MASSEY UNIVERSITY

An investigation into the application of microfluidics to the analysis of chromosome conformation

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Abstract

Ever since the discovery of DNA, biologists have been striving to unravel its mysteries. Many efforts have been made over the years to further our understanding of genes, what they do and how they function. Genomes exist as a 3D structure inside the nucleus and they are not randomly arranged. However, there are still many gaps in the knowledge of how the structure fills this 3D space. Using chromosome conformation capture (3C) and other methods based on proximity ligation, interactions between different sections on the chromosome can be captured. A computer simulated 3D chromosome model can then be created based on the interaction data. Currently, global interaction maps can only be created for populations of cells. The overall goal of this research is to develop a protocol that will enable the capture of chromosome interactions within a single cell. This requires the use of microfluidic chips due to the minute quantity of DNA within a single cell. Therefore the main objectives of this research are to: 1) build and test a microfluidic system (lab-on-a-chip or LOC) that will aid in the capture of inter- and intra- chromosomal interactions of a single cell; and 2) characterize the restriction and ligation of DNA that will be performed in a microfluidic system.

In order to assess the efficiency of DNA digestion within microfluidic chips, EcoRI and MspI digestion kinetics within microtubes is first characterized to establish a base line for comparison with digestion kinetics within microfluidic chips. The $K_{m,}$ V_{max} and K_{cat} for EcoRI within microtubes are 32 nM, 0.14 nM s^{-1} and 1.4 fmol s^{-1} U^{-1} respectively. The K_m, V_{max} and K_{cat} for MspI within microtubes are 125 nM, 1.46 nM s^{-1} and 29.2 fmol s^{-1} U^{-1} respectively.

On the other hand, the digestion kinetics within microfluidic chips is undetermined, because both restriction enzymes exhibit non-specific nuclease activity within microfluidic chips under the conditions tested. The exhibition of non-specific nuclease activity is unexpected and causes ligation of DNA performed in microfluidic chips to fail. The non-specific nuclease activity of EcoRI and MspI within microfluidic chips is also problematic for the overall goal of developing a protocol that will enable the capture of chromosome interactions within a single cell, because the non-specific nuclease activity would cause loss of template and random variations in results obtained.

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I would like to thank Justin O'Sullivan and John Harrison for the guidance provided and patience shown throughout this project.

I would also like to thank Chris Rodley for providing most of the primer sequences used in this project.

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List of abbreviations

[S]	Substrate concentration
3C	Chromosome conformation capture
3D	Three dimensional
4C	3C-on-chip or open-ended 3C, or 'olfactory receptor' 3C
5C	Chromosome conformation capture carbon copy
AB	Amplification bias
ADO	Allele drop out
bp	Base pair
BSA	Bovine serum albumin
FACS	Fluorescence-activated cell sorting
GCC	Genome conformation capture
LOC	Lab-on-a-chip
MDA	Multiple displacement amplification
Microtube	1.7ml Microfuge tube
PA	Preferential amplification
PCR	Polymerase chain reaction
PDMS	Poly-dimethylsiloxane
PGD	preimplantation genetic diagnosis
qPCR	Quantitative polymerase chain reaction or real-time polymerase chain reaction
SEM	Scanning electron microscope
VI	Virtual instrument
WGA	Whole genome amplification

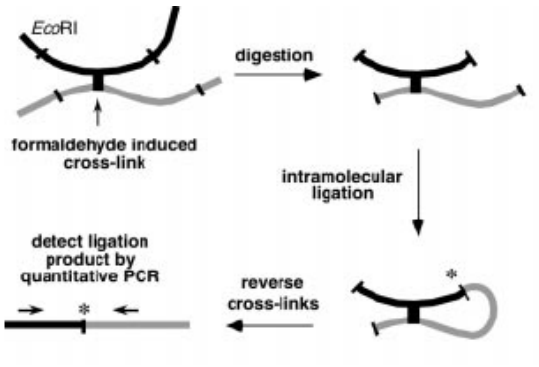
1. Introduction

This research is a vital component of a larger project that aims to capture the chromosome interaction of a single cell. The completion of the larger project will achieve two purposes: 1) determine variations between individuals instead of variations between populations; and 2) decrease resources spend. There are three parts to the overall project: 1) building and testing of lab-on-a-chip (LOC) that will be used to perform assay to capture chromosome interaction; 2) single-cell isolation; and 3) whole genome amplification (WGA) to create enough templates for sequencing. This research has two main objectives: 1) build and test a microfluidic system (lab-on-a-chip or LOC) that will aid in the capture of inter- and intra- chromosomal interactions of a single cell; and 2) characterize the restriction and ligation of DNA that will be performed in microfluidic system.

1.1. Methods of analyzing chromosome interactions

Ever since the discovery of DNA, biologists have been striving to unravel its mysteries. Many efforts have been made over the years to further our understanding of genes, what they do and how they function. Genomes exist as a three-dimensional (3D) structure inside the nucleus and they are not randomly arranged (Takizawa, Meaburn, & Misteli, 2008). However, there are still many gaps in the knowledge of how the structure fills this 3D space. Using chromosome conformation capture (3C) (Dekker, et al., 2002) and other methods (Dostie, et al., 2006; Duan, et al., 2010; Lieberman-Aiden, et al., 2009; Lomvardas, et al., 2006; O'Sullivan, Sontam, Grierson, & Jones, 2009; Ohlsson & Göndör, 2007; Rodley, et al., 2009; Simonis, et al., 2006; Wurtele & Chartrand, 2006; Zhao, et al., 2006) based on proximity ligation (Fredriksson, et al., 2002),

interactions between different sections on the chromosome can be captured and then mapped. Even though 3C is not the first technique that allowed the investigation of chromosome interactions, it is the first technique that allowed a snapshot of the chromosome interactions to be taken with relative ease (Fig. 1) (Dekker, et al., 2002). 3C is based on the proximity ligation technique (Dekker, et al., 2002) which, as the name suggests, relies on the ligation of sequences that are in close proximity (Dekker, et al., 2002; Fredriksson, et al., 2002). Subsequent techniques that were developed made various improvements or adaptations to 3C, but were still based on proximity ligation.

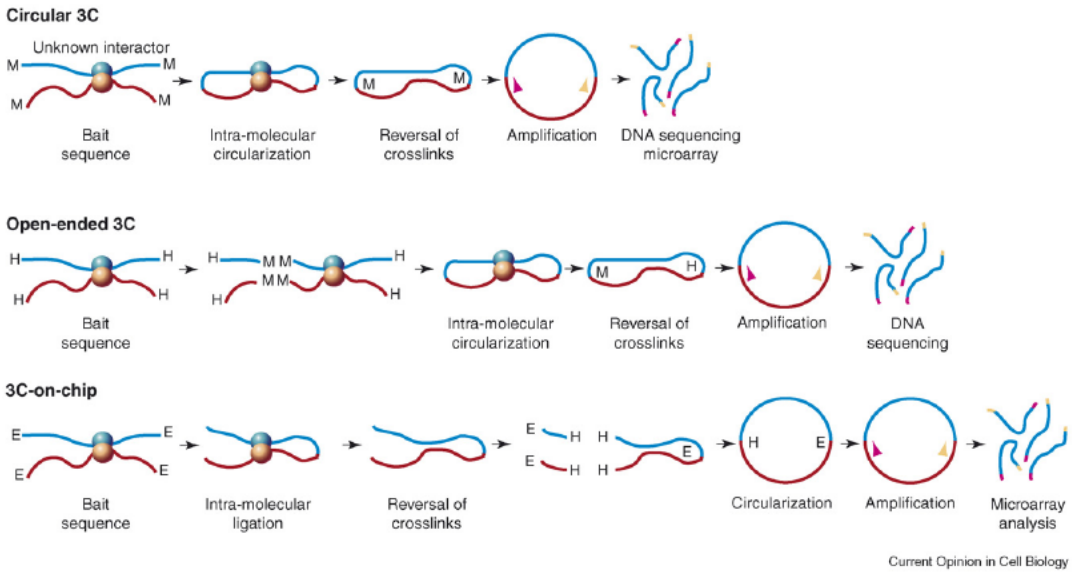


(Dekker, Rippe, Dekker, & Kleckner, 2002)

Figure 1. Schematic of the chromosome conformation capture (3C) methodology. Asterisk indicates newly formed restriction site after ligation.

All proximity ligation based techniques begin by cross-linking DNA that is in close proximity with other DNA or with other proteins. The cells are then lysed and the DNA is digested by a restriction endonuclease, usually with one that leaves a cohesive end. Cross-linked chromatin is then diluted to promote ligation of sequences in close proximity. The methods primarily differ after the removal of the cross-links. 3C detects the presence of ligated products using the polymerase chain reaction (PCR) or real-time PCR (qPCR) with primers that amplified across the ligated restriction site (Fig. 1) (Dekker, et al., 2002). Four

independent laboratories developed the 4C (Zhao, et al., 2006), 3C-on-chip (Simonis, et al., 2006), open-ended 3C (Wurtele & Chartrand, 2006), or 'olfactory receptor' 3C (Lomvardas, et al., 2006) method at approximately the same time (Fig. 2) in order to map DNA interactions with a bait sequence (Ohlsson & Göndör, 2007). The underlying methods for these four techniques are very similar. They first circularize DNA via ligation, before using primers that anneal to the ends of the bait sequence and amplify outwards (i.e. inverse PCR) from the bait sequences (Lomvardas, et al., 2006; O'Sullivan, et al., 2009; Simonis, et al., 2006; Wurtele & Chartrand, 2006; Zhao, et al., 2006). The amplified products are then sequenced or identified by hybridization to a microarray.

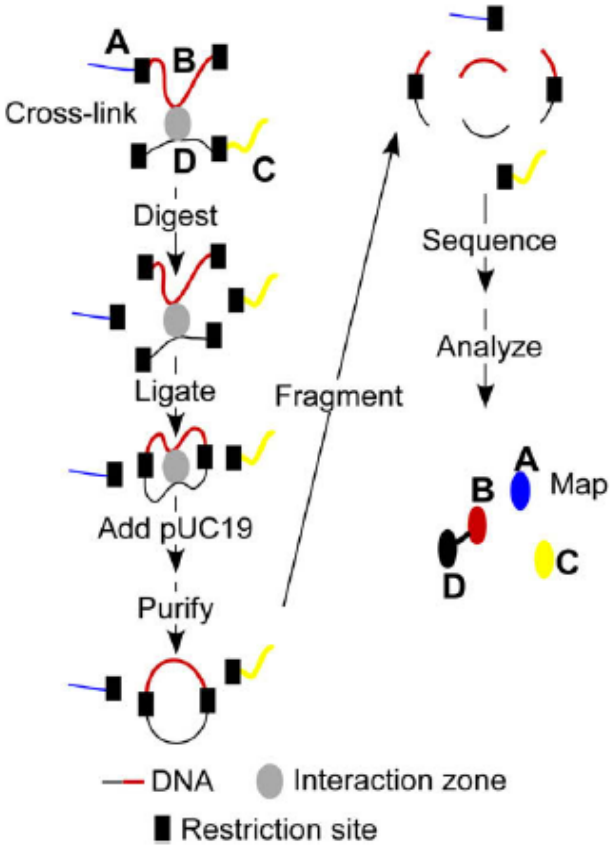


(Ohlsson & Göndör, 2007)

Figure 2. Comparison of various 4C methodologies. Restriction enzyme recognition sites: M = *Msp* I; H = *Hind* III; E = *Eco* RI. The 'olfactory receptor' 3C is not shown, but it is most similar to principles of Circular 3C.

Chromosome conformation capture carbon copy (5C) employs multiplex ligation-mediated amplification after the removal of cross-links. This requires the annealing and ligation of multiplexed nucleotides across the ligation sites of

interested sequence. The ligated multiplexed nucleotides are then amplified using universal primers. The amplified products are then analyzed by high-throughput sequencing or microarray (Dostie, et al., 2006).

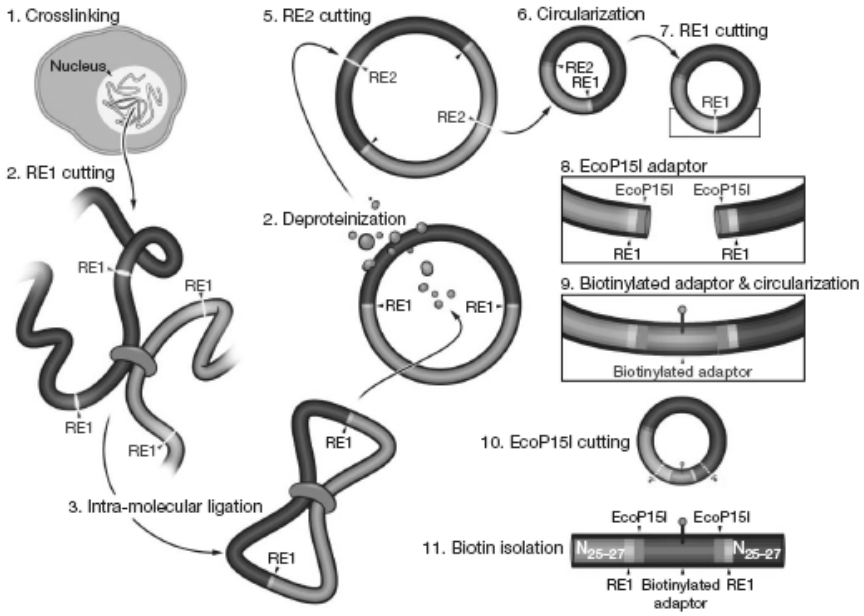


(Rodley, Bertels, Jones, & O'Sullivan, 2009)

Figure 3. Genome conformation capture (GCC) methodology. Hi-C (Lieberman-Aiden, et al., 2009) is similar to GCC with an additional step between digestion and ligation to fill the sticky ends and mark with biotin. Purification after ligation involves biotin pull down.

Genome conformation capture (GCC) was the first proximity ligation based method that did not require the use of specific primers, thus allowing global analysis of chromosome interactions (Rodley, et al., 2009). This is achieved by sequencing nebulised DNA fragments after the removal of cross-links (Fig. 3). Two other techniques, Hi-C (Fig. 3) (Lieberman-Aiden, et al., 2009) and the

Duan modified Hi-C method (Fig. 4) (Duan, et al., 2010), use similar strategies. However, the Hi-C based techniques both add biotinylated adaptors. Hi-C then shears the DNA and purifies using biotin pull down (Lieberman-Aiden, et al., 2009) . The modified H-C method ligates Eco15I adaptors to digested DNA before the ligation of biotinylated adaptors. Eco15I, a Type III restriction endonuclease, or other Type II restriction endonucleases, is then used to cleave the DNA and the interested sequence is purified using a biotin pull down (Fig. 4) (Duan, et al., 2010).



(Duan, et al., 2010)

Figure 4. The Duan modified Hi-C method. Similar to other proximity ligation methods, except three restriction endonucleases are used at various points: after whole genome extraction, after removal of cross-links and after circularization with the addition of biotinylated adaptors.

The proximity ligation based methods can be subdivided into two groups: sequence targeted methods and global sequencing methods. 3C (Dekker, et al., 2002), 4C (Lomvardas, et al., 2006; Simonis, et al., 2006; Wurtele & Chartrand,

2006; Zhao, et al., 2006) and 5C (Dostie, et al., 2006) are sequence targeted methods. These methods require previous knowledge of sequences in order to design primers or multiplex oligos to anneal to the DNA. These techniques target specific sequences to assess the interactions of these sequences, and as such are not useful for identifying global interactions. However, they are very useful for the confirmation of known interactions. 4C is slightly better than 3C and 5C at identifying unknown interactions, because 4C only requires knowledge of the bait sequences.

GCC (Rodley, et al., 2009), Hi-C (Lieberman-Aiden, et al., 2009) and the Duan modified Hi-C method (Duan, et al., 2010) do not require previous knowledge of the interacting sequences instead relying on global sequencing of the purified ligated DNA. Therefore, global identification of chromosome interactions can be achieved. However, loss of sequences can be a problem. Firstly, purification may cause loss of information. All global sequencing methods have purification steps. GCC has the least amount of purification, while both Hi-C and the Duan modified Hi-C method require multiple rounds of purification, including gel purification and biotin pull down. Additionally, Hi-C only uses sheared DNA fragments between 300 and 500 base pairs (bp) in the biotin pull down. It is possible that interactions are missed because they are outside the 300-500 bp size range. The Duan modified Hi-C method uses Eco15I to cut out DNA fragments containing both interacting partners and biotinylated adaptor. However, it has been shown that it is very difficult to completely digest DNA using Eco15I, even with 15 fold molar enzyme excesses to recognition site excess (Möncke-Buchner, et al., 2009). This would also cause loss of information.

Furthermore, the mapped interactions are averaged across the whole population. This is true for all proximity ligation based methods, not just the global sequencing methods. This is not due to any inherent fault of the methodology, but due to the fact that populations of cells are used. In order to remedy this problem and improve the efficiency of proximity ligation based methods, this research strives to decrease the number of cells used in the experiment, with the ultimate goal of using single cells only.

Single cell studies have been performed for various purposes. One of the main reasons for using a single cell is the scarcity of samples. For genetic mutation screenings, for example preimplantation genetic diagnosis (PGD), only a very minute amount of cells are available (Fiegler, et al., 2007; J. B. Geigl, et al., 2009; Handyside, et al., 2004; Hellani, et al., 2004; Y. Li, et al., 2005; Ling, et al., 2009; Nasri, Jamal, Abdullah, Razi, & Mokhtar, 2009; Ren, et al., 2007; Schowalter, Fredrickson, & Thornhill, 2007; J. Wei, Wang, Zheng, Zheng, & Huang, 2007). Microbiologists also use single cell techniques as a method to study unculture able microorganisms due to the scarcity of samples (Bouzid, et al., 2010; Kvist, Ahring, Lasken, & Westermann, 2007; Marcy, Ouverney, et al., 2007; Rodrigue, et al., 2009; Stepanauskas & Sieracki, 2007; Zhang, et al., 2006). Another reason for using single cell methods is to study variations between individuals (J. Geigl & Speicher, 2008). This includes variation of the genome (Fiegler, et al., 2007; J. B. Geigl, et al., 2009; Imle, Polzer, Alexander, Klein, & Friedl, 2009; Ling, et al., 2009; Nasri, et al., 2009), variations in chemical make-up (Mellors, et al., 2010), variations in enzymatic activity (McClain, et al., 2003), variation in expression patterns and variation in expression patterns

under different conditions (Tang, et al., 2009; Taylor, et al., 2009).

1.2. Background information on restriction enzymes used

As mentioned before, part of the GCC protocol for capturing chromosomal interactions involves the digestion and re-ligation of DNA. These two steps of the GCC protocol will be carried out in microfluidic chips. It is therefore important to characterize digestion and ligation inside microfluidic chips. EcoRI and MspI were used to characterize digestion within the microfluidic chips. EcoRI was chosen because there is a lot of data on EcoRI kinetics in the literature (Aiken, McLaughlin, & Gumport, 1991; Kettling, Koltermann, Schwille, & Eigen, 1998; Langowski, Urbanke, Pingoud, & Maass, 1981; X. M. Li, Song, Zhao, & Li, 2008; McLaughlin, Benseler, Graeser, Piel, & Scholtissek, 1987) and can therefore be used to validate the kinetic measurements.

MspI was chosen because previously published GCC analysis of *Saccharomyces cerevisiae* chromosomal interaction used MspI (Rodley, et al., 2009). Restriction enzymes with different specific recognition sites will produce different restriction maps. Therefore, using the same restriction enzyme will ensure the data collected from single-cell experiments can be correlated with previous data collected from GCC experiments performed on populations.

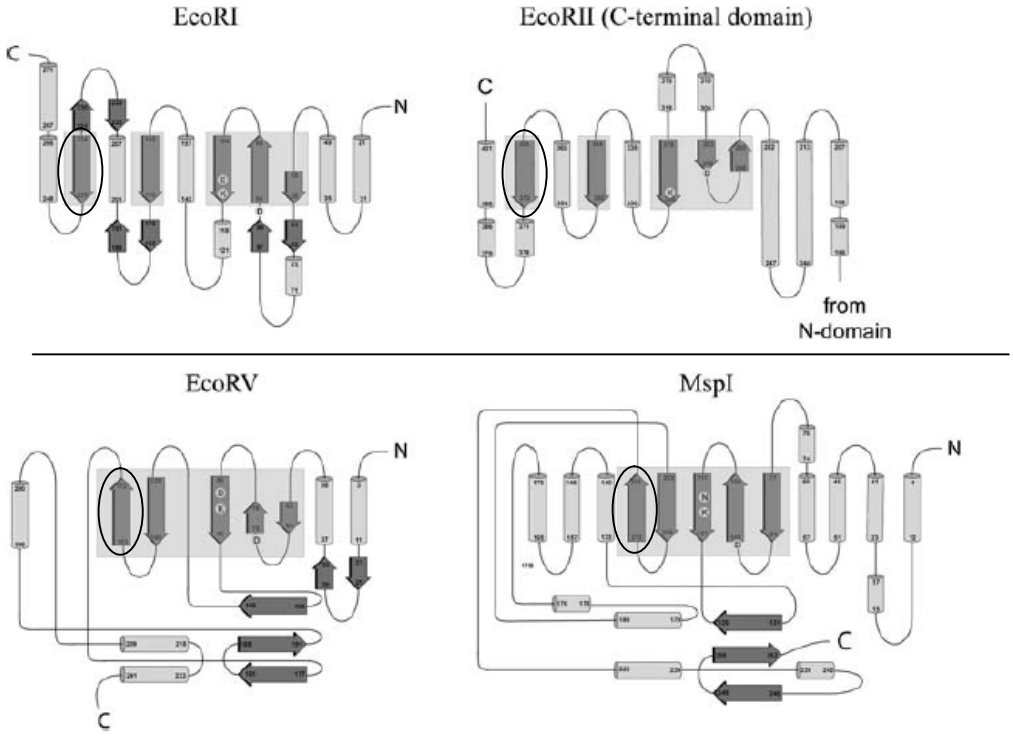
Due to problems encountered during characterization of DNA digestion within microfluidic chips, it is beneficial to have some background information on the two restriction enzymes. Both EcoRI and MspI belong to the Type IIP (palindromes) restriction enzyme family which recognizes symmetric sequences (Roberts, et al., 2003). There are a total of 11 subtypes in Type II.

