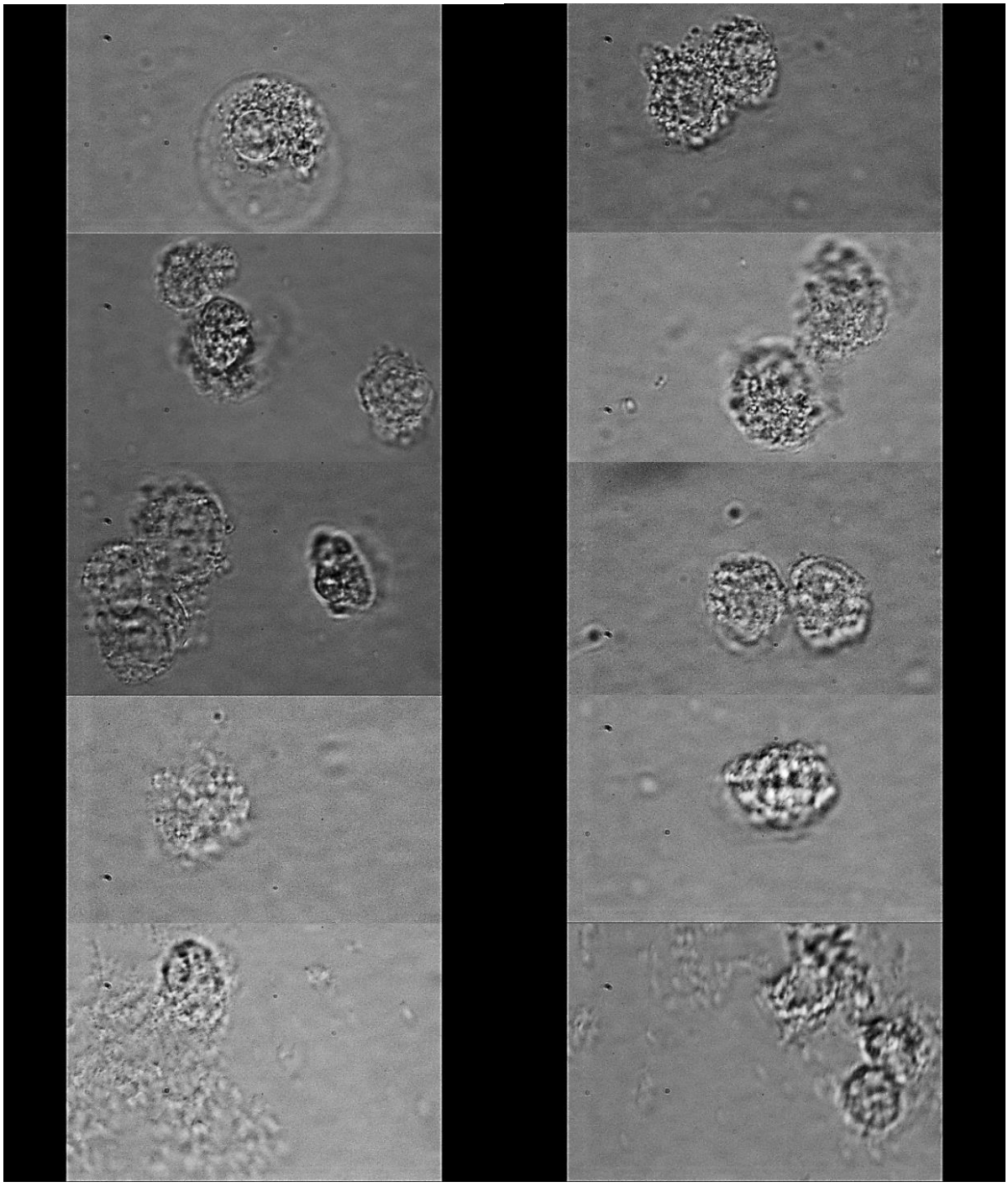


Figure 3.3.9 Cell structure with Different concentration of demecolcine and incubation time. The left side is cell incubated after 1 hour while the right side is cell incubated after 3 hours. The concentration of the demecolcine from top to the bottom image is (2.5µl/0.06ml, 5µl/0.06ml, 7.5µl/0.06ml, 10µl/0.06ml, 15µl/0.06ml)

4 Conclusion and Future Direction

The objective of this project was to develop a relatively inexpensive and simple single cell analysis method i.e. integrated micro & nanofluidic device to achieve a precise profile of the epigenetic modification.