Some are defined by their recognition sequences, such as Type IIP which recognizes a symmetrical sequence; others are defined by their structure, such as Type IIH which has a similar structure as Type I but acts as Type II. The subtypes are not mutually exclusive and some enzymes can belong to two subtypes, for example, DpnI is a Type IIP and Type IIM because it cleaves a symmetrical (Type IIP), methylated (Type IIM) target. Type II restriction enzymes can also be divided into two different branches based on the orientation of one of the β-sheets in the core structure, which is defined by five β-sheets flanked by one α-helix at each end (Venclovas, Timinskas, & Siksnys, 1994). The fifth β-sheet is parallel to the fourth β-sheet for enzymes in the EcoRI branch (Huai, et al., 2000) but anti-parallel for enzymes in the EcoRV branch (Anderson, 1993). EcoRI is the classic example of EcoRI branch, while MspI belongs to the EcoRV branch (Fig. 5).

Regardless of which branch or subtype a Type II restriction enzyme belongs to, the binding and cleavage mechanism is the same. The restriction enzyme first binds non-specifically to the DNA then searches for the recognition site by repeated dissociation and re-association, and/or linear diffusion along the DNA. Once the recognition site is found, a conformational change is induced and the DNA is cleaved at the specific site (A. Pingoud & Jeltsch, 1997, 2001).

Both EcoRI and MspI require the Mg^{2+} ion as cofactor for cleavage but not for binding (V. Pingoud, et al., 2009; Vipond, Baldwin, & Halford, 1995; Woodhead, Bhave, & Malcolm, 1981; Xu, Kucera, Roberts, & Guo, 2004). EcoRI binds DNA as a homodimer (Muir, et al., 1997; A. Pingoud & Jeltsch, 1997; Watrob, et al., 2001), while MspI is one of the few Type II restriction nucleases that binds DNA

as an asymmetric monomer at least in crystals (Xu, et al., 2004). However, it is currently uncertain whether MspI can dimerize on DNA similar to FokI (Wah, Bitinaite, Schildkraut, & Aggarwal, 1998; Wah, Hirsch, Dorner, Schildkraut, & Aggarwal, 1997) and Sau3AI (Friedhoff, Lurz, Luder, & Pingoud, 2001).



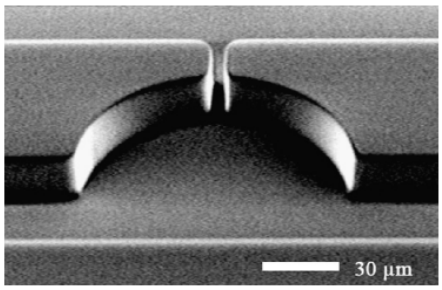
(A. Pingoud, Fuxreiter, Pingoud, & Wende, 2005)

Figure 5. Structure of EcoRI and EcoRV branches of Type II restriction enzymes. The α-helices are light gray. The β-sheets are dark gray. The 5 β-sheets of the core structure are in shaded gray boxes. The black circle showed the β-sheet that defined whether the enzyme is in the EcoRI branch or the EcoRV branch of Type II restriction enzymes. EcoRI and EcoRII are in the EcoRI branch of Type II restriction enzymes. EcoRV and MspI are in the EcoRV branch Type II restriction enzymes.

1.3. Isolating single cells

A prerequisite for studying single cells is the ability to isolate one cell from a population of cells. Several methods have been utilized to isolate a single cell from culture or cell mixture. For single-cellular organisms, these methods

generally fit into one of three groups: single cell isolation through serial dilution, single cell isolation aided by microfluidic chip and single cell isolation by micromanipulation. Serial dilution is the most labour intensive but it does not require complicated setups or the use of expensive machinery (Zhang, et al., 2006). The difficulty of serial dilution lies in confirming the presence of a single cell and then loading the single cell. Isolating single cells using microfluidic chips is usually aided by either microscopic methods, such as fluorescence-activated cell sorting (FACS) (Huang, et al., 2007; Marcy, Ishoey, et al., 2007; Marcy, Ouverney, et al., 2007; Rodrigue, et al., 2009), or electro-osmosis so the process can be automated (Mellors, et al., 2010). A particularly interesting study used custom designed poly-dimethylsiloxane (PDMS) chips that capture single cells by altering the flow rate of two connected micro-channels (Fig. 6) (Yamaguchi, et al., 2009). This design also allowed lysis and growth of the captured cell on the same chip.



(Yamaguchi, Arakawa, Takeda, Edagawa, & Shoji, 2009)

Figure 6. Scanning electron microscope (SEM) image of PDMS chip. Cells flow through the bottom channel at faster flow rate than top channel, trapping a cell at the thin channel connecting the top and bottom channels.

Micromanipulation centers on the use of a micromanipulator and the ability to 'pick up' a single cell (Imle, et al., 2009; Krylov, et al., 2000). For example, Krylov, et al. (2000) lined up the cell and capillary tube using a

micromanipulator, then transferred the cell from the plate to the capillary tube by siphoning or electro-osmosis. For multi-cellular organisms, laser assisted micro-dissection made it possible to isolate specific cells from tissues (Frumkin, et al., 2008). Laser-assisted micro-dissection is a more advanced, automated version of micromanipulation. It uses a computer-aided robot, microscope and laser to dissect a single cell from tissue with precision (Frumkin, et al., 2008).

1.4. Whole genome amplification

After acquiring a single cell the interaction has to be assessed using the proximity ligation based methods like 3C. Due to the small amount of DNA in one cell, whole genome amplification (WGA) has to be performed following DNA digestion and ligation to obtain sufficient template for DNA sequencing or PCR detection. Apart from the original PCR-based WGA method, several newer and more effective methods are available (Barker, et al., 2004; Bergen, et al., 2005; Dean, et al., 2002; Handyside, et al., 2004; Jiang, Zhang, Deka, & Jin, 2005; Y. Li, et al., 2005; Nelson, et al., 2002; Panelli, Damiani, Espen, Micheli, & Sgaramella, 2006; Schowalter, et al., 2007; Spits, et al., 2006a, 2006b). However, despite the improvements, these newer WGA methods still have some inherent problems when amplifying very small amount of samples (less than 1ng) (Bouzid, et al., 2010; Ling, et al., 2009; Marcy, Ishoey, et al., 2007; Rodrigue, et al., 2009; Zhang, et al., 2006). These problems can affect the quality of the results obtained.

Bouzid, et al., (2010) tested the performance of several commercial WGA kits using *Cryptosporidium* clinical isolates. Two different WGA methods were employed by different kits: Multiple displacement amplification (MDA) and

linker ligation mediated amplification (OmniPlex). Out of the parameters tested, the results for amplification success rate and product fidelity are of particular interest. Results obtained suggested that MDA had the potential to perform better than OmniPlex. Out of the two MDA-based kits tested, GenomiPhi and REPLI-g, GenomiPhi had the highest success rate and product fidelity out of all the commercial kits tested. However, REPLI-g did not perform as well as the OmniPlex kits. The difference in performance may be due to the organism or cell type used, or because the manufacturers' protocol is not optimized for a particular cell-type or for use on a small amount of DNA (Glentis, et al., 2009; Ren, et al., 2007). Furthermore, Bouzid, et al., (2010) did not test the kits using single cells; they tested using the minimum amount of DNA recommended by the manufacturers (1~10ng). Therefore, the problems encountered may be more severe when the DNA content of one cell is amplified.

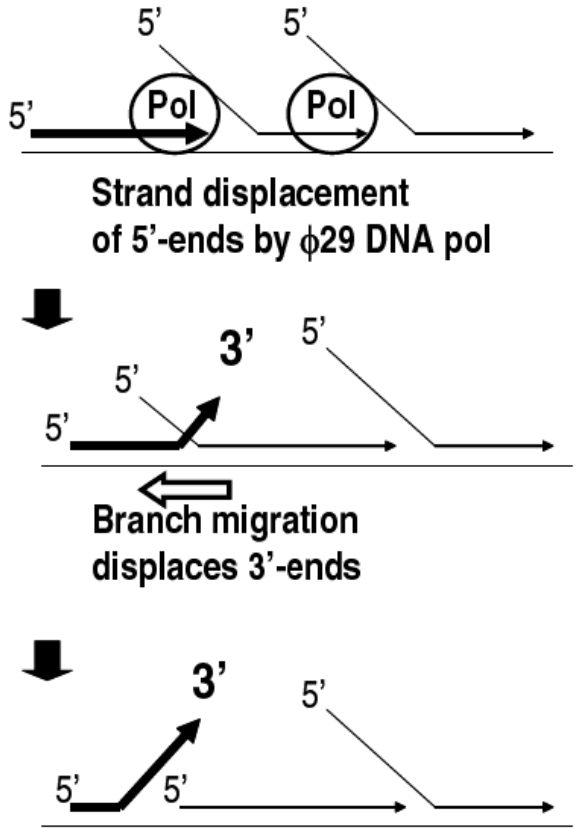
Regardless of the difference in performance, OmniPlex is not a good choice for the purpose of studying single-cell chromosome interaction, because this required the ligation of adaptor sequences to the DNA fragments created by random chemical cleavage (Barker, et al., 2004; Bergen, et al., 2005). As some DNA fragments generated from the steps before WGA may be circular fragments, the addition of adaptors would be difficult. On the other hand, MDA is a type of isothermal amplification using phage DNA polymerase, Phi 29, and degenerate hexamers (Bouzid, et al., 2010; Dean, et al., 2002; Nelson, et al., 2002). Phi 29 was first characterized by Blanco & Salas (1984). It produces a higher yield from single cell material than Taq polymerase, has higher processivity and has a much lower error rate than Taq polymerase due to proof-reading ability (Handyside, et al., 2004; Nelson, et al., 2002). However,

even though MDA produces less non-specific amplification than other PCR-based methods, non-specific amplification is still present, especially when very small amounts of DNA template are used (less than 1ng) (Marcy, Ishoey, et al., 2007; Rodrigue, et al., 2009). As shown by Marcy, et al. (2007), as the reaction volume reduces, the amount of non-specific amplification also decreases, therefore, it is possible to further reduce non-specific amplification by minimizing the total reaction volume. The smallest total reaction volume tested was 60 nl. Rodrigue, et al. (2009) also suggested using duplex-specific nuclease to degrade abundant sequences. This would act as a type of internal normalization and, at the same time, free up resources so the less abundant sequences can be amplified.

Additionally, amplification bias (AB), preferential amplification (PA), and allele drop out (ADO) can reduce the representativeness of the results or cause false positives (Hellani, et al., 2004; Ling, et al., 2009; Nasri, et al., 2009). These problems are generally due to unequal amplification of template near the beginning of the amplification cycle, caused by different binding efficiency or random chance (Hellani, et al., 2004; Ling, et al., 2009; Nasri, et al., 2009) . More template copies will increase the likelihood of polymerase binding. Both AB and PA are situations where certain sequences are over-amplified when compared with other sequences in the same amplification process. This can drown out signals from other sequences and cause false results. ADO is a form of under-amplification, which can lead to the failure to identify heterozygous alleles, hence the name "allele drop out". For example, the ADO rate for 40 single cells amplified using MDA was 10.25% when assessed by sequencing a known mutation in the beta globin gene and 5% when assessed by fluorescent

PCR analysis of heterozygous loci in the short tandem repeats (STRs) (Hellani, et al., 2004). Both over- and under- amplification can cause problems in WGA of single cells by producing false results (Rodrigue, et al., 2009). However, despite the difficulty in recovering a complete genome from a single cell using MDA; it is possible to obtain a near complete genome sequence using MDA products (Jiang, et al., 2005; Rodrigue, et al., 2009; Zhang, et al., 2006).

Furthermore, it had been reported that MDA can produce chimeric products (Lasken & Stockwell, 2007; Rodrigue, et al., 2009; Zhang, et al., 2006). Lasken & Stockwell (2007) had deciphered the mechanism of chimera formation during MDA (Fig. 7). Phi 29 displaces the 5′ end of the newly synthesized DNA strand in front of it while it is replicating DNA, allowing several polymerases to attach and replicate on the same template, one after another (Dean, et al., 2002). This is the foundation of the high processivity of Phi 29, but this is also the cause of chimera formation. The displaced strands would compete with the previous strand for association with the template strand. If the displaced strand re-anneals, the 5′ end of the displaced strand would dislocate the 3′ end of the previous strand. The loose 3′ end can then prime other already displaced 5′ ends, forming chimeras (Fig. 7). Due to this chimera forming mechanism, the chimeras formed are generally between up-stream and down-stream sequences and are rarely formed between different strands. Resolving the mechanism offered some options for reducing chimera formation (Lasken & Stockwell, 2007) and it has been confirmed that the addition of S1 nuclease can reduce chimera formation (Zhang, et al., 2006).



(Lasken & Stockwell, 2007)

Figure 7. Mechanism of chimera formation of phage polymerase phi 29. Branch migration displaces 3′ end. The displaced 3′ end can prime other 5′ ends, creating a chimera.

1.5. Microfluidic chips

Another important aspect of studying single-cell chromosome interaction is the utilization of microfluidic chips (Fig. 8) due to the minute quantity of DNA in a single cell. There are a large number of applications for microfluidic chips, ranging from aiding in the construction of nano- and micro-structures to the diagnosis of diseases in developing countries (Fig. 9) (Gai, Li, Silber-Li, Ma, & Lin, 2005; Lee, et al., 2010; Lenshof & Laurell, 2010; Malic, Brassard, Veres, & Tabrizian, 2010; Mark, Haeberle, Roth, von Stetten, & Zengerle, 2010; Marre & Jensen, 2010; Yang, Xu, & Wang, 2010). Despite the wide range of applications,

the main reason for adopting microfluidic chips in different methods is remarkably similar. Microfluidic chips provide an easy, time-efficient, and cost-efficient alternative for various tests. Microfluidic chips can achieve this due to three general reasons.

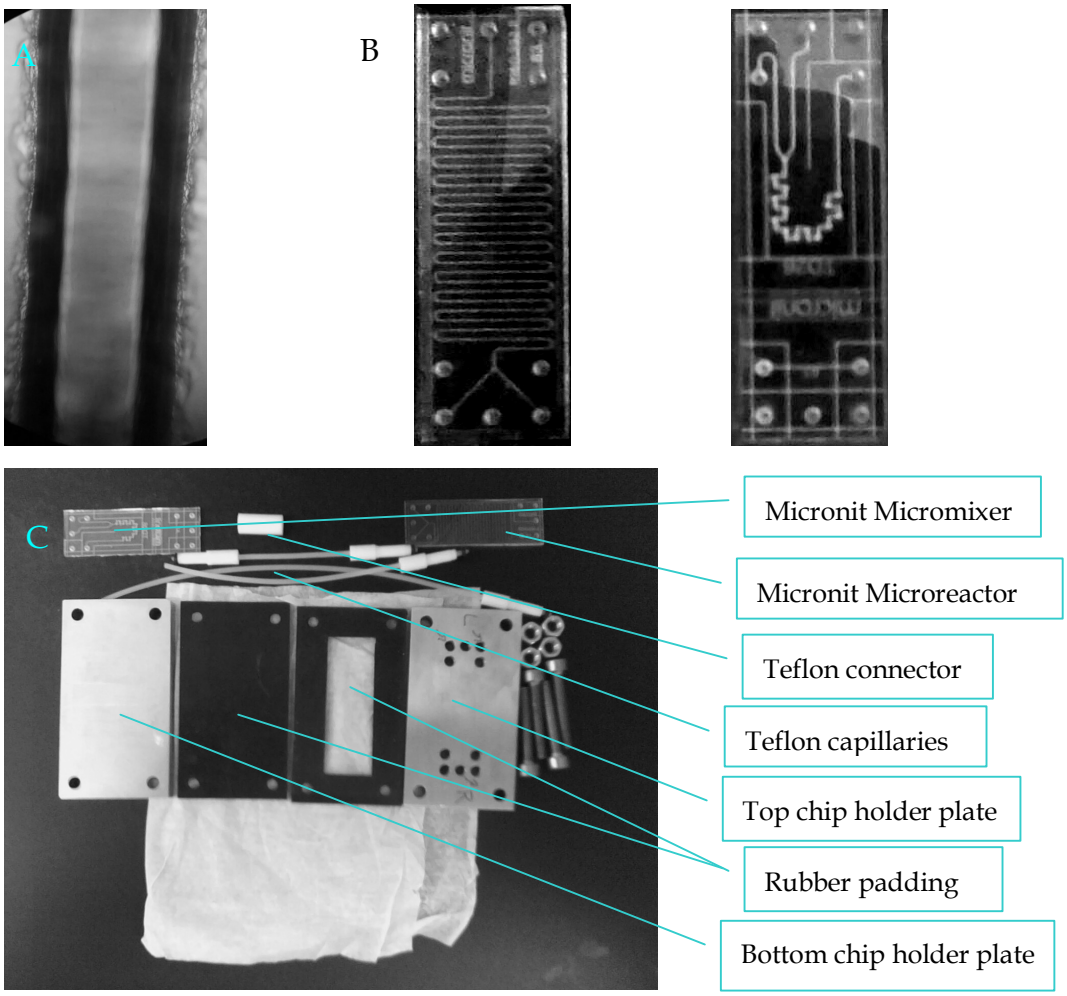


Figure 8. Microfluidic chips used in this project. **A,** Channel of Microreactor at 400x magnification. **B**, From left to right: Microreactor; Micromixer. **C**, Components of lab-on-a-chip used in this project.

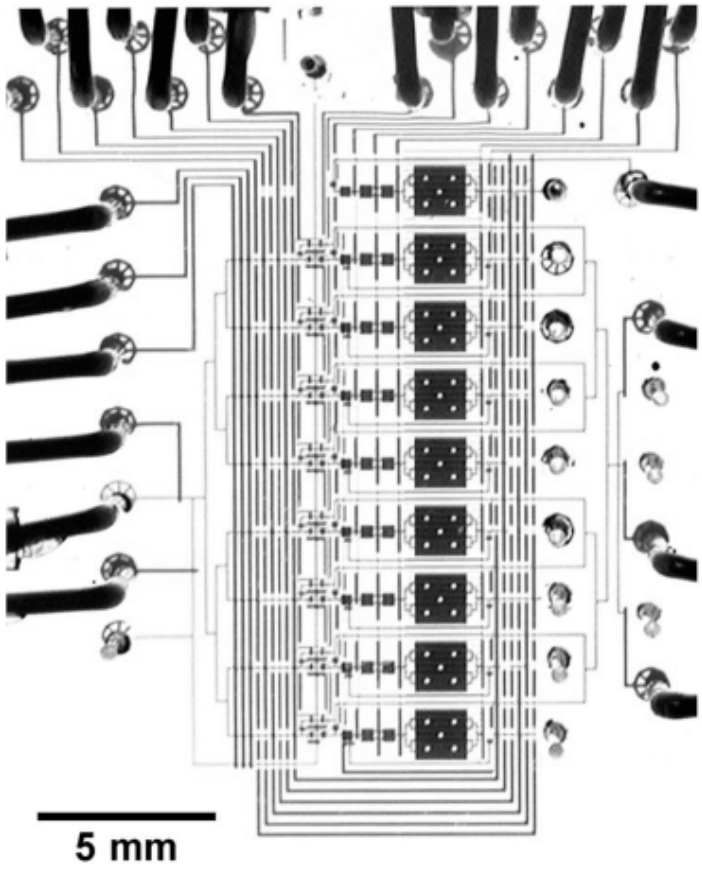
(Lee, Kim, Chung, Demirci, & Khademhosseini, 2010)
Figure 9. Disposable microfluidic device. A example of an automated, disposable, and small diagnostic microfluidic chip that allows fast primary and / or urgent medical diagnostic to be carried out on the spot. Wireless technology can be fitted to enable wireless interface for monitoring. This can be especially useful in developing countries.

Firstly, almost all microfluidic chip designs allow for the automation of experiments (Huang, et al., 2007; Marcy, Ishoey, et al., 2007; Marcy, Ouverney, et al., 2007; McClain, et al., 2003; Mellors, et al., 2010; Ros, Hellmich, Regtmeier, Duong, & Anselmetti, 2006; Taylor, et al., 2009; Wang & Lu, 2006; Yamaguchi, et al., 2009; Yin, et al., 2007). Simply load the samples and reagents into various computer controlled pumps, then activate the preset program that coordinates the flow rate, and wait. Even without a computer, the pumps can be easily controlled manually using pre-determined times or start signals.

Secondly, microfluidic chips can streamline experiments by allowing the user to run several experiments in parallel (Fig. 10) or in series (Fig. 11, and 7). Running parallel experiments only requires multiple channels and the ability to allow one cell to flow through each channel separately (Huang, et al., 2007; Marcy, Ishoey, et al., 2007; Marcy, Ouverney, et al., 2007; Taylor, et al., 2009). Alternatively, running experiments in series is carried out continuously in the same channel. Therefore, they first require the dilution of cells so that there is sufficient time between the arrival of each cell for the reaction and subsequent

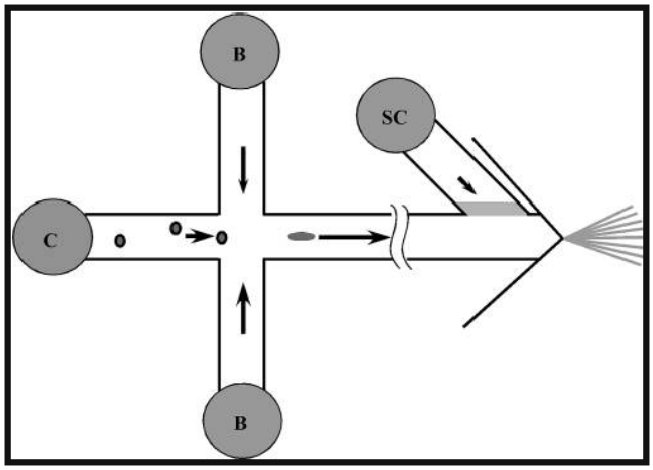
detection to take place (McClain, et al., 2003; Mellors, et al., 2010; Ros, et al., 2006; Wang & Lu, 2006; Yamaguchi, et al., 2009; Yin, et al., 2007).