It can be concluded that the procedure of using a microfluidic device to extract the genetic material is successful and it can be easily used for other cells such as human cells. The optimizations of the amount of fluorescence dye and the concentration of demecolcine that will help in achieving the objective of developing a simple and efficient methodology were achieved in this project. However, linearization of chromatin in the nanofluidic device, one of the significant parts of the project could not be achieved due to unforeseen circumstances. More time will be needed to develop a newer nanofluidic device such that linearization of chromatin can be done in the nanofluidic device.

One of the significant part of the project is not achieved but once the linearization of the chromatin is done, the success of creating an integrated micro & nanofluidic device as seen in Figure 4.1 (on the next page for epigenetics profiling) can soon be achieved. It is improvised such that buffer channel are shorter to reduce waiting time, and only one side channel per reservoir so to maximise the suction. The nanochannel is integrated with the tapered section as shown.

The use of different antibodies to profile different epigenetic modification marks should be looked into in the future. As different antibodies with different fluorescence dye might change the chromatin structure slightly thus affecting the linearization of chromatin in the nanochannel.

After the integrated micro & nanofluidic device is developed, other optimization such as size of channel, concentration of buffer should be looked into to achieve the objective of a simple and efficient methodology. All these future works could create the cheapest epigenetic profiling device that could help us better understand living organisms and pave a way to foresee any diseases that can be seen from epigenetic profile.