Thirdly, the volume in the microfluidic chip channel and reaction chamber is very small, minimizing the quantities of both reagents and samples required. This is very important in single cell studies because the quantity of DNA in a single cell is very small and conventional methods using microfuge tubes will dilute the sample too much.



(Marcy, Ouverney, et al., 2007)

Figure 10. Photograph of a single cell isolation and genome amplification chip that can run up to 9 samples in parallel. Each reaction chamber (three small black squares and one large black square) is separated by valves that open systematically to enable further reactions.

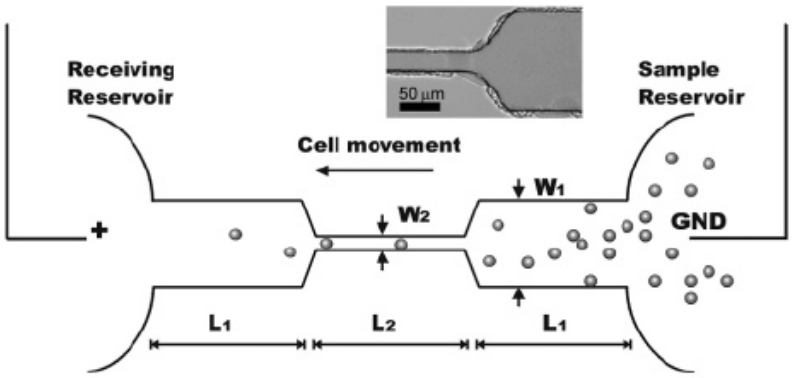


(Mellors, Jorabchi, Smith, & Ramsey, 2010)

Figure 11. Schematics of a cross chip connected to electrospray orifice. "**C"** contains cell reserve,"**B"** contains buffer. While electrodes are connected to **B's** and **C**. Electrolysis occurs at cross junction. As illustrated in the diagram, single-cells can be analyzed in series.

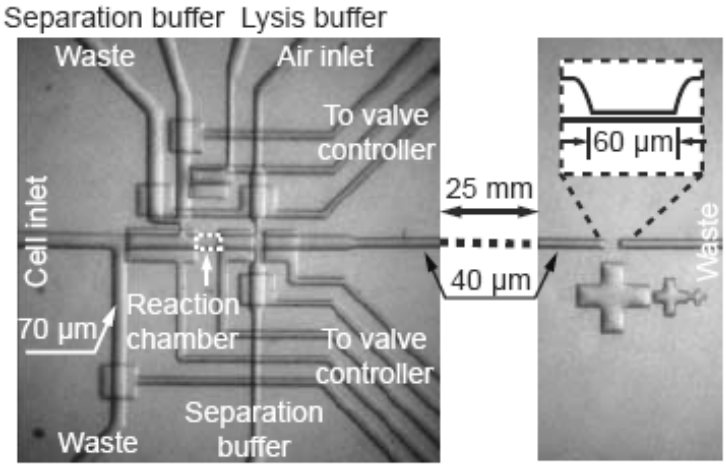
Furthermore, just as there are many applications for microfluidic chips, there are as many different microfluidic chip setups. This is because each setup is tailored to the needs of the experiment. The diversity is partly due to the ease with which PDMS chips can be customized (Love, Wolfe, Jacobs, & Whitesides, 2001) and partly due to the wide range of applications for the chips. For some setups used in single cell experiments, a general cross chip is incorporated (Fig. 11). The cross chip allows electrodes to be applied at various points so that the electro-current at the junction would be strong enough to lyse the cell. The electro-current can also be used to direct the cell or cell content to the desired destination via electro-osmosis (McClain, et al., 2003; Mellors, et al., 2010; Ros, et al., 2006). Other simpler designs only have a single channel and observe the effects of various stimuli applied on the single cells (Fig. 12) (Wang & Lu, 2006; Yin, et al., 2007). Some have reaction chambers where samples are systematically treated with various reagents (Fig. 10 and 8) (Huang, et al., 2007;

Marcy, Ishoey, et al., 2007; Marcy, Ouverney, et al., 2007). For example, one design pushes the sample back and forth between different chambers or channels and fills them with appropriate reagents (Fig. 13) (Huang, et al., 2007), while another design has several chambers in series and systematically opens up later chambers one by one, filling them with the required reagents (Fig. 9) (Marcy, Ishoey, et al., 2007; Marcy, Ouverney, et al., 2007).



(Wang & Lu, 2006)

Figure 12. A schematic of a simple one channel microfluidic chip. Electrodes enabled electoporation and observation of cells at L2.



(Huang, et al., 2007)

Figure 13. An example of a more complex microfluidic chip design. Cell manipulation occurs on the left, molecule counting section on the right. The cell is pushed around the various chambers and channels around the reaction chamber while the reaction chamber is flushed then refilled with reagents.

1.6. Mapping chromosomal interactions within a single cell

Determining the 3D arrangement of chromosomes in a single cell using LOC has several theoretical advantages over similar experiments performed on populations of cells. The most obvious advantage is in the decrease in the resources that are required. There is not just a substantial decrease in reagents used, once automated, human resources can also be conserved. Furthermore, the decrease in human handling also leads to a decrease in the introduction of human errors. Currently, billions of yeast cells are used in bulk and it is only possible to receive an average map across the whole of the population. Utilizing single cell methods, it is possible to focus on a single individual and determine the degree of randomness in chromosome interactions. Similar to other single cell studies, this would allow further research into the variations between individuals, giving a less generalized view of the 3D genome interaction. Additionally, interactions will not be lost during the purification process because no purification will be required. However, a major disadvantage in using single cells in the study is that only a maximum of two interactions can be captured for each DNA fragment. If one particular sequence interacts with 2 or more DNA fragments, only one of these interactions may be captured due to the capturing method used. This is less of a problem when similar experiments were conducted in bulk, because the other interactions would also be present within the population of ligated products. Despite this disadvantage, mapping the chromosomal interactions of a single cell can still be used to confirm the presence of interactions and reveal more information about chromosome interactions.

This research is a part of a larger project to map the 3D chromosome interactions of a single cell. This requires isolation of a single cell and the scaling down of GCC, 3C or other proximity ligation based methods to capture and map the chromosome interaction. In order to achieve this overall goal, LOC will be used to analyze the minute quantity of DNA within a single cell. This research aims to build and test a LOC that will aid in the capture of inter- and intra- chromosomal interactions of a single cell and to characterize the restriction and ligation of DNA that will be performed in LOC. The methodology developed in this research will be applicable to all organisms, even organisms with limited samples such as embryonic stem cells or uncultureable organisms.

2. Equipment

2.1. Microfluidic chips

Microfluidic chips were purchased from Micronit (Enschede, Netherlands). Two different models were obtained: Microreactor (6µl internal volume; Micronit, FC_R150.332.2_PACK) and Micromixer (2µl internal volume; Micronit, FC_TD26_PACK). Teflon capillaries with an (inner diameter [ID]: 250µm; internal volume: 0.491µl/cm; Micronit, FC_TF_KIT) were also purchased. The ends of the Teflon capillaries connecting to the microfluidic chips were machined at an angle to fit into the wells of the microfluidic chips (Fig. 14). O-rings (ID = 1mm, cross section = 1mm, MR001X1, Seal Imports Ltd., New Zealand.) were used to create a leak-proof seal around the capillary-chip junction. A small groove was machined into the capillaries to prevent the o-ring from moving (Fig. 14). The chip holders used were manufactured in-house with the stainless steel plates manufactured from 6mm thick sourced and laser machined by ETech industry New Zealand Ltd. according to the dimensions shown in Fig. 15. Two butyl rubber paddings were also made (Fig. 16): one to fit between the microfluidic chip and the base plate to cushion the microfluidic chip (base padding); and a second one to fit around the microfluidic chip and hold the microfluidic chip in place (top padding).

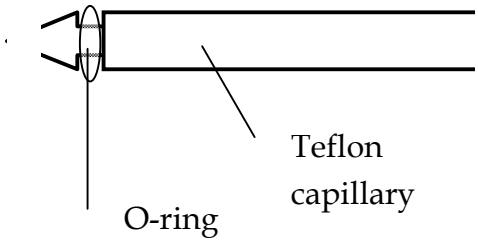


Figure 14. Diagram illustrating the modification of the Teflon capillary ends. The Teflon was machined with an indent to prevent o-ring movement. Not drawn to scale.

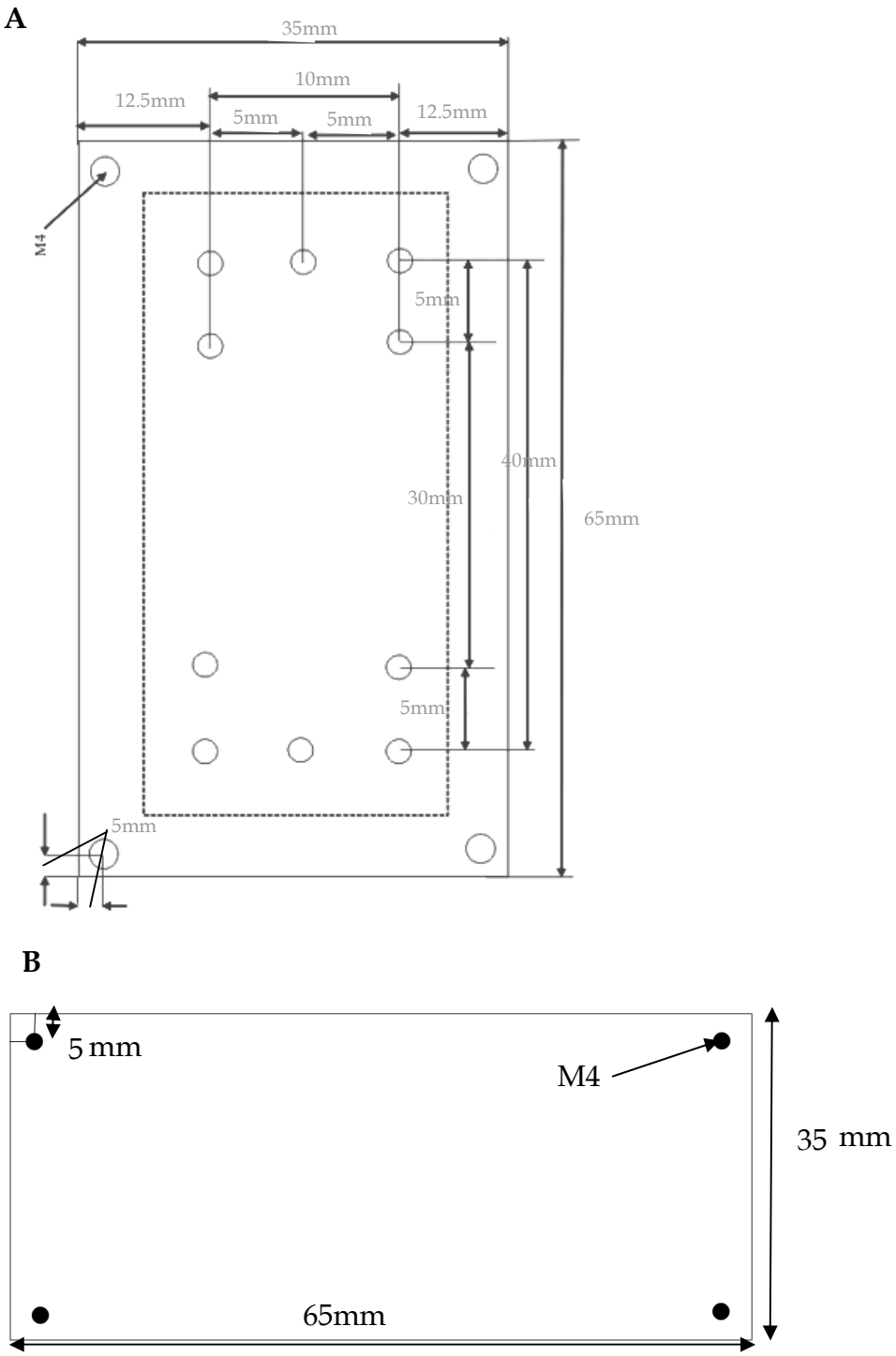


Figure 15. Schematics of chip holders. Bolts (M4x25) are positioned at the four corners to secure the chip holder together. Not drawn to scale. **A** Top plate. The dotted lines represent the approximate size of the microfluidic chip. The 10 circles in the middle of the plate correspond to the wells on the chip, which were half threaded to secure the capillaries in place. **B** Base plate.

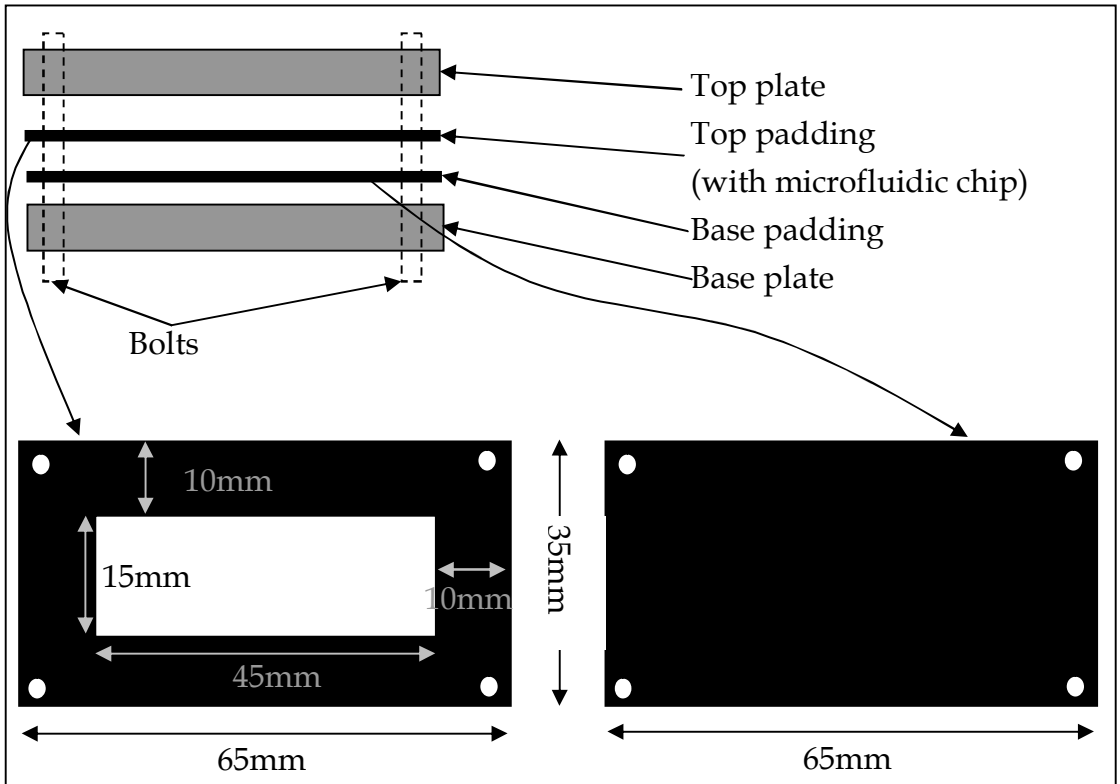


Figure 16. Assembly of chip holder with dimensions for top and base padding. Bolts (M4x25) are positioned at four corners to secure the chip holder together.

Different chips were connected in series and were used in various experiments. The following series were used as standard:

Reaction-mixing-reaction chip series (R-M-R series):

This series contained three microfluidic chips. The outflow of the first microreactor chip was connected to a micromixer chip, which was subsequently connected to a second microreactor chip (Fig. 17).

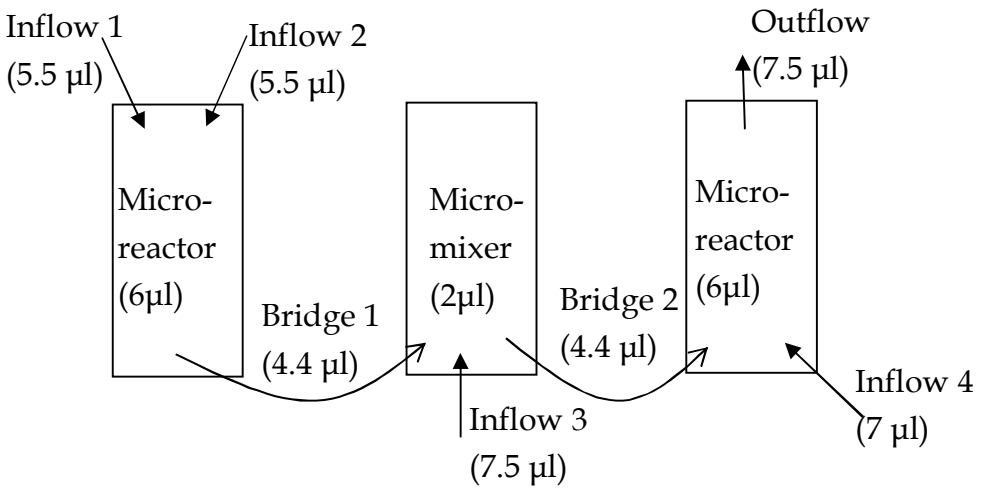


Figure 17. Schematic of reaction-mixing-reaction chip series (R-M-R series). Not drawn to scale.

Two-chip setup:

Three different versions of a two-chip setup were used (Fig. 18):

1. The reaction-reaction series (R-R series) contained two microreactor chips in series.
2. The reaction-mixing series (R-M series) contained one microreactor chip flowing into a micromixer chip.
3. The mixing-mixing series (M-M series) contained two micromixer chips in series.

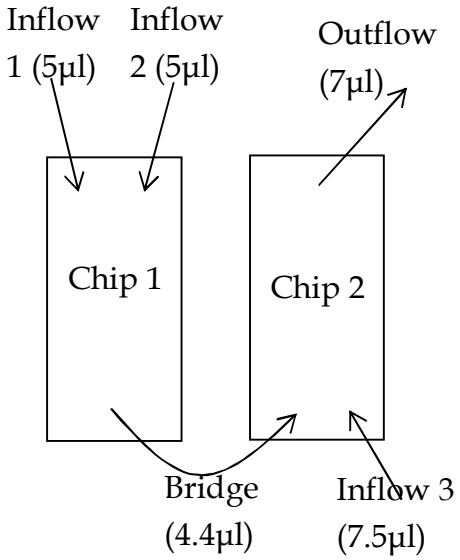


Figure 18. Schematic of two-chip setup. In R-R series: Both chips were microreactor chips (6µl). In R-M series: Chip 1 was a microreactor chip (6µl), and chip 2 was a micromixer chip (2µl). In M-M series: Both chips were micromixer chips (2µl).

Single chip:

Single chip setups were also used (Fig. 19).

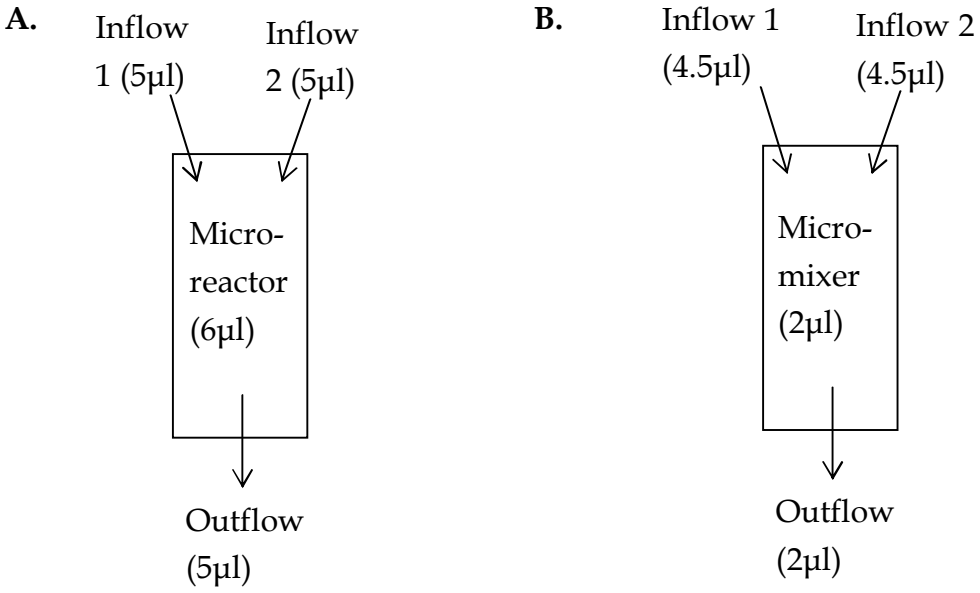


Figure 19. Schematic of single chip setup. Not drawn to scale. **A.** Single microreactor chip. **B.** single micromixer chip.

Cleaning protocol

The microfluidic chips were cleaned by first pumping 1ml of 70% ethanol then pumping 1ml of 5% bleach through the chips. The microfluidic chips were then rinsed with 2ml of Milli-Q water twice. The wash cycles were driven by Becton, Dickinson and company (B.D) luer lock 3 c.c syringes. Refer to section 2.2 and Table 1 for more details on syringes.

2.2. Syringes

The syringes used in this study are listed in Table 1.