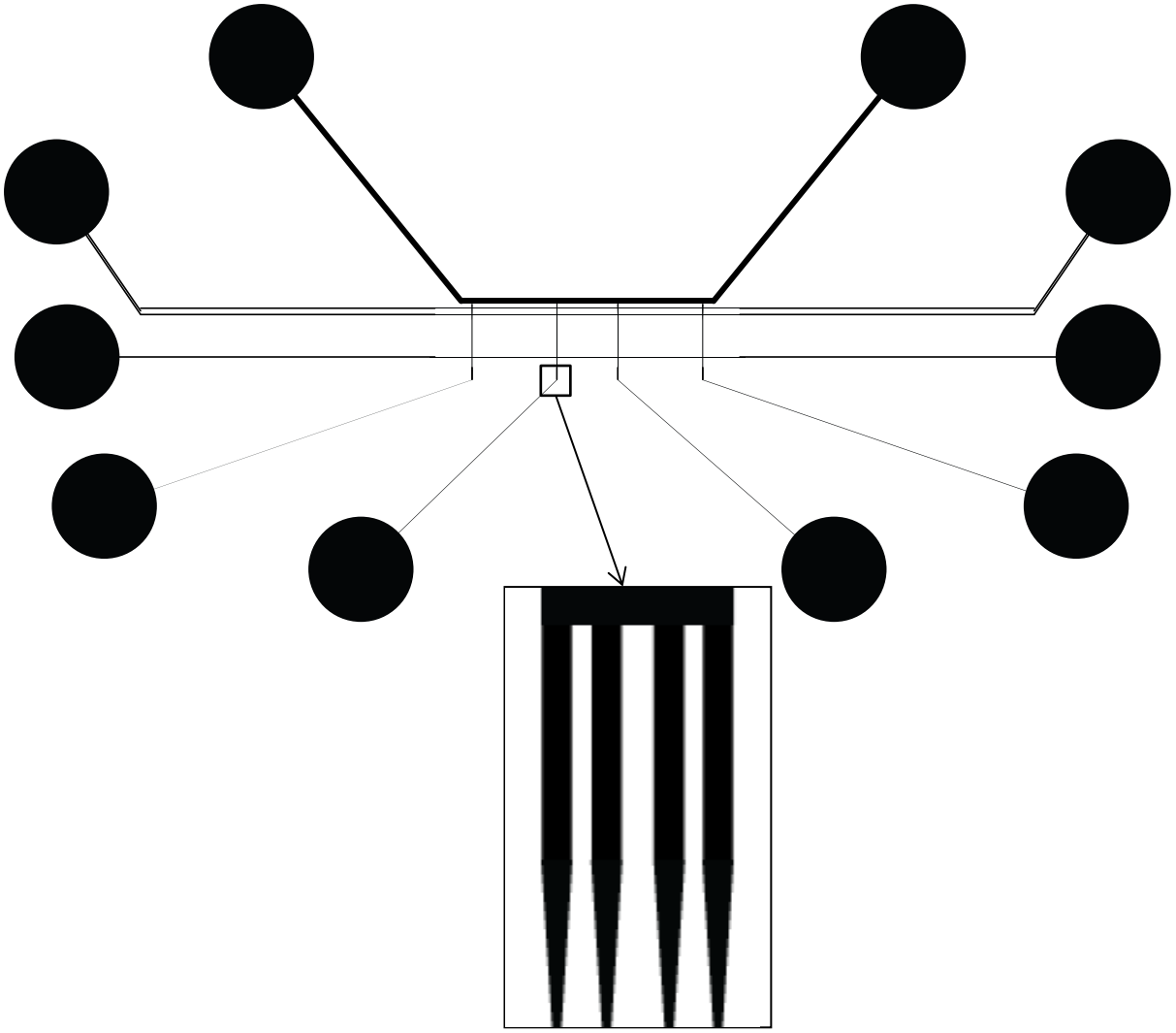


Figure 4.1 Integrated micro & nanofluidic device

Appendix A: Microscope Image of the Microfluidic and Nanofluidic Device