Company	Syringe model	Internal volume	Inner diameter
Hamilton [a]	RN701	10 µl	0.485 mm
Hamilton [a]	RN702	25 µl	0.729 mm
Hamilton [a]	RN710	100 µl	1.457 mm
Hamilton [a]	RN750	500 µl	3.256 mm
Becton , Dickinson and Company [b] (B.D)	Luer lok 3c.c (309585)	3 ml	8.585 mm

Table 1. Syringes used in this study. [a.] Hamilton (Nevada, USA). [b.] Becton, Dickinson and Company (Franklin Lakes, NJ, USA)

Hamilton syringes containing reaction mixtures were connected to the Teflon capillaries by RN adaptors (dual ferrule, Hamilton, catalogue No. 55751-01). The B.D syringes containing washing liquid were connected to the Teflon capillaries by Teflon connecters manufactured in-house from segments (20 to 40mm) of ¼" Teflon rod with a $^{1}/_{16}$" hole drilled through the centre.

2.3. Syringe control

NE-1000 Multi-Phaser pumps (New Era Pump Systems Inc., Farmingdale, NY, USA) were used to control the syringes. Refer to the NE-1000 Multi-Phaser pump manual for manual controls.

For all experiments in this work, pumps were computer controlled. Pumps were connected to the computer using a USB to RS-232 converter (USB-232; National Instruments, Austin, TX, USA) to convert a USB port on the computer

to a RS-232 port. Virtual instruments (VI) were developed using LabView 2009 (National Instruments) and were used to control the pumps (Appendix A and B). The VI's were developed with the aid of NE-500 drivers found at http://sine.ni.com/apps/utf8/niid_web_display.model_page?p_model_id=8502.

2.4. Protocols for testing lab-on-a-chip

2.4.1. Testing for leakages

Four methods were used to check for leakages and proper sealing between the capillaries and chips. (A) After the lab-on-a-chip (LOC) was filled with water, a quick push/pull test was first conducted by attaching and sealing all inflows and outflow with syringes half loaded with water. If the microfluidics system did not have any leaks or air bubbles inside, infusing or withdrawing from one syringe would create a similar response in another syringe. (B) Next, all inflows and outflows were attached with syringes loaded with water and an air bubble. Pulling or pushing on one plunger would expand or contract the air bubble. If the system was sealed properly, the air bubble would remain expanded or contracted after only one plunger is pulled or pushed. (C) Thirdly, a paper towel was placed under the LOC and 3ml of Milli-Q water (in B.D 3c.c syringes) from each inflow was infused simultaneously at 6ml/hour. If there were no leaks, there would not be visible signs of wetness on the paper towel. The Teflon connectors connecting the B.D syringes to the LOC acted as safety valves that released excess pressure in the system. (D) Lastly, real-time polymerase chain reaction (qPCR) was used to quantify DNA collected from the microfluidic chips and compared with the input.

2.4.2. Characterization of microfluidic chips

The R-M-R chip series (Fig. 17) was used to test-pump reagents through in order to characterize the system.

2.4.2.1. Phenol red indicator test

Phenol red (pH 5.5, 1% w/v; Gibco laboratories, catalogue # 870-1160IJ) was loaded into 500 µl Hamilton syringes and connected to inflow 1 (Fig. 17). Potassium hydroxide (KOH, pH 12) was loaded into two 10 µl Hamilton syringes and connected to inflows 2 and 4 (Fig. 17). Hydrochloric acid (HCl, pH 2) was loaded into 50 µl Hamilton syringes and connected to inflow 3 (Fig. 17). Each inflow was primed (the Teflon capillaries were filled with reagents). The pumping from each inflow was timed so that the pH indicator would mix with the acid or base from each inflow to cause an observable colour change. Specifically, 13 µl from inflow 1 (phenol red, pH 5.5; 1 µl/minute) and 1 µl from inflow 2 (KOH, pH 12, 1 µl/minute) were simultaneously injected. 2 µl from inflow 3 (HCl, pH 2, 1 µl/minute) was injected 9 minutes after the inflow from 1 and 2 was started. Finally, 13.5 µl from inflow 1 (phenol red, pH 5.5; 1 µl/minute) and 2 µl from inflow 4 (KOH, pH 12; 2 µl/minute) were simultaneously injected. Colour changes of phenol red were used to indicate proper mixing of the reagents.

2.4.2.2. Blue Dextran test:

Blue Dextran (2000kDa; Sigma-Aldrich, catalogue # D5751-1G) was used to mimic DNA (average molecular weight of 660 Da/bp) being pumped through the chips from inflow 1. Blue Dextran was added to Milli-Q water until the blue colour could be seen through the capillary (10-20% w/v). 2 µl of Blue Dextran

solution was then pumped through the R-M-R chip series (Fig. 17) with Milli-Q water before and after the Dextran solution and the spread of the blue dye was measured (Table 2). Dextran spread by 0.05 µl/cm of channel travelled (spread 1.8 µl in total) when flown through a microreactor chip (6 µl) and increased by 0.1µl/cm of channel travelled (spread 1.2µl in total) when flown through a micromixer chip (2µl). In the Teflon capillary, the Dextran increased by 0.06 µl/cm. The reason that the Dextran increased more in volume when moving through the micromixer chip was because the micromixer chip is designed to mix the solutions. Therefore, the lagging end of Dextran will be mixed with the Milli-Q water following the Dextran. Estimate is shorter than actual because the opacity of the Teflon tube masked fainter colours in the lagging tail of the Dextran.

	Before the first chip (micro-reactor chip)	Before the second chip (micromixer chip)	Before the third chip (micro-reactor chip)	After the third chip (micro-reactor chip)
Mean (n=5)	2.1±0.39 µl	4.1±0.84 µl	5.3±0.50 µl	6.9±0.76 µl

Table 2. Volume taken up by blue Dextran through R-M-R chip series (Fig. 17).

2.4.2.3. Using a gas bubble to stop expansion of blue Dextran

Dextran was pumped through a water-filled R-M-R chip series (Fig. 17) with gas bubbles separating the Dextran solution from the surrounding water. The presence of gas bubbles did limit Dextran expansion, however the gas bubbles were observed to move backwards in an apparently random manner. The random nature of the movement would cause problems in synchronizing the delivery of reagents into each of the microfluidic chips. The exact cause of the

movement was undeterminable; however it was observed that the gas bubble inside the syringes, especially for syringes with larger than 25 µl internal volume, would often shift backwards in a similar fashion.

Dextran was also pumped into a completely air-purged R-M-R chip series (Fig. 17) followed by a gas bubble then water. Upon the Dextran entering the micromixer chip, enough pressure was created to cause the gas bubble between Dextran and water to compress. This caused the fluid to exhibit a start-stop movement instead of a smooth pumping action. Attempts at confining reaction mixtures by using gas bubbles to prevent diffusion were hence abandoned due to the unreliability exhibited in these trials.

3. Materials and Methods

3.1. Enzymes and buffers

Enzyme	Enzyme order information	Buffer used with enzyme
EcoRI (5000 U. 15 U/µl)	Invitrogen, catalogue # 15202-013	500 mM Tris-HCl (pH 7.5) 100 mM $MgCl_2$ 10 mM Dithiothreitol (DTT) 1,000 mM NaCl
T4 DNA Ligase (500 U. 1 U/µl)	Invitrogen, catalogue # 15224-025	50 mM Tris-HCl (pH 7.6) 10 mM $MgCl_2$ 1 mM ATP 1 mM DTT 25% (w/v) polyethylene glycol-8000 (PEG) PEG was replaced with Milli-Q water in no PEG T4 DNA ligase buffer.
MspI (5000 U. 20 U/µl)	New England Biolabs, catalogue # R0106S	20 mM Tris-acetate (pH 7.9 at 25°C) 50 mM potassium acetate 10 mM Magnesium Acetate 1 mM Dithiothreitol
Taq DNA Polymerase with ThermoPol Buffer (400 U. 5 U/µl)	New England Biolabs, catalogue # M0267S	20 mM Tris-HCl (pH 8.8 at 25°C) 10 mM $(NH_4)_2SO_4$ 10 mM KCl 2 mM $MgSO_4$ 0.1% Triton X-100

3.2. Primers

The primers used in this study (Table 3) were designed using Primer 3 (http://frodo.wi.mit.edu/primer3/). Primers were manufactured by Sigma-Aldrich or IDT.

Primers were designed for two purposes: 1) to amplify the components for the restriction and ligation assay; and 2) to amplify across restriction sites to quantify the amount of restriction enzyme and ligase activity.

Primer name	Sequence
Across MspI restriction site:	
gDNAAcross7827_F*	TGTTTTAATGAAGGCACGTCAG
gDNAAcross7827_R*	AAGAGGCGCTTTCCGATAAT
PlasAcross14086_F*	TCAGTCCTTCCTTCCAACTCA
PlasAcross14086_R*	CGACATTGAAACAGCCAAGA
Across re-ligated MspI restriction site:	
PlasgDNA3CF (4586-240262)*	TTCTCGATCCCAGAAGTGCT
PlasgDNA3CR (4586-240262)*	TTAGTCCCACCAGCTCCAAC
Across EcoRI restriction site:	
pUC19overEco#2F*	GTTTTCCCAGTCACGACGTT
pUC19overEco#2R*	ATTAGGCACCCCAGGCTTTA
Ran1RPA135*	GCGCACAAGTGTCGTATATCA
Ran2RPA135*	CATGAGAGACCCAGCCAATAA
For DNA strand without EcoRI or MspI restriction site:	
gDNAoverBglII(8682)F	TTCATTCCCGAAACGTAAGC
gDNAoverBglII(8682)R	AGGCCATGGTGGAGATTTTT

Table 3. Table of primers used in this study. Primers pUC19overEco#2F and Ran2RPA135 are also used to amplify across re-ligated EcoRI restriction site.
* Primers designed by Chris Rodley (Massey University)

3.3. Polymerase chain reaction (PCR)

PCR reactions were performed for 2 purposes:

1. Prepare templates for digestion and ligation assays.
2. Quantify reaction products by real-time PCR (qPCR).

End point PCR was performed on a Palm Cycler (Corbett Research) using the protocol: 95°C, 7minutes; 30 x (95°C, 30 seconds; 58°C, 30 seconds; 72°C, 30 seconds); 72°C, 3 minutes; hold at 20°C. The reagents were listed in Table 4

qPCR was performed on a ABI prism 7000 (Applied Bioscience) using the protocol: 50°C, 2 minutes; 95°C, 2 minutes; 30 x (95°C, 15 seconds; 58°C, 30 seconds; 72°C, 30 seconds [detection step]); 55°C, 1 minute; with dissociation stage of 95°C, 15 seconds; 60°C, 20 seconds; 95°C, 15 seconds. The reagents were listed in Table 5

PCR reagents	x1 (ul)
10 X Thermal Taq Buffer	5.00
DMSO	1.50
Tween 20 (10%)	0.50
dNTP (1.25 mM)	8.00
Milli-Q H_2O	27.00
Primers (10 mM)	3.00
Taq (5 U/μl)	4.00
PCR template	1.00
Total	50.00

Table 4. Reagents for end point PCR reaction.

Reagents	X1 (ul)
iTaq SYBR Green Supermix with ROX (Bio Rad, Catalog# 172-5850)	7.5
Primer (10mM)	1.5
Milli-Q H_2O	4.0
DNA	2.0
Total	15.0

Table 5. Reagents for qPCR reaction.

Pre-EcoRI template 1:

PCR products from primer pairs pUC19overEco#2F + pUC19overEco#2R (template: pUC19)

Pre-EcoRI template 2:

PCR products from primer pairs Ran1RPA135 + Ran2RPA135 (template: gnomic DNA of *Saccharomyces cerevisiae*, BY4741)

EcoRI ligation template:

Pre-EcoRI template 1 and 2 were mixed with equal molar ratio (2.5 ng: 2.5 ng in 1 µl) before they were digested with EcoRI (1.5 U/µl, 37°C, overnight). EcoRI was then inactivated (65°C, 20 minutes).

EcoRI template:

EcoRI ligation template (2.5 ng/µl) was ligated with T4 DNA ligase (0.1 U/µl, 37°C, overnight). The ligated products were used as templates in a PCR reaction (primers: pUC19overEco#2F + Ran2RPA135) to produce the EcoRI template.

EcoRI template is 154 base pairs (bp) in length and contains one EcoRI restriction site and one MspI restriction site.

Pre-MspI template 1:

PCR products from primer pairs PlasAcross14086_F + PlasAcross14086_R (template: plasmid of *S. cerevisiae*, BY4741)

Pre-MspI template 2:

PCR products from primer pairs gDNAAcross7827_F + gDNAAcross7827_R (template: genomic DNA of *S. cerevisiae*, BY4741)

MspI ligation template:

Pre-MspI template 1 and 2 were mixed in equal molar ratio (2.5 ng: 5 ng in 1 µl) before they were digested with MspI (2 U/µl, 37°C, overnight). MspI was then inactivated (80°C, 20 minutes).

MspI template:

MspI ligation template (3.75 ng/µl) was ligated with T4 DNA ligase (0.1 U/µl, 37°C, overnight). The ligated products were used as templates in a PCR reaction (primers: PlasgDNA3CF (4586-240262) + PlasgDNA3CR (4586-240262)) to produce the MspI template.

MspI template is 162 bp in length and contains no EcoRI restriction site and one MspI restriction site.

No-restriction-site template:

PCR products from primer pairs gDNAoverBglII(8682)F + gDNAoverBglII(8682)R (template: genomic DNA of *S. cerevisiae*, BY4741). No-restriction-site template is 207bp in length and contains no EcoRI restriction site and no MspI restriction site.

Presence of all PCR products were confirmed by 1% agarose gel electrophoresis and visualized with ethidium bromide on Gel Doc system (BioRad). All PCR products were purified using DNA clean & concentrator (Zymo Research, catalogue number D4013) according to the manufacturer's instructions.

Quantification of template by qPCR was done by comparing the sample collected with a standard curve generated from known serial dilutions of PCR products of EcoRI template (0.01 to 1000 pg/µl, in steps of 10 fold dilution), MspI template (0.02 to 2000 pg/µl, in steps of 10 fold dilution) or no-restriction-site template (0.02 to 2000 pg/µl, in steps of 10 fold dilution).

3.4. Restriction enzyme digestion in microtubes

Digestions were carried out in microtubes (Axygen, MCT-175-C; 1.7 ml) with EcoRI (Invitrogen) or MspI (NEB) in their respective 1x buffer. MspI (2 U/µl, 0.2 U/µl, 0.02 U/µl, 0.002 U/µl, or 0.0004 U/µl) was used to digest MspI template (9 nM [1 ng/µl]; 37°C, 2 hours). EcoRI (0.05 U/µl, 0.005 U/µl, or 0.0005 U/µl) was used to digest EcoRI template (5 nM [0.5 ng/µl]; 37°C, 2 hours). The amount of undigested substrate was quantified by qPCR.

3.5. Restriction enzyme digestion in microreactor chips

EcoRI template (final concentration 5 nM [0.5 ng/µl]) or MspI template (final concentration 9 nM [1ng/µl]) was digested with EcoRI (final concentration 0.05 U/µl, 0.025 U/µl, 0.005 U/µl or 0.0005 U/µl) or MspI (final concentration 0.02 U/µl, 0.002 U/µl, or 0.0004 U/µl), respectively, within a single microreactor chip (Fig. 19 A). The template was loaded to inflow 1 (Fig. 19 A). Restriction enzyme in 2x buffer was loaded to inflow 2 (Fig. 19 A). Both were injected

simultaneously (1 µl/minute, 1 µl each) and incubated at 37°C for 2 hours. 20 µl was collected from the LOC and products were quantified by qPCR.

3.6. EcoRI digestion kinetics within microtubes

The reaction time and the concentration of EcoRI and EcoRI template for measuring the initial rate were estimated based on previously published EcoRI kinetics data (Kettling, et al., 1998; McLaughlin, et al., 1987). 0.3 nM ~ 10 nM of EcoRI template, 0.1 U of EcoRI and 1 x reaction buffer were reacted in a total volume of 10 µl and incubated at 37°C for 1 minute. The reaction was quenched by snap-freezing at -78°C in a 100% ethanol and dry ice bath. The original template concentration was determined from a reaction that did not contain EcoRI. EcoRI template concentrations in the samples collected were determined by qPCR of a 100 fold dilution of the samples collected. The initial rate of the reaction was determined by first subtracting the original concentration from the substrate concentration at 1 minute then dividing by 60 seconds.

3.7. MspI digestion kinetics within microtubes

The reaction time and the concentration of MspI and MspI template that would produce the initial rate in microtube was estimated using the definition of an enzyme Unit from the manufacturer's web site (1 U of restriction enzyme will digest 1 µg of DNA in one hour in a 50 µl reaction; http://www.neb.com/nebecomm/tech_reference/restriction_enzymes/setting_up_reaction.asp). For MspI kinetics, 3 nM ~ 300 nM of MspI template, 0.5 U of MspI and 1 x reaction buffer were reacted in a total reaction volume of 10 µl and incubated at 37°C for 1 minute. The reaction was quenched by snap-freezing at -78°C in a 100% ethanol and dry ice bath. The original template

concentration was determined from a reaction that did not contain MspI. MspI template concentration in samples collected was determined by qPCR assay of 1000 fold dilution of sample. Initial rate of reaction was determined by first subtracting the original concentration from the substrate concentration at 1 minute then dividing by 60 seconds.

3.8. EcoRI digestion time course in micromixer chips

A single micromixer chip was used (Fig. 19 B). 10 µl of EcoRI template was loaded into a 10 µl Hamilton syringe connected to inflow 1 (Fig. 19 B) and 10 µl of EcoRI in 2 x reaction buffer was loaded into a 20 µl Hamilton syringe connected to inflow 2 (Fig. 19 B). Four combinations of EcoRI and EcoRI template were tested within a micromixer chip (concentration recorded as final concentration within the micromixer chip): 1) EcoRI = $5x10^{-3}$ U/µl, EcoRI template = 0.05 ng/µl; 2) EcoRI = $1x10^{-3}$ U/µl, EcoRI template = 0.05 ng/µl; 3) EcoRI = $5x10^{-5}$ U/µl, EcoRI template = 0.005 ng/µl; and 4) EcoRI = $5x10^{-6}$ U/µl, EcoRI template = 0.5 ng/µl. The two inflows were primed at 6 µl/minute for 45 seconds. The pump rate was then changed to control the reaction time inside the micromixer chip (incubated at 37°C): 2 µl/minute for 60 seconds; 1.5 µl/minute for 80 seconds; 1.2 µl/minute for 100 seconds; 1.0 µl/minute for 120 seconds; 0.857 µl/minute for 140 seconds; 0.667 µl/minute for 180 seconds; 0.571 µl/minute for 210 seconds; 0.444 µl/minute for 270 seconds. The reactions were quenched directly by collection into a microtube on 100% ethanol and dry ice bath (-78°C). The LOC was washed with Milli-Q water before each timed reaction then purged with air.

3.9. EcoRI digestion kinetics in micromixer chips

A single micromixer chip was used (Fig. 19 B). EcoRI template between 0.7 nM ~ 25 nM were loaded onto inflow 1 (Fig. 19 B) and 1 x 10^{-3} U/µl of EcoRI in 2 x buffer was loaded onto inflow 2 (Fig. 19 B). Both solutions were pumped simultaneously into the micromixer chip at 4 µl/minutes each, while inside a 37°C incubator (total reaction time of 30 seconds). The reaction was quenched directly by collection into a microtube on 100% ethanol and dry ice bath (-78°C). The original template concentration was determined from the same DNA stock solution loaded onto inflow 1 diluted 2 fold to match concentration of sample collected from the chip. Template concentration was determined by qPCR assay of 10 fold dilution of sample collected. The LOC was washed with Milli-Q water and then purged with air between each reaction.

3.10. MspI digestion kinetics within micromixer chips

A single micromixer chip was used (Fig. 19 B). MspI template between 0.3 nM ~ 80 nM were loaded onto inflow 1 (Fig. 19 B) and 8 x 10^{-4} U/µl of MspI in 2 x buffer was loaded onto inflow 2 (Fig. 19 B). Both solutions were pumped simultaneously into the micromixer chip at 2 µl/minutes each, while inside a 37°C incubator (total reaction time of 1 minute). The reaction was quenched directly by collection into a microtube on dry ice ethanol bath (-78°C). The original template concentration was determined from the same DNA stock solution loaded onto inflow 1 diluted 2 fold to match concentration of sample collected from the chip. Template concentration was determined by qPCR assay of 10 fold dilution of sample collected. The LOC was washed with Milli-Q water and then purged with air between each reaction.

3.11. Determination of non-specific activity

Vitalization of non-specific activity on agarose gel:

45 ng of EcoRI template or 40 ng of no-restriction-site template (lacking MspI and EcoRI restriction site) were reacted separately with or without EcoRI (15 U) in a microtube at 37°C for 4 hours in total volume of 10 µl. 40 ng of no-restriction-site template was also reacted with or without EcoRI (0.04 U) in a micromixer chip (Fig. 19 B) at 37°C for 1 minute in total volume of 4 µl. All reactions were quenched directly by collection into a microtube on dry ice ethanol bath (-78°C). Products were run on a 1% agarose gel electrophoresis and visualized with ethidium bromide on Gel Doc system (BioRad).

40 ng of MspI template and 40 ng of no-restriction-site template (lacking MspI and EcoRI restriction site) were reacted separately with or without MspI (20 U) in a microtube at 37°C for 4 hours in total volume of 10 µl. 40 ng of no-restriction-site template was also reacted with or without MspI (0.04 U) in a micromixer chip (Fig. 19 B) at 37°C for 1 minute. All reactions were quenched directly by collection into a microtube on dry ice ethanol bath (-78°C). Products were run on a 1% agarose gel electrophoresis and visualized with ethidium bromide on Gel Doc system (BioRad).

Quantification of non-specific activity:

A single micromixer chip was used (Fig. 19 B) to trigger non-specific nuclease activity of EcoRI and MspI. DNA and restriction enzyme were loaded onto different inflows (Fig. 19 B). MspI template (1 ng/µl; lacking EcoRI restriction site) was digested with EcoRI (0.001 U/µl) at 37°C for 1 minute. No-restriction-site template (0.5 ng/µl; lacking MspI and EcoRI restriction site)

was digested with MspI (0.0005 U/µl) at 37°C for 1 minute. All reactions were quenched directly by collection into a microtube on dry ice ethanol bath (-78°C). The amount of undigested template was quantified by qPCR and compared with the amount of templates from the same stock solution that was pumped through the micromixer without restriction enzymes.

3.12. Restriction enzyme digestion in microtubes with glass beads

Four different conditions were tested for EcoRI within microtubes: 1) EcoRI digestion of no-restriction-site template with glass beads (acid-washed, 425-600 µm; Sigma-Aldrich, Sl09511); 2) no-restriction-site template with glass beads (no EcoRI); 3) EcoRI digestion of no-restriction-site template without glass beads; and 4) EcoRI digestion of EcoRI template without glass beads. All digestions were incubated at 37°C overnight. Products were run on a 1% agarose gel electrophoresis and visualized with ethidium bromide on Gel Doc system (BioRad).

Four different conditions were tested for MspI within microtubes: 1) MspI digestion of no-restriction-site template with glass beads; 2) no-restriction-site template with glass beads (no MspI); 3) MspI digestion of no-restriction-site template without glass beads; and 4) MspI digestion of MspI template without glass beads. All digestions were incubated at 37°C overnight. Products were run on a 1% agarose gel electrophoresis and visualized with ethidium bromide on Gel Doc system (BioRad).

3.13. Ligation of EcoRI or MspI digested template by T4 DNA ligase

When carried out in microtubes, EcoRI ligation template (2.5 ng/µl) or MspI ligation template (3.75 ng/µl) was ligated by T4 DNA Ligase (Invitrogen, 0.1 U/µl) in 1 x ligase buffer. When carried out in a microreactor chip (Fig.19 A), the reaction mixture (EcoRI ligation template [2.5 ng/µl] or MspI ligation template [3.75 ng/µl] with T4 DNA Ligase [0.1 U/µl], and 1 x ligase buffer) was pre-mixed just before loading into the syringe. 2 µl of the reaction mixture was pumped into a microreactor chip (Fig. 19 A) at 1 µl/minute and then incubated at 37°C for 2 hours.

4. Results

The successful construction of LOC (i.e. pumps, syringes, Teflon capillaries and microfluidic chips) concluded one of the objectives of this research: the building and testing of LOC. LOC will be used to perform two of the important steps in the capture of chromosomal interactions: 1) digestion of cross-linked DNA from a single cell; and 2) the subsequent ligation of digested, cross-linked DNA. Therefore the next step was to characterize DNA digestion and ligation within LOC. Cross-linking of the chromatin and lysis of the cell will not be performed with the current LOC arrangement because it was not possible to confirm the injection of a single cell or single-cell lysate into the microfluidic chips, following single-cell isolation by serial dilution (data not shown). Other downstream reactions, such as reverse cross-linking and whole genome amplification (WGA), will be performed after sample collection from LOC.

4.1. Preliminary comparison between MspI digestion in microfluidic chip and MspI digestion in microtube.

In order to determine whether there were any differences between the performances of MspI in microtubes and microfluidic chips, MspI template (9 nM [1 ng/µl]) was digested with MspI (NEB) in a microtube (Fig. 20A; MspI final concentration 2 U/µl , 0.2 U/µl, 0.02 U/µl, 0.002 U/µl, or 0.0004 U/µl) or a single microreactor chip (Fig. 20B; MspI final concentration 0.02 U/µl, 0.002 U/µl, or 0.0004 U/µl) at 37°C for 2 hours. An identical final concentration of MspI template (9 nM [1 ng/µl]) was used in the microtube and microreactor chip digestions. When low concentration of MspI was used, over 80% of substrate was undigested for reaction within microtube but only less than 3% of substrate was undigested for reaction within microfluidic chips. This suggested

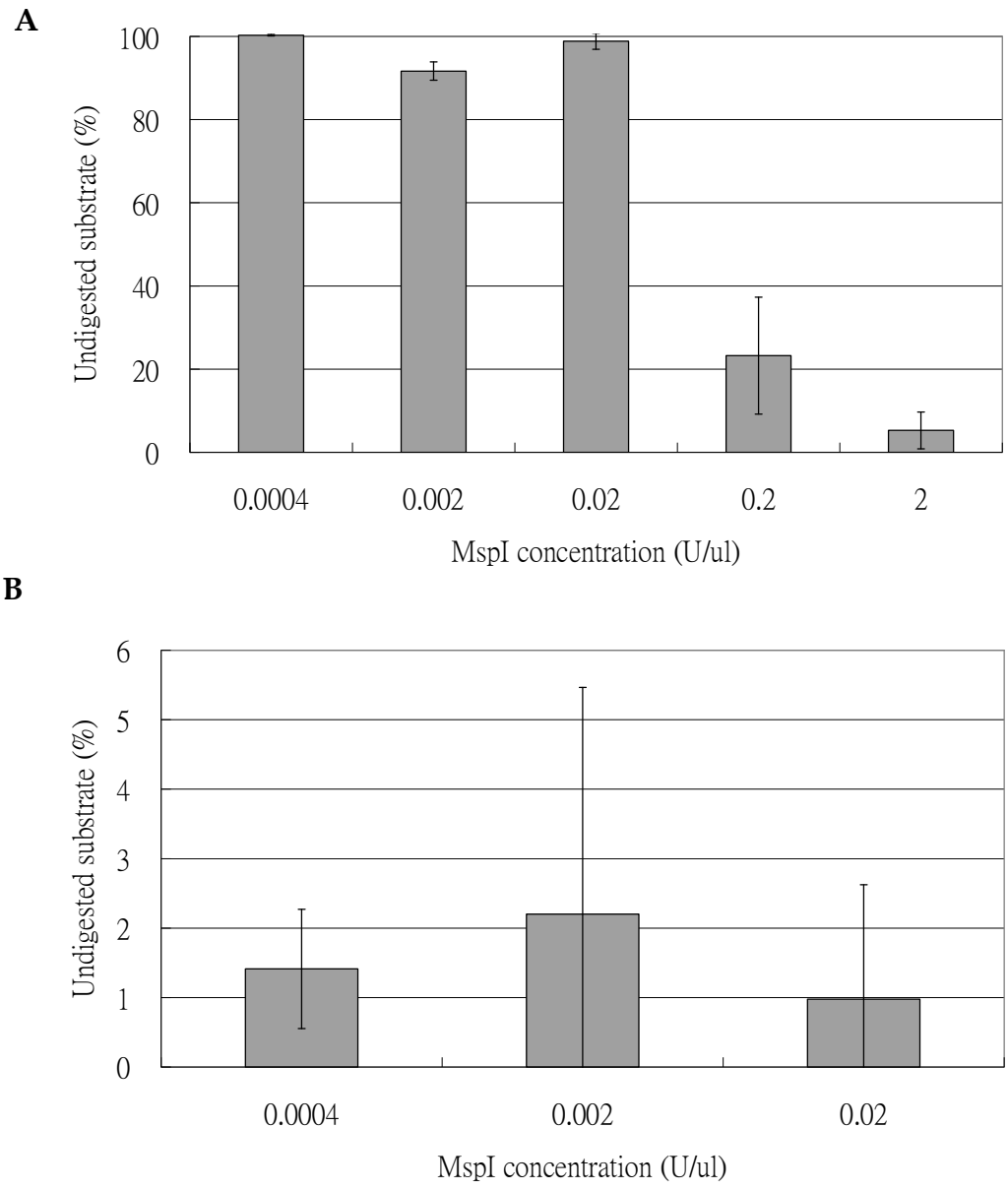


Figure 20. MspI digestion is more efficient in microfluidic chip than in microtubes. MspI concentration was quantified by qPCR. **A**. MspI did not reliably fully digest templates containing the MspI restriction site (9 nM [1 ng/µl]) at concentrations of 0.02 U/µl or lower within a microtube (2 hours, 37°C; total reaction volume: 10 µl). **B** MspI digested templates containing MspI restriction site (9 nM [1 ng/µl]) at all concentration tested within microreactor chips (2 hours, 37°C; total reaction volume: 2 µl). Result shown was the mean ± standard error of 2 experimental repeats (n=2).

that MspI was digesting efficiently at low concentration within microfluidic chips but not within microtubes (Fig. 20A). The digestion in the microfluidic chip was so efficient that the amount of DNA digested with 2 U MspI per 1 µl in microtubes was the similar to the amount of DNA digested with 0.0004 U MspI per 1 µl in the microfluidic chips.

4.2. Preliminary comparison between EcoRI digestion in microfluidic chip and EcoRI digestion in microtube.

In order to determine if another restriction enzyme exhibited a similar increase in activity within the microfluidic chip, EcoRI was subjected to similar experiments. EcoRI was chosen because it is a well characterized, commonly used restriction enzyme. A quick initial test was conducted to compare the activity of EcoRI in microtube with its activity in microfluidic chip. EcoRI template (5 nM [0.5 ng/µl]) was digested by EcoRI (Invitrogen) in microtubes (0.05 U/µl, 0.005 U/µl, or 0.0005 U/µl) or single microreactor chips (0.05 U/µl, 0.025 U/µl, 0.005 U/µl, or 0.0005 U/µl). In a result that was similar to that observed for MspI, a significant proportion of substrate (over 50%) remain undigested after 2 hours with low concentration of EcoRI in microtube, but the concentration of undigested substrate (less than 10%) decreased substantially after 2 hours with low concentration of EcoRI in microfluidic chips (Fig. 21).

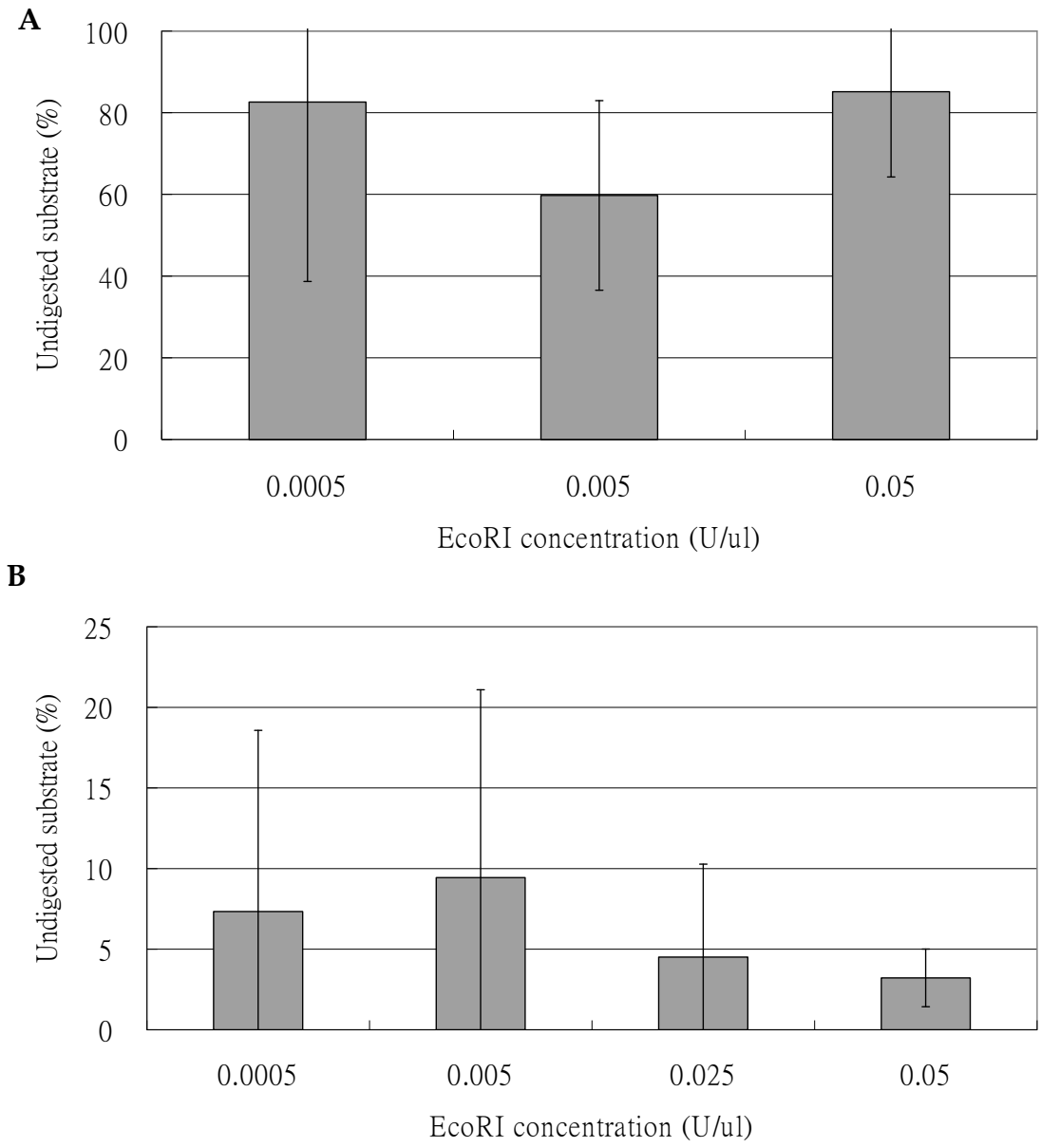


Figure 21. EcoRI digestion is more efficient in a microfluidic chip than in microtubes. Substrate concentration was quantified by qPCR. **A**. Low concentration of EcoRI did not fully digest template containing EcoRI restriction site (5 nM [0.5 ng/µl]) within microtubes (37°C, 2 hours; total reaction volume: 10 µl). **B.** EcoRI digestion occurred at all concentration tested within microreactor chips (template concentration: 5 nM [0.5 ng/µl]; 37°C, 2 hours; total reaction volume: 2 µl). Result shown was the mean ± standard error of 2 experimental repeats (n=2).

4.3. EcoRI kinetics within microtubes

The preliminary test results (section 4.1 and 4.2) suggested there was a significant difference in the activity of restriction enzymes depending on the reaction vessel in which the digestion was performed. This difference was previously observed but the increase in digestion kinetics was not characterized (Fu & Lin, 2007). Since one of the objectives of this research is to characterize DNA digestion within microfluidic chips, it is logical that the difference in digestion kinetics was investigated. EcoRI digestion kinetics within the microtube was estimated from the original substrate (EcoRI template) concentration and initial rate of digestion (Fig. 22). The rate of digestion was determined by change in substrate concentration over the change in time. The percentage of undigested substrate for various starting concentration measured at 1 minute (>70%) confirmed that the initial rate of reaction was sampled (Fig. 22A). The $K_{m,}$ V_{max} and K_{cat} for EcoRI in a microtube were 32 nM, 0.14 nM s^{-1} and 1.4 fmol s^{-1} U^{-1} respectively. Lineweaver-Burk plot was used to estimate the kinetics values. The plot was produced by plotting inverse of substrate concentration against inverse of initial rate of reaction. The inverse of x-intersect is the K_m, the inverse of y-intersect is the V_{max}, and K_{cat} is determined by $V_{max}/(K_m$ x concentration of enzyme).

A

Substrate concentration (nM)	Undigested substrate
9.55	80%
7.23	82%
5.28	81%
2.94	70%
1.34	74%
0.367	74%

B

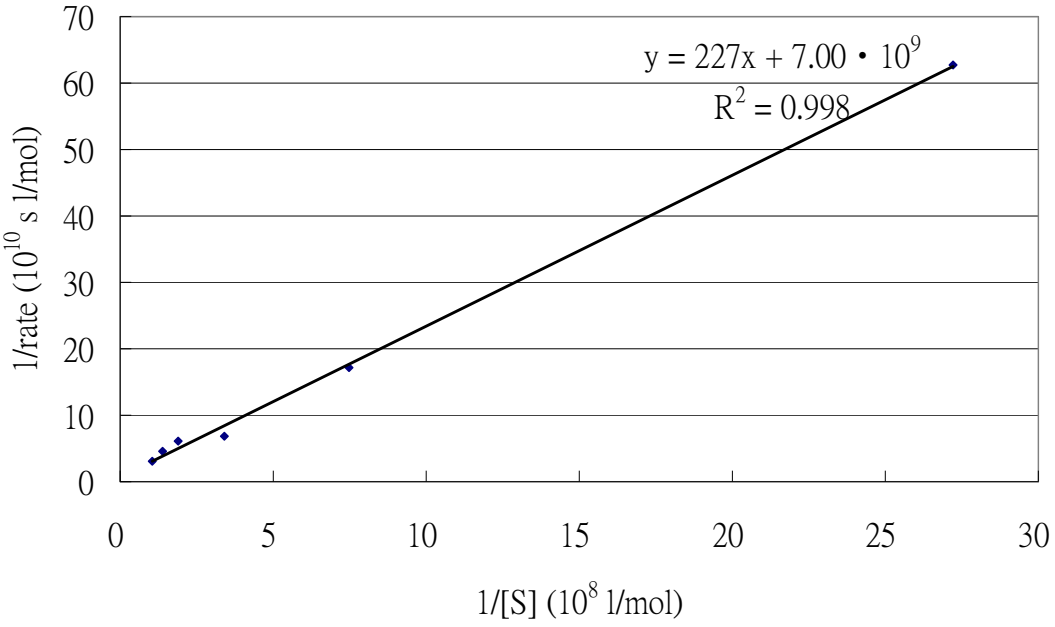


Figure 22. EcoRI template concentration and initial rate of digestion was used to estimate EcoRI kinetics in a microtube. Substrate concentration was measured using qPCR. **A**. Original EcoRI template concentration and percentage of undigested substrate after digestion (EcoRI concentration: 0.1 U/ul; 37°C, 1 minute; total reaction volume: 10 µl). Result shown was the mean of two repeats. **B**. Lineweaver-Burk plot of EcoRI digestion in a microtube. [S] = substrate concentration. Rate = initial rate of digestion.

4.4. EcoRI kinetics within microfluidic chips

Enzyme kinetics can not be determined without an initial reaction rate. Various different combinations of EcoRI and EcoRI template concentrations were tested, but none resulted in the initial rate at the shortest time that the digested sample could be reliably collected (Fig. 23). Even at an incubation time of 30 seconds, the initial rate was unable to be measured (Table 6). The inability to sample at the initial rate of reaction was later discovered to be caused by non-specific nuclease activity of EcoRI in microfluidic chip (section 4.7).

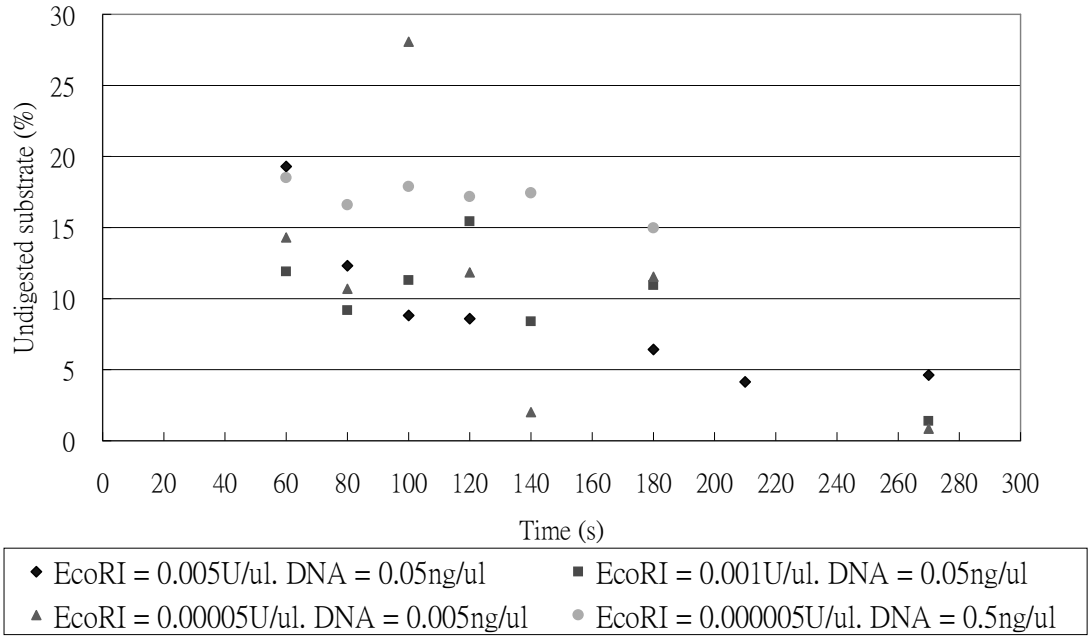


Figure 23. The parameters for assaying the initial rate of EcoRI digestion were not found. Four different EcoRI and EcoRI template concentrations were used (see above) to produce a digestion time course. However, none produced a measurable initial rate of digestion even at the shortest time that the digested sample could be reliably collected. Substrate concentration was quantified by qPCR. Concentration of undigested substrate was normalized to original substrate concentration (i.e. concentration of substrate when reaction time = 0 second).

Substrate concentration (nM)	Undigested substrate
12.3	2.5%
10.3	25.1%
5.95	4.5%
3.59	7.0%
1.39	5.7%
0.276	12.3%
0.345	0.3%

Table 6. The initial rate of EcoRI digestion was not measured for digestion within single micromixer chip. EcoRI final concentration: $5x10^{-4}$ U/µl; 37°C, 30 seconds; total volume collected: 4.5 µl. Result shown was the mean of 2 repeats. Substrate concentration was quantified by qPCR.

4.5. MspI enzyme kinetics within microtubes

MspI kinetics within microfluidic chips was characterized by comparing with MspI digestion kinetics within microtubes. MspI was characterized because MspI was the chosen restriction enzyme for performing GCC using LOC. MspI kinetics within microtubes was first measured to provide a point of comparison. The Lineweaver-Burk plot was generated for MspI digestion using the original DNA substrate (MspI template) concentration and the initial rate of digestion within microtubes (Fig. 24). The rate of digestion was determined by change in substrate concentration over the change in time. The percentage of undigested substrate (over 50% for majority of samples) at the time of sampling (1 minute) confirmed that the initial rate of reaction was sampled for the digestion reaction performed within a microtube (Fig. 24A). K_m, V_{max} and K_{cat} for MspI in microtube reactions were calculated to be 125 nM, 1.46 nM s^{-1} and 29.2 fmol s^{-1} U^{-1} respectively.