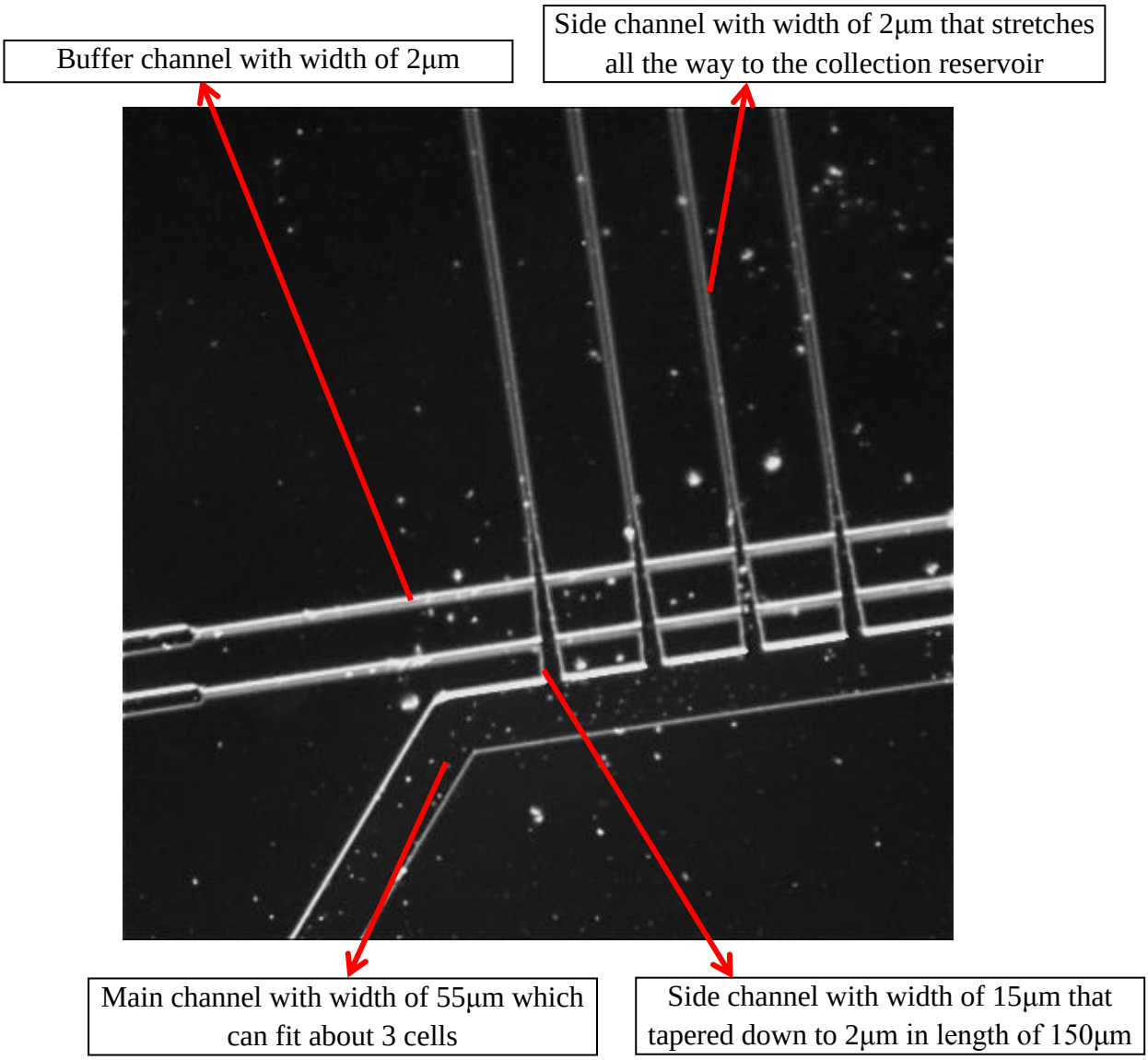


Figure A.1 Microscope image of microfluidic device with some of its dimension

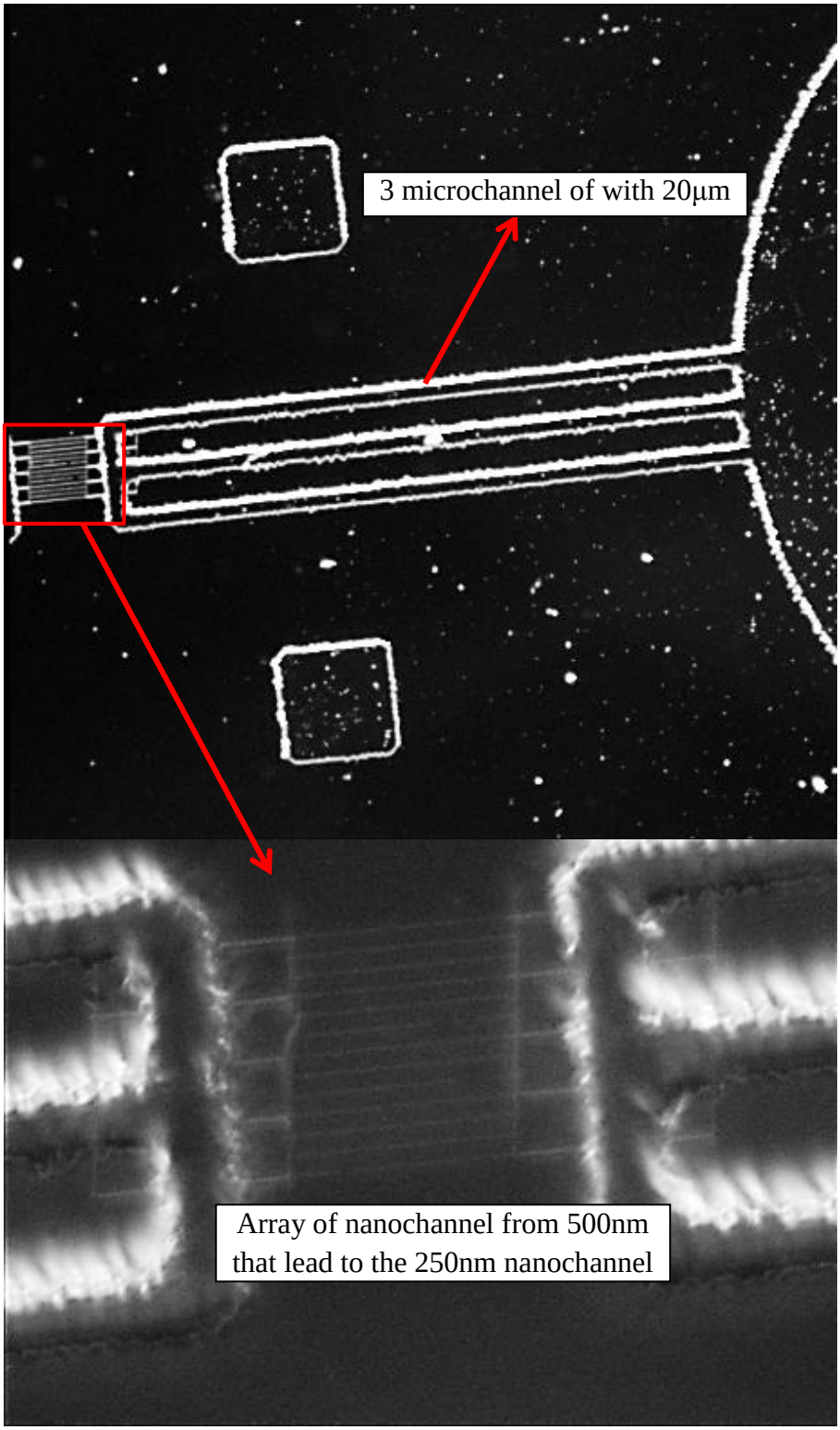
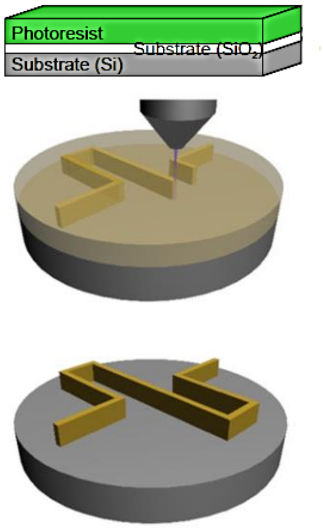