A

Substrate concentration (nM)	Undigested substrate
296	73%
207	62%
97.5	59%
59.3	62%
18.5	41%
5.71	32%

B

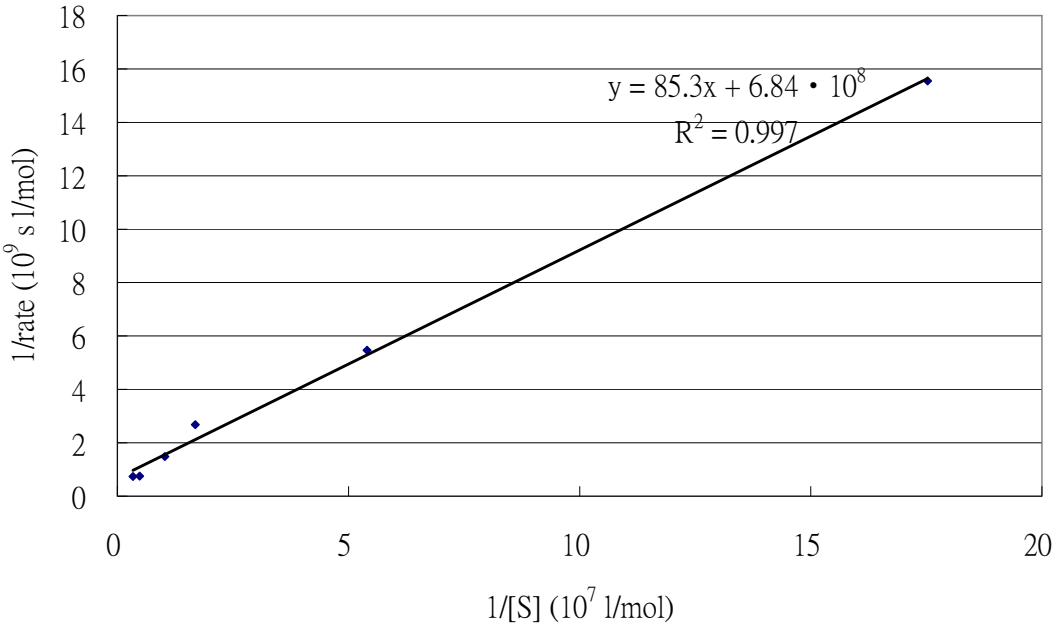


Figure 24. Substrate concentration and initial rate of digestion were used to estimate MspI kinetics in microtube. MspI concentration was 0.5 U/µl. Digestion was carried out at 37°C for 1 minute. Substrate concentration was quantified by qPCR. Two repeats (n=2) **A**. Table with original substrate concentration and percentage of undigested substrate after digestion. **B**. Lineweaver-Burk plot of MspI digestion in microtube. [S] = substrate concentration. Rate = initial rate of digestion.

4.6. MspI kinetics within microfluidic chips

The initial rate of MspI digestion within a micromixer chip was unable to be assayed (Table 7). The initial reaction rate must be assayed to determine the enzyme kinetics. The undigested substrate (MspI template) collected after digestion within micromixer chip showed that more than 70% of the DNA substrate had been digested by MspI ($4x10^{-4}$ U/µl; 37°C, 1 minute; total volume collected: 4.5 µl). The inability to sample the initial rate of reaction was later discovered to be caused by non-specific nuclease activity of MspI in a microfluidic chip (section 4.7).

Substrate concentration (nM)	Undigested substrate
39.3	28%
20.0	23%
3.49	17%
0.368	13%
0.151	14%

Table 7. The initial rate of MspI digestion was not measured for digestion within single micromixer chip. MspI final concentration: $4x10^{-4}$ U/µl; 37°C, 1 minute; total volume collected: 4.5 µl. Substrate concentration was quantified by qPCR. Result shown was the mean of 2 repeats.

4.7. EcoRI and MspI exhibit non-specific nuclease activity within microfluidic chips

The fact that the initial rate of reaction for EcoRI and MspI within the micromixer chips could not be measured, even after decreasing the concentration of EcoRI many fold, may be due to the restriction enzymes not functioning properly in the micromixer chips. From earlier results (section 4.1 and 4.2) it was obvious that EcoRI was digesting, therefore it was theorized that the digestion was not specific. To test this theory, the products of EcoRI digestions performed within microtubes and micromixer chips with EcoRI template (containing EcoRI restriction site) or no-restriction-site template (lacking EcoRI or MspI restriction site) was separated on a 1% agarose gel (Fig. 25A). Reactions lacking the EcoRI enzyme were used as negative controls. Product of the correct size (116bp and 38bp [size was too small to see on gel]) was observed for EcoRI digestion of EcoRI template within the microtubes and there was no obvious digestion of the no-restriction-site template (Fig. 25A). This demonstrated that EcoRI was functioning properly within microtubes without non-specific nuclease activity. However, the absence of a band for the sample collected from an EcoRI micromixer chip digestion of no-restriction-site template confirmed the presence of non-specific nuclease activity for EcoRI within micromixer.

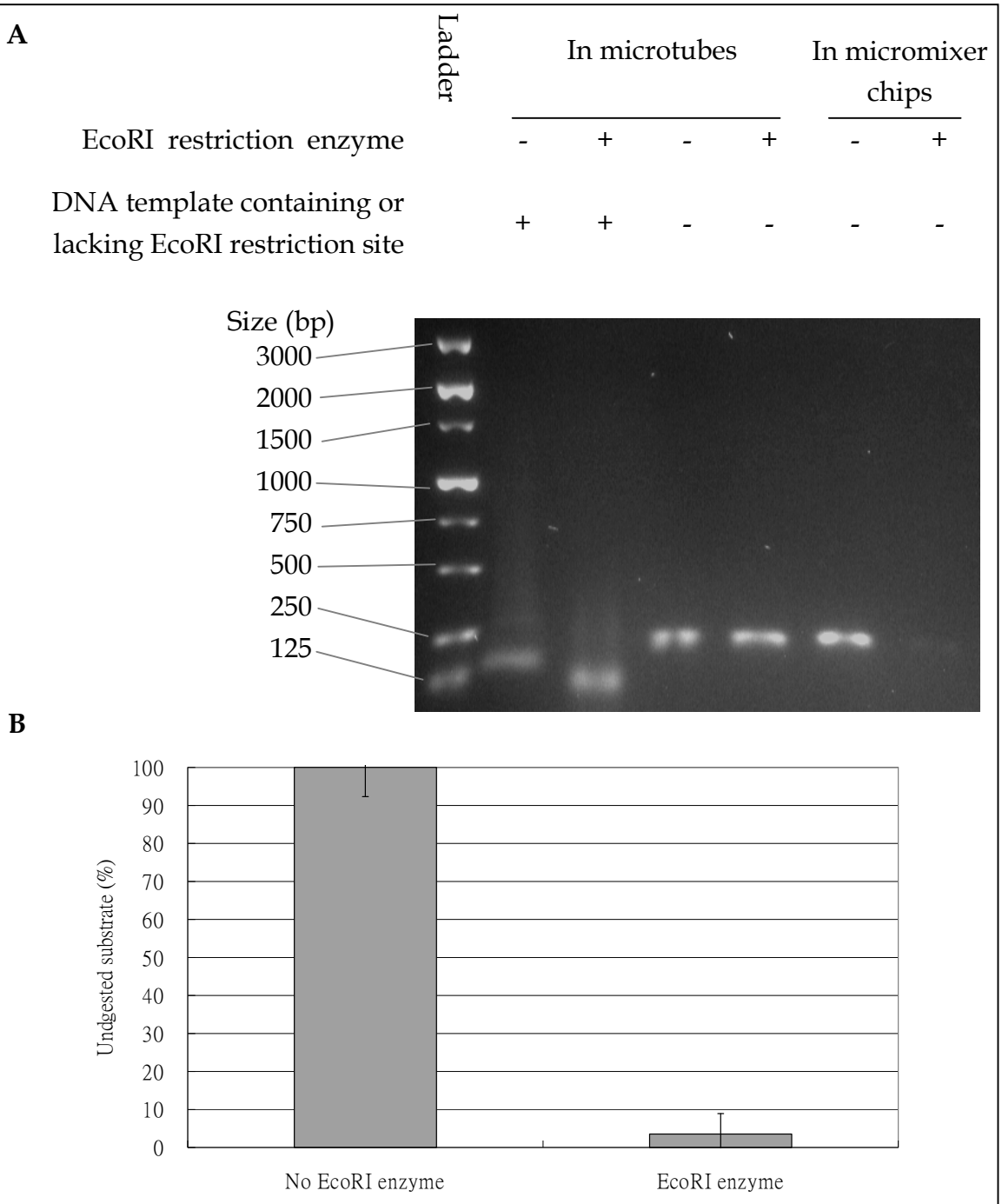


Figure 25. EcoRI exhibited non-specific nuclease activity when acting within the micromixer chip. **A**. EcoRI digestion was performed within microtubes (37°C, overnight) or micromixer chips (37°C, 1 minute) with EcoRI template or no-restriction-site template. Digestion products were separated on a 1% agarose gel and visualized with ethidium bromide. **B**. Quantification of MspI template (lacking EcoRI restriction site) by qPCR after incubation within a micromixer chip with or without EcoRI restriction enzyme (37°C, 1 minute). qPCR assay showed that digestion caused by non-specific nuclease activity was responsible for >90% of reduction in template. Result shown as mean of 3 repeats ± standard errors.

The extent of the non-specific nuclease activity exhibited by EcoRI within micromixer chips was quantified by qPCR (Fig. 25B). EcoRI template (containing EcoRI restriction site) and MspI template (lacking EcoRI restriction site) was incubated with the EcoRI restriction enzyme in a micromixer at 37°C for 1 minute and the result was compared to template incubated without the EcoRI restriction enzyme in the same micromixer under the same conditions. The result showed that more than 90% of the template was digested (Fig. 25B).

A similar experiment was performed with MspI to determine if it also showed non-specific activity nuclease activity when active within a micromixer chip (Fig. 26). Similar to results observed with EcoRI, both the pattern of bands on agarose gel (Fig. 26A) and qPCR quantification (Fig. 26B) of MspI digestion of no-restriction-site template (lacking MspI restriction site), demonstrated that MspI also exhibited non-specific nuclease activity in micromixer chips, but not in microtubes.

Furthermore, to determine if the increase in surface area to volume ratio inside the micromixer chip is the cause of the observed non-specific nuclease activity, acid-washed glass beads (425-600 µm; Sigma-Aldrich, Sl09511) were incubated in microtubes with EcoRI (1.5 U/µl) or MspI (2 U/µl) with no-restriction-site template (3.8 ng/µl) at 37°C overnight. The products were separated on a 1% agarose gel and visualised with ethidium bromide. Both enzymes did not exhibit non-specific nuclease activity within microtubes with glass beads (Fig. 27).

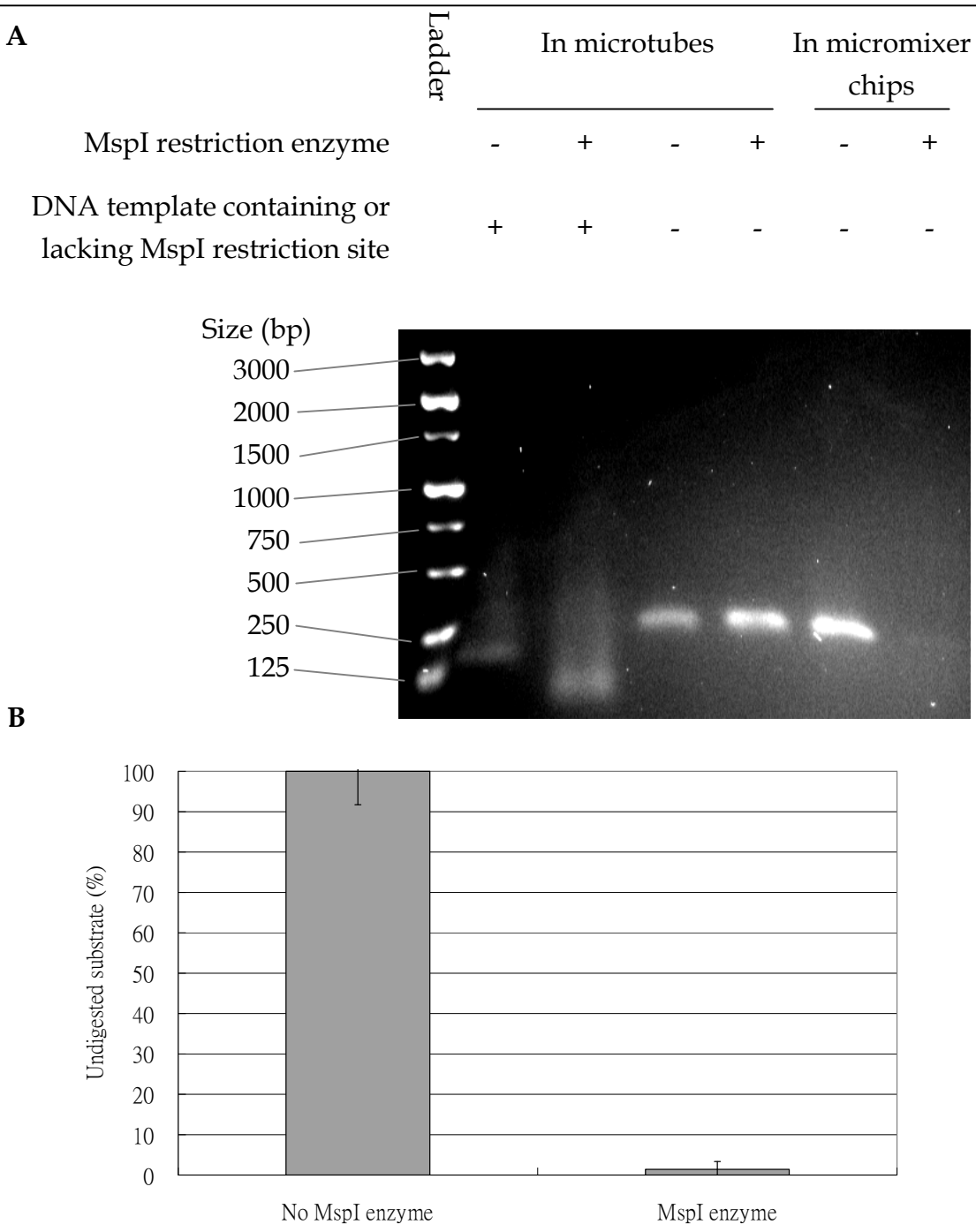


Figure 26. MspI exhibited non-specific nuclease activity when acting within the micromixer chip. **A**. MspI digestion was performed within microtubes (37°C, overnight) or micromixer chips (37°C, 1 minute) with MspI template or no-restriction-site template. Digestion products were separated on a 1% agarose dell and visualized with ethidium bromide. **B**. Quantification of no-restriction-site template (lacking MspI restriction site) by qPCR after incubation within a micromixer chip with or without MspI restriction enzyme (37°C, 1 minute). qPCR assay showed that digestion caused by non-specific nuclease activity was responsible for >90% of reduction in template. Result shown as mean of 3 repeats ± standard errors.

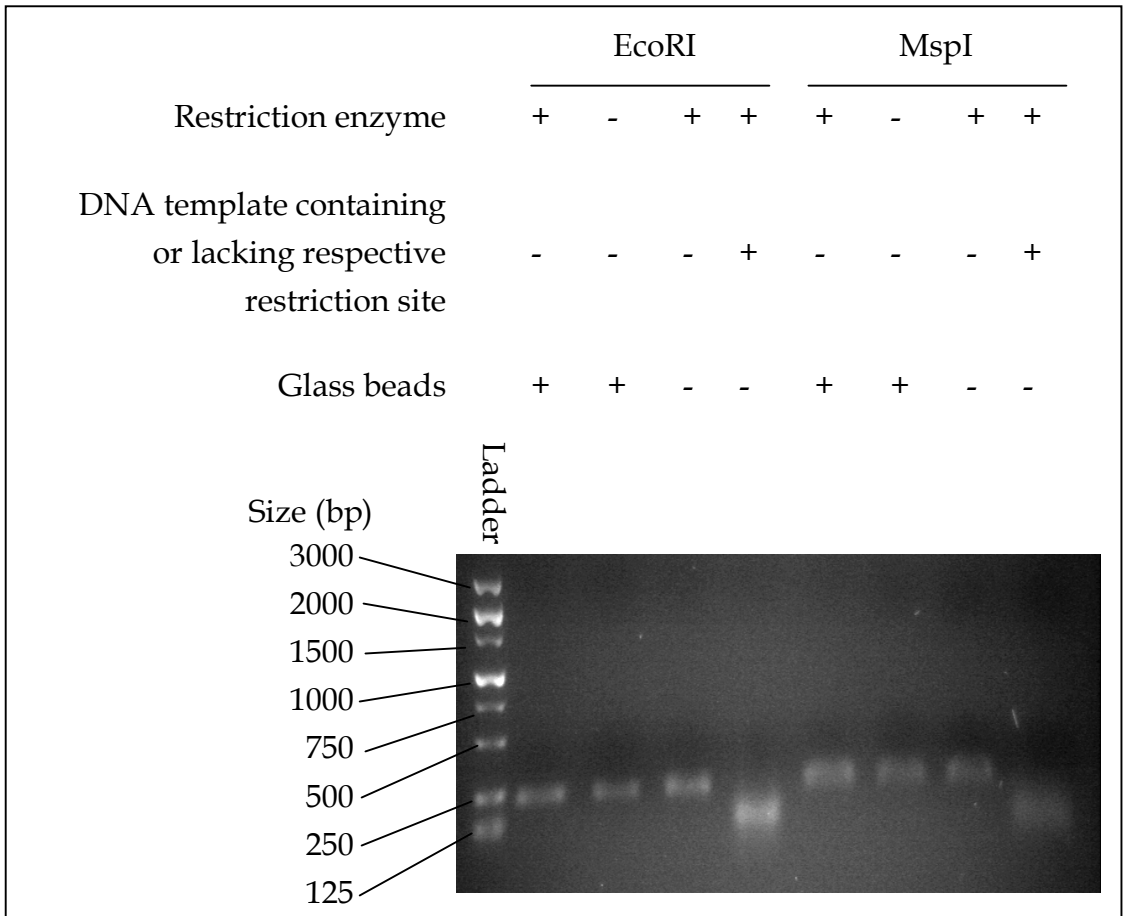


Figure 27. No non-specific nuclease activity was observed when EcoRI or MspI was incubated with glass beads. EcoRI or MspI digestion within microtubes with or without glass beads (425 µm-600 µm) of DNA templates containing or lacking the respective restriction site (37°C, overnight).

4.8. Ligation in lab-on-a-chip

As noted earlier, another step of the GCC assay that must be performed in the LOC was ligation. However, the newly characterized non-specific nuclease activity of MspI and EcoRI when digesting within microfluidic chips caused failures in attempts to characterize the ligation activity when following an in-microfluidic-chip digestion (data not shown), where the ligated product produced in LOC was less than 0.5% of the ligated product produced in microtube under the same conditions.

Experiments were performed where the ligation reaction solution (MspI ligation template, T4 DNA ligase and ligase buffer) was pre-mixed and incubated in either a microtube or a microreactor chip at 37°C for 2 hours (Fig. 28). Under these conditions, ligation within a microfluidic chip produced less than 25% of the re-ligated products than ligation carried out in a microtube (Fig. 28). The LOC was pre-incubated with bovine serum albumin (BSA) to test whether coating of LOC with BSA will increase ligation efficiency within microfluidic chips.

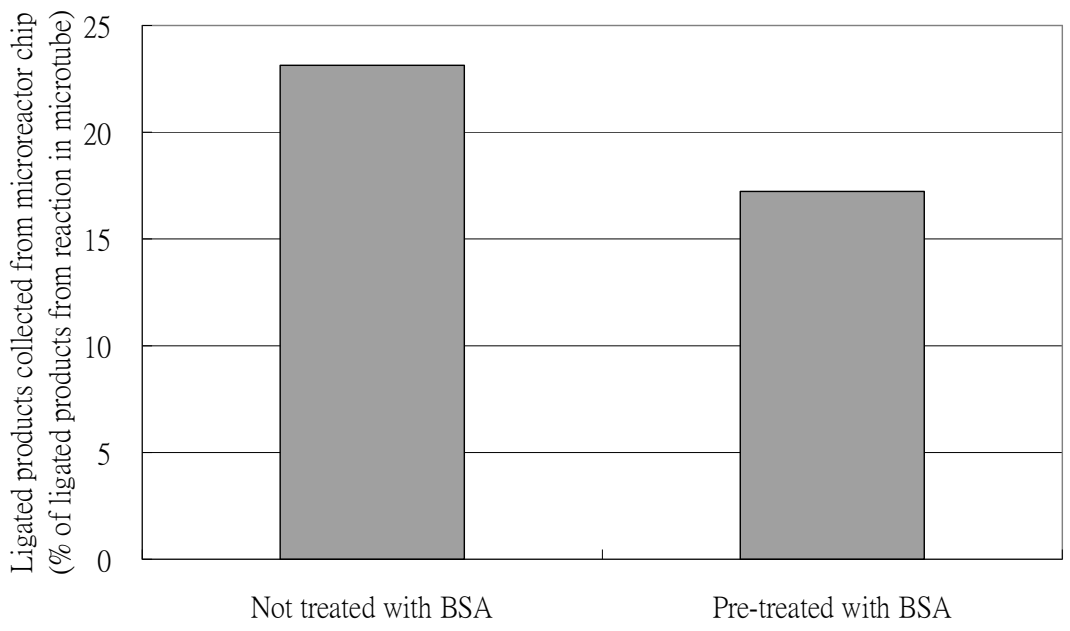


Figure 28. Ligation within the microfluidic chip was not as efficient as ligation within the microtube. T4 DNA ligase ligation of MspI digested pre-MspI template 1 and 2 inside a microreactor chip that was pre-washed with 150µg/ml of bovine serum albumin (BSA). Ligation reaction solution was pre-mixed prior to incubation then incubated in either 1) microtube or 2) microreactor chip (37°C, 2 hours). Ligated product from microreactor chip was normalized to ligated product from microtube (one repeat). Concentration of ligated products was quantified by qPCR.

In order to determine whether the difference in the amount of re-ligated product was due to residual activity of the restriction enzyme used to prepare the ligation templates, EcoRI was subjected to an increasing number of inactivation steps following digestion of mixed pre-EcoRI template 1 and 2 within microtubes. The digested products were then re-ligated within a microtube or a microreactor chip. EcoRI was selected for this experiment because according to the manufacture's web site, MspI is not sensitive to heat inactivation (http://www.neb.com/nebecomm/products/productR0106.asp) but EcoRI is sensitive to heat inactivation (http://products.invitrogen.com/ivgn/product/15202039). Even though there were notable increases in the microtube ligation following the increasing steps of stringent inactivation, there were no notable increases in the amount of re-ligated product produced by ligation within the microreactor chip (Fig. 29). Furthermore, the amount of re-ligated product produced within the microreactor chip was less than 20% of the re-ligated product produced from the microtube under similar conditions.

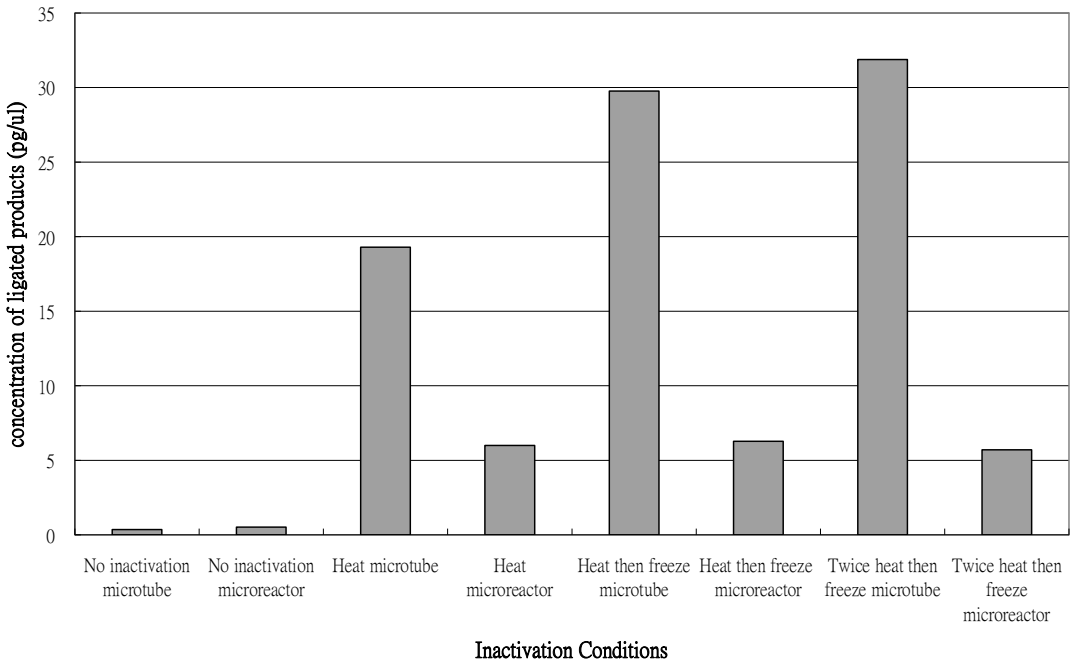


Figure 29. Concentrations of re-ligated products were affected by the stringency of the EcoRI inactivation steps for ligation performed in microtubes but not in the microreactor chip. T4 DNA ligase catalyzed ligation of two different EcoRI digested PCR products (pre-EcoRI template 1 and 2) within a microtube or microreactor chip. EcoRI inactivation was performed with increasing stringency: 1) no inactivation; 2) 65°C for 20 minutes (heat); 3) 65°C for 20 minutes then freeze at -78°C (heat then freeze); or 4) 2 cycles of 65°C for 20 minutes then freeze at -78°C (twice heat then freeze). Concentration of ligated products was quantified by qPCR.

5. Discussion

Chromosome interactions and detailed analyses of the 3D structure of DNA inside a cell is an area of recent biology that has been intractable until recently (Rodley, et al., 2009). As a result of this, chromosomal interactions at a single-cell level have never been reported. The main objectives of this research are to build and test a microfluidic system (LOC) that will aid in the capture of inter- and intra- chromosomal interactions of a single cell and to characterize the restriction and ligation of DNA that will be performed in LOC. This is a part of a larger research project which aims to capturing chromosome interactions in single-cells with the aid of LOC. Using LOC methodologies to assay chromosome interactions in single-cells would achieve two outcomes: 1) focus on cell to cell variation instead of global, averaged variation; and 2) decrease the resources required, including time, to perform proximity based ligation methodologies (Dekker, et al., 2002; Fredriksson, et al., 2002). This seemed like an uncomplicated project when it was first proposed, because the three steps of this project, single-cell isolation, capture of chromosome interactions and whole genome amplification (WGA), had all be successfully done before, albeit separately. Firstly, serial dilution of cell culture would be used to isolate a single cell. Secondly, the techniques that would be implemented to capture chromosome interactions had been trialed, tested and published in previous studies (Dekker, et al., 2002; Lomvardas, et al., 2006; O'Sullivan, et al., 2009; Ohlsson & Göndör, 2007; Rodley, et al., 2009; Simonis, et al., 2006; Wurtele & Chartrand, 2006; Zhao, et al., 2006). The key differences in the study were: (a) where the reactions were to be performed (in a microfluidic chip) and (b) the amount of DNA and reagent used in each reaction. Lastly, WGA kits are commercially available and had been successfully used to amplify DNA from a

single cell (Fiegler, et al., 2007; J. B. Geigl, et al., 2009; Glentis, et al., 2009; Handyside, et al., 2004; Hellani, et al., 2004; Imle, et al., 2009; Jiang, et al., 2005; Kvist, et al., 2007; Ling, et al., 2009; Marcy, Ishoey, et al., 2007; Marcy, Ouverney, et al., 2007; Ren, et al., 2007; Rodrigue, et al., 2009; Schowalter, et al., 2007; Spits, et al., 2006a). This project should simply link three established methodologies into one process with the aid of a LOC. However, similar to other seemingly simple tasks, unexpected obstacles were hidden out of sight. Even though the enzyme kinetics of EcoRI and MspI within microtubes was successfully estimated, the enzyme kinetics of both enzymes within the microfluidic chips was not measurable because both enzymes exhibited a non-specific nuclease activity under the conditions tested. This non-specific nuclease activity explained why digestion followed by re-ligation in LOC failed, but it did not explain the reduction in the amount of re-ligated DNA in the microfluidic chip when only ligation was carried out. However, ligase contaminated with nuclease might be the reason for the reduction of re-ligated products (http://www.neb.com/nebecomm/products/productm0202.asp). Problems were also encountered in single-cell isolation. Specifically, while a single-cell could be easily acquired by serial dilution, it was not possible to confirm the loading of single-cells or lysate from single-cells into the LOC. Due to the unexpected set-backs and time restrictions WGA was unable to be tested.

5.1. Restriction enzyme digestion

A key step in single-cell GCC involves the digestion of DNA inside the LOC. Therefore, it is important to characterize the activity of restriction enzymes in a microfluidic chips. The increase in restriction enzyme activity originally observed in the microfluidic chips was quite exciting, as one of the main

reasons for using LOC was to decrease resources, especially time, spent on GCC protocol (Fig. 20 and 21). This observation led to the characterization of the enzyme kinetics of two restriction enzymes, EcoRI and MspI. EcoRI was characterized because there was already a lot of published data on EcoRI kinetics (Aiken, et al., 1991; Kettling, et al., 1998; Langowski, et al., 1981; X. M. Li, et al., 2008; McLaughlin, et al., 1987). MspI was characterized because single-cell GCC analysis of *S. cerevisiae* would use MspI in the digestion step so the chromosome interactions of a single-cell could then be compared with previously published data (Rodley, et al., 2009).

5.1.1. EcoRI kinetics within microtubes

A wide range of kinetics values have been reported for EcoRI (Aiken, et al., 1991; Hager, et al., 1990; Kettling, et al., 1998; Langowski, et al., 1981; X. M. Li, et al., 2008) and it is clear that the kinetics of EcoRI depend upon template length, the position of the recognition site and the flanking sequence (McLaughlin, et al., 1987). The reported K_m values range from 4.8 nM to 226 nM (Aiken, et al., 1991; Hager, et al., 1990; Kettling, et al., 1998; Langowski, et al., 1981; X. M. Li, et al., 2008). The kinetic parameters that were measured in this study for EcoRI digestion of a 152bp sequence within microtubes (K_m = 32 nM; V_{max} = 0.14 nM s^{-1}; Fig. 22) were comparable with published data using a DNA fragment of similar size (sequence length = 66bp; K_m = 14 nM; V_{max} = 0.74 nM s^{-1}; Kettling, et al., 1998). The observed variation may have been due to: 1) the difference in sequence lengths; 2) the positions of the restriction site (center of 66bp fragment or 38bp from the 5′ end of 152bp fragment); and 3) the difference in the buffer system used (Kettling, et al., 1998; X. M. Li, et al., 2008).

5.1.2. MspI kinetics within microtubes

The kinetics of MspI digestion within microtubes was measured using the same method as that described for EcoRI kinetics within microtubes. MspI kinetics is not as well studied as EcoRI kinetics. The kinetic parameters measured (K_m = 125 nM; K_{cat} = 29.2 fmol s^{-1} U^{-1}; Fig. 22) are comparable to previously published data (K_m = 260 nM; K_{cat} = 0.262 fmol s^{-1} U^{-1}; Kim, Kang, & Yoo, 1994). The variation observed was likely due to similar reasons for the variation observed in the EcoRI digestion kinetics. The length of the digested substrate, the position of the restriction site and buffer system can all affect the digestion kinetics MspI (Kettling, et al., 1998; X. M. Li, et al., 2008; McLaughlin, et al., 1987). The difference in the incubation temperatures would also have an impact on the MspI kinetics. Kim et al. (1994) incubated the digestion reaction at 20°C, whereas the digestion reaction was incubated at 37°C in this study. The K_{cat} value reflects how fast the substrate is converted to product for a given amount of enzyme. Therefore, it is not surprising that MspI has a much higher value of K_{cat} at 37°C than 20°C.

5.1.3. Non-specific nuclease activity within microfluidic chips

The discovery of non-specific nuclease activity of EcoRI and MspI within microfluidic chips was unexpected, as there was no indication that this would occur. From real-time PCR (qPCR) results (Fig. 25 and 26), it was unclear how much specificity was lost for EcoRI and MspI inside the microfluidic chip because the digestion template used was short. Fu & Lin (2007) reported an

increase in EcoRI activity inside a self-fabricated glass microfluidic device without observing non-specific nuclease activity. However, they (Fu & Lin, 2007) did not quantify their products; therefore it is difficult to determine if there was any non-specific nuclease activity.

There were two major differences in the methods. Firstly, the buffer composition was different. The major difference was Fu & Lin (2007) carried out the digestion at pH of 9.2 instead of 7.5 used in this study. This was surprising because restriction enzymes can completely lose specificity at high pH (Izsvak & Duda, 1989). Secondly, Fu & Lin (2007) used electrokinetics to drive the DNA and EcoRI through their device, whereas the reaction solutions in this study were driven by pumps. It is unclear what effect the method of driving the fluids through the device can have on restriction enzyme digestion within a microfluidic device. Furthermore, there was no evidence that supported the presence of non-specific nuclease activity; there was also no evidence that denied its presence within the microfluidics device made by Fu & Lin (2007). The digestion products were analyzed by the gel electrophoresis and the result was not quantitative. Visual comparisons of the intensity of the product bands on the gel electrophoresis indicate a decrease in intensity as digestion time increases. However, without accurate product quantification, it is impossible to determine whether there is a real decrease in the amount of DNA as digestion time increases. By contrast, the study presented here quantitatively showed, by qPCR, that EcoRI and MspI exhibit non-specific nuclease activity when digestions are carried out within a microfluidic chip (Fig. 25, 26 and 27).

EcoRI has star activity under specific buffer conditions that decrease water

activity (Izsvak & Duda, 1989; Robinson & Sligar, 1993, 1998; H. Wei, Therrien, Blanchard, Guan, & Zhu, 2008); however MspI had not previously been reported to show star activity. Restriction enzyme star activity is defined as a relaxation of specificity and is not to be confused with a complete loss of specificity (Izsvak & Duda, 1989). Star activity in EcoRI was previously discovered to be caused by the loss of water molecules that facilitate the binding of EcoRI and DNA (Robinson & Sligar, 1993, 1998). This is caused by buffer conditions, such as high glycerol concentration, that increase osmotic pressure, inducing the release of water molecules bound to the restriction enzyme – DNA complex. As the recognition of a specific restriction site is mediated by water molecules, the loss of water molecules caused a relaxation of specificity. It is unlikely that there was an increase in osmotic pressure inside a microfluidic chip because the buffer concentration used inside the microfluidic chips was identical to that used in the microtubes.

Another hypothesis that must be considered is the possibility that the structures of the EcoRI and MspI restriction enzymes were affected by an unknown factor inside the microfluidic chips. The main difference between digestion within microfluidic chips and microtubes is the presence of the large glass surface area relative to the overall reaction volume; therefore, the most likely factor that could influence the restriction enzymes specificity is the increased surface to volume ratio. The surface area to volume ratio for a microtube is approximately 110 cm^2: 1 cm^3, whereas for the micromixer chip this ratio is approximately 1600 cm^2: 1 cm^3. Since glass is more hydrophobic than the solution containing the enzyme, it is possible that hydrophobic sections of the enzyme associate with the glass surface, and thus disturb the structure of the enzyme, causing a loss of

specificity. Furthermore, the movement of the solution plus the association between restriction enzyme and glass surface may cause a shearing force to be applied onto the enzyme, further distorting the restriction enzyme structure.

The hypothesis that the increased glass surface area caused the non-specific nuclease activity of EcoRI and MspI was tested by incubating the restriction enzymes with no-restriction-site template within microtubes with glass beads (Fig. 27). The addition of glass beads increased the surface area to volume ratio inside a microtube (to approximately 400 cm^2: 1 cm^3). Neither enzyme exhibited non-specific nuclease activity within microtubes with glass beads. This may be due to the surface area to volume ratio being too small. Additionally, it may be a combination of factors, including surface area, which triggered the non-specific nuclease activity of restriction enzymes within the microfluidic chips. The solution within a microtube with glass beads was static whereas the solution within a microfluidic chip was moving. As mentioned before, it is possible that a combination of large surface area to volume ratio in addition to movement is required to induce non-specific nuclease activity of restriction enzymes. The association between glass surface and restriction enzyme is not enough to trigger the non-specific nuclease activity; the restriction enzyme may have to be sheared across the glass surface.

How the structure of EcoRI and MspI may be affected is currently undetermined. Restriction enzymes have two main different conformations: the binding and the cleaving conformation (A. Pingoud & Jeltsch, 1997). The binding conformation allows the enzyme to loosely associate with DNA to search for a specific restriction site. The specific binding of a restriction enzyme

to a restriction site induces a conformational change that allows it to cleave DNA. Theoretically, deformation or relaxation of the restriction enzyme structure could relax the recognition process and thus cause the restriction enzyme to change to the cleavage conformation without first binding to the specific recognition site. Additionally, the restriction enzyme structure could be altered such that the enzyme is locked in the cleavage conformation, unable to return to the binding conformation. This would cause the restriction enzyme to cleave as soon as it binds the DNA template. Changes to one subunit of the EcoRI homodimer (Muir, et al., 1997; A. Pingoud & Jeltsch, 1997; Watrob, et al., 2001) are likely to affect the activity of the whole enzyme. It has been shown for EcoRV, that a change to one subunit of the homodimer affects the activity of the complete enzyme (Stahl, Wende, Jeltsch, & Pingoud, 1996).

It has also been shown that DNA can bind to glass microscope slides in the presence of chaotropic salts at high concentrations (Nanassy, Haydock, & Reed, 2007). Even though there were no chaotropic salt in the digestion buffer used in this study, it was still possible that the DNA interacted weakly with the surface of the glass. Under normal conditions, it is the conformational change of restriction enzyme from binding to cleavage conformation that induces the DNA to bend (A. Pingoud & Jeltsch, 1997, 2001). The interaction between DNA and glass surface in addition to the movement of the solution, may apply enough force onto the DNA to bend the DNA and induce a conformational change of the restriction enzyme bounded, causing the restriction enzyme to cleave.

5.1.4. Implications of non-specific activity of EcoRI and MspI within microfluidic chips

Regardless of the cause, the presence of non-specific nuclease activity of EcoRI and MspI within the microfluidic chips was a setback to this project. GCC and other similar assays are based on the proximity-ligation technique (Dekker, et al., 2002; Fredriksson, et al., 2002; Rodley, et al., 2009). Briefly, cell content is first cross-linked before cell lysis to preserve the chromosomal interactions. The DNA from the cell lysate is digested then re-ligated in a diluted solution to promote the re-ligation of cross-linked DNA. If a restriction enzyme with non-specific nuclease activity is used in the digestion step, the DNA fragments produced by digestion will be different each time and there will be considerable loss of template. Non-specific nuclease activity will also prevent the formation of sticky ends on digested DNA. This will also hinder the re-ligation of cross-linked DNA. This will lead to the formation of different ligation products, causing variations in the data. Therefore, unless the issue of non-specific nuclease activity of restriction enzymes within microfluidic chips is resolved, chromosome interaction data generated using LOC to carry out the digestion step will be unreliable.

5.2. Ligation by T4 DNA ligase

Another important step in single-cell GCC that would be performed in the microfluidic chips is the ligation of digested DNA with T4 DNA ligase. The yield of ligated products from ligations performed in microfluidic chips was less than 25% of that produced by ligation within microtubes (Fig. 28 and 29). The decrease in yield was observed regardless of which restriction enzyme, EcoRI or MspI, was used to create the substrate for ligation. The increasing

amount of deactivation steps after EcoRI digestion also did not improve the yield of ligated products from the microfluidic chips, though it did slightly improve the yield from microtubes (Fig. 29). It is possible that the endonuclease activity that co-purified with the T4 DNA ligase, as described by the manufacturer, has increased activity inside microfluidic chips (http://www.neb.com/nebecomm/products/productm0202.asp) similar to that observed for the restriction enzymes.

The non-specific nuclease activity of EcoRI and MspI within the microfluidic chips also explains the low yields of ligated products from experiments where the digestion and ligation steps were sequentially performed in LOC (ligated products from reactions in microfluidic chips was less than 0.05% of ligated products from reactions in microtubes; data not shown). Since qPCR could only assay one specific ligated product, the non-specific nuclease activity of EcoRI and MspI would substantially decrease the yield.

5.3. Single cell isolation

In order to capture the chromosomal interaction pattern in a single cell, single-cells must be isolated. Even though a cell can be isolated by serial dilution, it was not possible to confirm how many cells were actually injected into the LOC. Both types of microfluidic chip (micromixer and microreactor) were too thick, and the Teflon capillary was too opaque, to see through using a microscope (Olympus CH30; data not shown). Since the concentration of a cell suspension is a measure of the mean number of cells per unit volume of solution, it is possible that any one sample does not contain a cell. Injecting no cells into the LOC is problematic; however PCR amplification can be used to

ultimately confirm injection. However, too great an incidence of blank injection will have a large impact on the efficiency of the protocol. Conversely, injecting more than one cell means that multiple cells would be analyzed at once and thus it would be impossible to tell which interactions occur within a single cell. Loading the single-cell content of pre-lysed single-cell into the LOC could be suggested as a way to overcome these limitations. However, this method relies upon 100% efficient transfer of the cell components into the LOC.

6. Conclusion and future work

This research is a part of a larger project that aims to capture inter- and intra-chromosomal interactions of a single cell. The main objectives of this research are to build and test a microfluidic system (LOC) that will aid in the capture of inter- and intra- chromosomal interactions of a single cell and to characterize the restriction and ligation of DNA that will be performed in LOC. LOC was successfully assembled and tested. Computer interface was also developed with LabView 2009 (National Instruments) to control the pumps (Appendix A and B).

Digestion kinetics of EcoRI and MspI was measured to characterize digestion of DNA within microfluidic chips. EcoRI and MspI digestion kinetics were used as benchmarks and were successfully obtained for digestion within a microtube. However, the quantification of digestion kinetics within microfluidic chips was unsuccessful due to non-specific nuclease activity of EcoRI and MspI under the conditions tested. It is hypothesized that the non-specific nuclease activity is caused by the increase in surface area. However, this was not confirmed through digestion reaction within a microtube with glass beads. Therefore, it is hypothesized that the movement of solution or other factors may also need to be present in conjunction with the increased surface area to trigger the non-specific nuclease activity. The non-specific nuclease activity of EcoRI and MspI within microfluidic chips is problematic for carrying out single-cell GCC with LOC because it would cause loss of template, random variations in ligated products and failure to produce sticky ends required for re-ligation.