Figure A.2 Microscope image of nanofluidic device with some of its dimension

Appendix B: Development and Preparation of Microfluidic Device

Microfluidic device is cast from a master stamp. The master stamp can be used to cast up to hundreds of replicas such that a new chip can be used in every experiment. The master stamp is made using a laser lithography process. The process can be seen from the illustration shown in Figure B.1.



-5µm negative photoresist, mr-DWL is coated onto the substrate

-Direct writing laser @ 405nm was exposed to the designated area (structure that is wanted)

-Non-exposed photoresist will be washed off with developer such that the design remain

Figure C.1 Procedure to produce the microfluidic device master stamp

To ensure quality of master stamp, Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM) is used to assure its dimension before using. As to provide a longer lifespan to the intricate design, a Teflon coating may be applied on the master stamp.

The microfluidic device used in the experiment is casted using a PDMS elastomer, SylgardTM 84 by Dow Corning Co. The PDMS base and curing agent is thoroughly mixed in a ratio of 10:1 before putting it into the master stamp to cast. Vacuum is applied such that no air bubble is presence in the cast and it will be left to cure for a minimal of 5 hours. After curing, the cast is released from the master stamp and reservoir holes are created. After which, it will be sent for plasma oxidisation for 30 second with a PDMS coated coverslip (coverslip is coated with PDMS is to provide a full PDMS environment for the cell as cell will tend to stick to the glass surfaces). Lastly, the cast will be placed on the PDMS coated coverslip and placed on a hotplate at 95°C for bonding for 2.5 minutes. The cast will be prepared for experiment such that cells, chromatin or buffer solution may be introduced into the device. Nanofluidic device is created in almost the same manner except the resist material used and lithography process with MeV proton.

Appendix C: Preparation of Mammalian Chromatin Protocol

1. Cell lysis buffer (total 50 mL)

250 mM PIPES	1mL
3M KCL	1.417 mL
20% NP40	1.25 mL
Dd H2O	46.3 mL
1M DTT (add fresh)	1:2000
0.1M PMSF (add fresh)	1:200

2. ChIP dilution buffer (total 5.545 mL)

10 % SDS	50 uL
20% Triton X-100	2.75 mL
250 mM EDTA	0.24 mL
1M Tris-HCL pH 7.3	0.835 mL
5M NaCl	1.67 mL

3. 1X PBS buffer

Protocol:

1. Take the cell, add 5mL 1% cold para formaldehyde, put on ice for 10 min, add 0.5 mL 2.5 mM glycine to quench the reaction. Suck out the solution.

2. Wash twice with cold PBS buffer (add PBS, re-suspend pellet by aspiration, centrifuge at 900 g to pellet again)

3. Add 1mL cell lysis buffer first before adding another 1mL cell lysis buffer

4. Keep on ice for 5 min

5. Spin down at 2000g to remove the cell debris from chromatin

6. Add 300-400uL ChIP dilution buffer, mixed it under ice and aliquot it into tubes and keep in -20/80°C

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