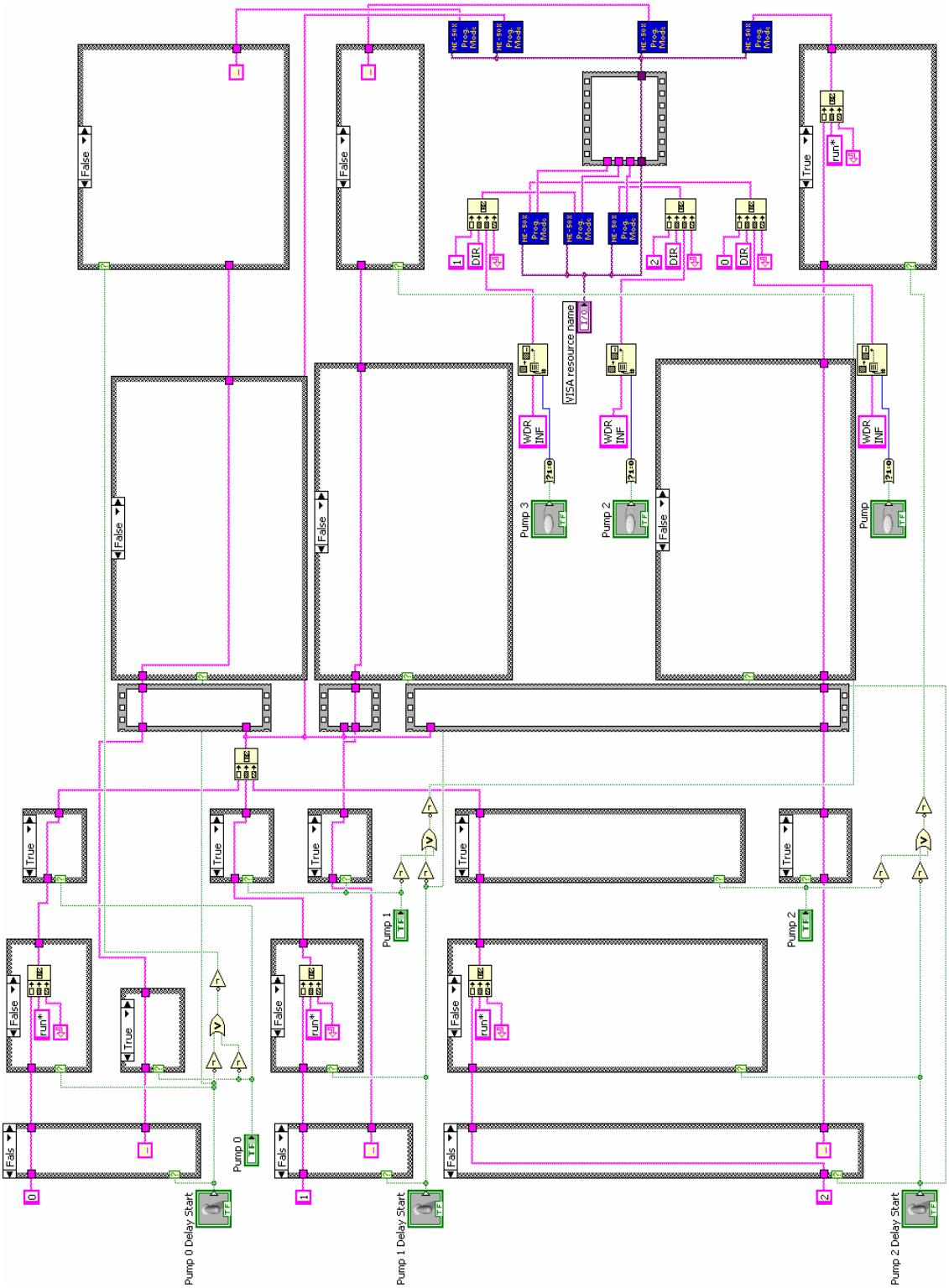
Future work will involve testing digestion conditions that negates the non-specific nuclease activity of restriction enzyme within microfluidic chips. Additionally, ligation within microfluidic chips will also need to be investigated further to determine if the yield of ligated products from ligation within microfluidic chips can be increased.

Furthermore, single-cell isolation protocol needs to be developed that allows the injection of a single cell or cell content from a single cell into the LOC. A single-cell trap made from PDMS was fabricated by Bryon Wright (University of Auckland). However, the single-cell trap arrived too late and will still need to be tested.

In conclusion, one of the two objectives of this research was fully met. LOC was constructed and tested to be fully functional and free of leakages. The attempt to characterize the digestion and ligation of DNA within microfluidic chips met unexpected complications due to non-specific nuclease activities exhibited by EcoRI and MspI within microfluidic chips. This research spearheads the research into chromosome interaction of a single cell and will provide some guide for future research in this area. Resolution of problems identified in this research will lead to the successful capture of chromosomal interactions within a single cell.

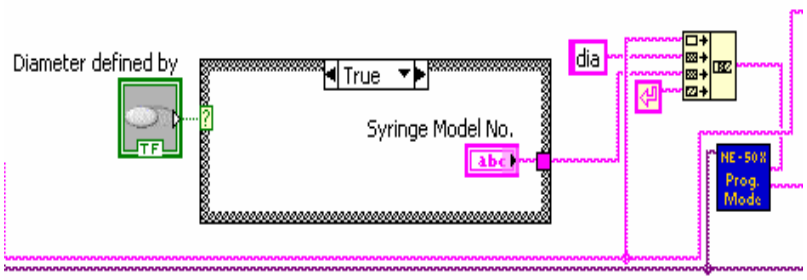
Appendices

Appendix A. Block diagram of VI for simultaneous activation of pumps.

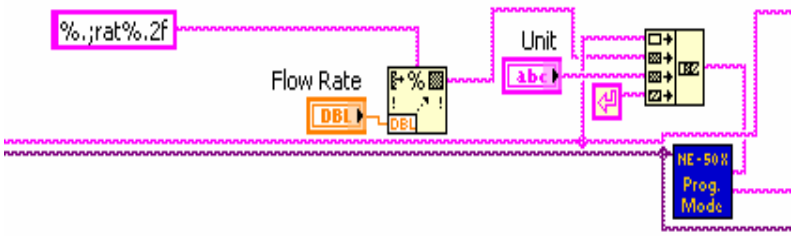


Appendix B. Core block diagram of VI to change pump settings.

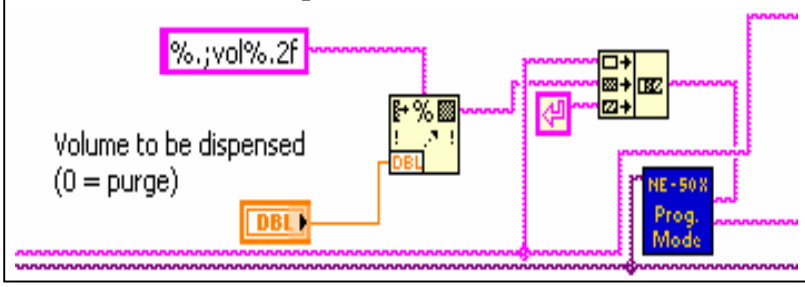
Set syringe inner diameter



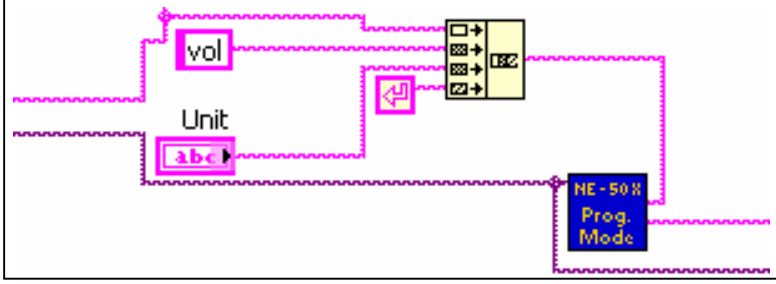
Set flow rate and unit for flow rate



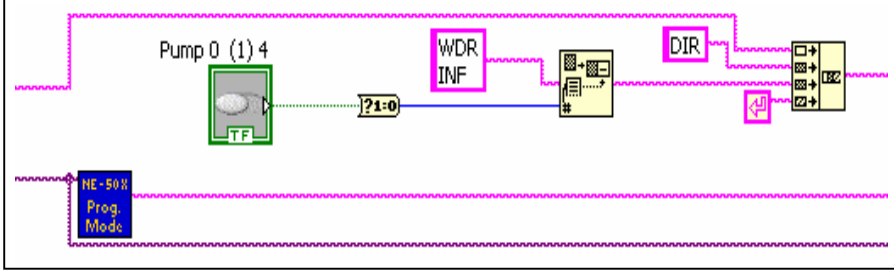
Set volume to be dispensed



Set unit for volume to be dispensed



Set pumping direction



References:

Aiken, C. R., McLaughlin, L. W., & Gumport, R. I. (1991). The highly homologous isoschizomers RsrI endonuclease and EcoRI endonuclease do not recognize their target sequence identically. *Journal of Biological Chemistry, 266*(28), 19070-19078.

Anderson, J. E. (1993). Restriction Endonucleases and Modification Methylases. *Current Opinion in Structural Biology, 3*(1), 24-30.

Barker, D. L., Hansen, M. S. T., Faruqi, A. F., Giannola, D., Irsula, O. R., Lasken, R. S., et al. (2004). Two methods of whole-genome amplification enable accurate genotyping across a 2320-SNP linkage panel. *Genome Research, 14*(5), 901-907. doi: Doi 10.1101/Gr.1949704

Bergen, A. W., Haque, K. A., Qi, Y., Beerman, M. B., Garcia-Closas, M., Rothman, N., et al. (2005). Comparison of yield and genotyping performance of multiple displacement OmniPlex (TM) whole genome amplified DNA generated from multiple DNA sources. *Human Mutation, 26*(3), 262-270. doi: Doi 10.1002/Humu.20213

Blanco, L., & Salas, M. (1984). Characterization And Purification Of A Phage Phi-29-Encoded DNA-Polymerase Required For The Initiation Of Replication. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences, 81*(17), 5325-5329.

Bouzid, M., Heavens, D., Elwin, K., Chalmers, R. M., Hadfield, S. J., Hunter, P. R., et al. (2010). Whole genome amplification (WGA) for archiving and genotyping of clinical isolates of Cryptosporidium species. [Article]. *Parasitology, 137*(1), 27-36. doi: 10.1017/s0031182009991132

Dean, F. B., Hosono, S., Fang, L. H., Wu, X. H., Faruqi, A. F., Bray-Ward, P., et al. (2002). Comprehensive human genome amplification using multiple displacement amplification. *Proceedings of the National Academy of Sciences of the United States of America, 99*(8), 5261-5266.

Dekker, J., Rippe, K., Dekker, M., & Kleckner, N. (2002). Capturing chromosome conformation. *Science, 295*(5558), 1306-1311.

Dostie, J., Richmond, T. A., Arnaout, R. A., Selzer, R. R., Lee, W. L., Honan, T. A., et al. (2006). Chromosome Conformation Capture Carbon Copy (5C): A massively parallel solution for mapping interactions between genomic elements. [Article]. *Genome Research, 16*(10), 1299-1309. doi: 10.1101/gr.5571506

Duan, Z., Andronescu, M., Schutz, K., McIlwain, S., Kim, Y. J., Lee, C., et al. (2010). A three-dimensional model of the yeast genome. *Nature, advance online publication*. doi: 10.1038/nature08973

Fiegler, H., Geigl, J. B., Langer, S., Rigler, D., Porter, K., Unger, K., et al. (2007). High resolution array-CGH analysis of single cells. *Nucleic Acids Research, 35*(3). doi: 10.1093/nar/gkl1030

Fredriksson, S., Gullberg, M., Jarvius, J., Olsson, C., Pietras, K., Gustafsdottir, S. M., et al. (2002). Protein detection using proximity-dependent DNA ligation assays. *Nature Biotechnology, 20*(5), 473-477. doi: Doi 10.1038/Nbt0502-473

Friedhoff, P., Lurz, R., Luder, G., & Pingoud, A. (2001). Sau3AI, a monomeric type II restriction endonuclease that dimerizes on the DNA and thereby induces DNA loops. *Journal of Biological Chemistry, 276*(26), 23581-23588.

Frumkin, D., Wasserstrom, A., Itzkovitz, S., Harmelin, A., Rechavi, G., & Shapiro, E. (2008). Amplification of multiple genomic loci from single cells isolated by laser micro-dissection of tissues. *Bmc Biotechnology, 8*. doi: 10.1186/1472-6750-8-17

Fu, L. M., & Lin, C. H. (2007). A rapid DNA digestion system. *Biomedical Microdevices, 9*(2), 277-286. doi: DOI 10.1007/s10544-006-9036-0

Gai, H. W., Li, Y., Silber-Li, Z., Ma, Y. F., & Lin, B. C. (2005). Simultaneous measurements of the flow velocities in a microchannel by wide/evanescent field illuminations with particle/single molecules. *Lab on a Chip, 5*(4), 443-449. doi: Doi 10.1039/B416476h

Geigl, J., & Speicher, M. (2008). New methods of single-cell analysis in research and diagnostics. *Medizinische Genetik, 20*(4), 407-415. doi: 10.1007/s11825-008-0138-3

Geigl, J. B., Obenauf, A. C., Waldispuehl-Geigl, J., Hoffmann, E. M., Auer, M., Hormann, M., et al. (2009). Identification of small gains and losses in single cells after whole genome amplification on tiling oligo arrays. *Nucleic Acids Research, 37*(15). doi: 10.1093/nar/gkp526

Glentis, S., SenGupta, S., Thornhill, A., Wang, R., Craft, I., & Harper, J. C. (2009). Molecular comparison of single cell MDA products derived from different cell types. *Reproductive Biomedicine Online, 19*(1), 89-98.

Hager, P. W., Reich, N. O., Day, J. P., Coche, T. G., Boyer, H. W., Rosenberg, J. M., et al. (1990). Probing the Role of Glutamic Acid-144 in the EcoRI Endonuclease Using Aspartic-Acid and Glutamine Replacements. *Journal of Biological Chemistry, 265*(35), 21520-21526.

Handyside, A. H., Robinson, M. D., Simpson, R. J., Omar, M. B., Shaw, M. A., Grudzinskas, J. G., et al. (2004). Isothermal whole genome amplification from single and small numbers of cells: a new era for preimplantation genetic diagnosis of inherited disease. *Molecular Human Reproduction,*

10(10), 767-772. doi: DOI 10.1093/molehr/gah101

Hellani, A., Coskun, S., Benkhalifa, M., Tbakhi, A., Sakati, N., Al-Odaib, A., et al. (2004). Multiple displacement amplification on single cell and possible PGD applications. *Molecular Human Reproduction, 10*(11), 847-852. doi: 10.1093/molehr/gah114

Huai, Q., Colandene, J. D., Chen, Y. Q., Luo, F., Zhao, Y. D., Topal, M. D., et al. (2000). Crystal structure of NaeI - an evolutionary bridge between DNA endonuclease and topoisomerase. *Embo Journal, 19*(12), 3110-3118.

Huang, B., Wu, H. K., Bhaya, D., Grossman, A., Granier, S., Kobilka, B. K., et al. (2007). Counting low-copy number proteins in a single cell. *Science, 315*(5808), 81-84. doi: DOI 10.1126/science.1133992

Imle, A., Polzer, B., Alexander, S., Klein, C. A., & Friedl, P. (2009). Genomic Instability of Micronucleated Cells Revealed by Single-Cell Comparative Genomic Hybridization. *Cytometry Part A, 75A*(7), 562-568. doi: 10.1002/cyto.a.20733

Izsvak, Z., & Duda, E. (1989). Star Activity and Complete Loss of Specificity of CeqI Endonuclease. *Biochemical Journal, 258*(1), 301-303.

Jiang, Z. W., Zhang, X. Q., Deka, R., & Jin, L. (2005). Genome amplification of single sperm using multiple displacement amplification. *Nucleic Acids Research, 33*(10), -. doi: Doi 10.1093/Nar/Gni089

Kettling, U., Koltermann, A., Schwille, P., & Eigen, M. (1998). Real-time enzyme kinetics monitored by dual-color fluorescence cross-correlation spectroscopy. *Proc. Natl. Acad. Sci. USA, 95*, 1416–1420.

Kim, D. S., Kang, Y. K., & Yoo, O. J. (1994). Effects of Base Analog Substitutions in the Sequence, ccgg, on the Cleavage and Methylation Reactions of HpaII and MspI Endonucleases and Their Cognate Methylases. *Biochemistry and Molecular Biology International, 32*(3), 507-514.

Krylov, S. N., Starke, D. A., Arriaga, E. A., Zhang, Z. R., Chan, N. W. C., Palcic, M. M., et al. (2000). Instrumentation for chemical cytometry. *Analytical Chemistry, 72*(4), 872-877.

Kvist, T., Ahring, B. K., Lasken, R. S., & Westermann, P. (2007). Specific single-cell isolation and genomic amplification of uncultured microorganisms. *Applied Microbiology and Biotechnology, 74*(4), 926-935. doi: 10.1007/s00253-006-0725-7

Langowski, J., Urbanke, C., Pingoud, A., & Maass, G. (1981). Transient Cleavage Kinetics of the EcoRI Restriction Endonuclease Measured in a Pulsed Quench-Flow Apparatus - Enzyme Concentration-Dependent Activity Change. *Nucleic Acids Research, 9*(14), 3483-3490.

Lasken, R. S., & Stockwell, T. B. (2007). Mechanism of chimera formation during the Multiple Displacement Amplification reaction. *BMC Biotechnol, 7*, 19.

Lee, W. G., Kim, Y. G., Chung, B. G., Demirci, U., & Khademhosseini, A. (2010). Nano/Microfluidics for diagnosis of infectious diseases in developing countries. *Advanced Drug Delivery Reviews, 62*(4-5), 449-457. doi: DOI 10.1016/j.addr.2009.11.016

Lenshof, A., & Laurell, T. (2010). Continuous separation of cells and particles in microfluidic systems. *Chemical Society Reviews, 39*(3), 1203-1217. doi: Doi 10.1039/B915999c

Li, X. M., Song, C., Zhao, M. P., & Li, Y. Z. (2008). Continuous monitoring of restriction endonuclease cleavage activity by universal molecular beacon light quenching coupled with real-time polymerase chain reaction. *Analytical Biochemistry, 381*(1), 1-7. doi: Doi 10.1016/J.Ab.2008.06.027

Li, Y., Di Naro, E., Vitucci, A., Zimmermann, B., Holzgreve, W., & Hahn, S. (2005). Detection of paternally inherited fetal point mutations for beta-thalassemia using size-fractionated cell-free DNA in maternal plasma. *Jama-Journal of the American Medical Association, 293*(7), 843-849.

Lieberman-Aiden, E., van Berkum, N. L., Williams, L., Imakaev, M., Ragoczy, T., Telling, A., et al. (2009). Comprehensive Mapping of Long-Range Interactions Reveals Folding Principles of the Human Genome. [Article]. *Science, 326*(5950), 289-293. doi: 10.1126/science.1181369

Ling, J. W., Zhuang, G. L., Tazon-Vega, B., Zhang, C. H., Cao, B. Q., Rosenwaks, Z., et al. (2009). Evaluation of genome coverage and fidelity of multiple displacement amplification from single cells by SNP array. *Molecular Human Reproduction, 15*(11), 739-747. doi: 10.1093/molehr/gap066

Lomvardas, S., Barnea, G., Pisapia, D. J., Mendelsohn, M., Kirkland, J., & Axel, R. (2006). Interchromosornal interactions and olfactory receptor choice. *Cell, 126*(2), 403-413. doi: 10.1016/j.cell.2006.06.035

Love, J. C., Wolfe, D. B., Jacobs, H. O., & Whitesides, G. M. (2001). Microscope projection photolithography for rapid prototyping of masters with micron-scale features for use in soft lithography. *Langmuir, 17*(19), 6005-6012.

Malic, L., Brassard, D., Veres, T., & Tabrizian, M. (2010). Integration and detection of biochemical assays in digital microfluidic LOC devices. *Lab on a Chip, 10*(4), 418-431. doi: Doi 10.1039/B917668c

Marcy, Y., Ishoey, T., Lasken, R. S., Stockwell, T. B., Walenz, B. P., Halpern, A. L., et al. (2007). Nanoliter reactors improve multiple displacement amplification of genomes from single cells. *Plos Genetics, 3*(9), 1702-1708.

doi: 10.1371/journal.pgen.0030155

Marcy, Y., Ouverney, C., Bik, E. M., Losekann, T., Ivanova, N., Martin, H. G., et al. (2007). Dissecting biological "dark matter" with single-cell genetic analysis of rare and uncultivated TM7 microbes from the human mouth. *Proceedings of the National Academy of Sciences of the United States of America, 104*(29), 11889-11894. doi: 10.1073/pnas.0704662104

Mark, D., Haeberle, S., Roth, G., von Stetten, F., & Zengerle, R. (2010). Microfluidic lab-on-a-chip platforms: requirements, characteristics and applications. *Chemical Society Reviews, 39*(3), 1153-1182. doi: Doi 10.1039/B820557b

Marre, S., & Jensen, K. F. (2010). Synthesis of micro and nanostructures in microfluidic systems. *Chemical Society Reviews, 39*(3), 1183-1202. doi: Doi 10.1039/B821324k

McClain, M. A., Culbertson, C. T., Jacobson, S. C., Allbritton, N. L., Sims, C. E., & Ramsey, J. M. (2003). Microfluidic devices for the high-throughput chemical analysis of cells. *Analytical Chemistry, 75*(21), 5646-5655. doi: Doi 10.1021/Ac0346510

McLaughlin, L. W., Benseler, F., Graeser, E., Piel, N., & Scholtissek, S. (1987). Effects Of Functional-Group Changes In The EcoRI Recognition Site On The Cleavage Reaction Catalyzed By The Endonuclease. *Biochemistry, 26*(23), 7238-7245.

Mellors, J. S., Jorabchi, K., Smith, L. M., & Ramsey, J. M. (2010). Integrated Microfluidic Device for Automated Single Cell Analysis Using Electrophoretic Separation and Electrospray Ionization Mass Spectrometry. *Analytical Chemistry, 82*(3), 967-973. doi: 10.1021/ac902218y

Möncke-Buchner, E., Rothenberg, M., Reich, S., Wagenführ, K., Matsumura, H., Terauchi, R., et al. (2009). Functional Characterization and Modulation of the DNA Cleavage Efficiency of Type III Restriction Endonuclease EcoP15I in Its Interaction with Two Sites in the DNA Target. *Journal of Molecular Biology, 387*(5), 1309-1319. doi: 10.1016/j.jmb.2009.02.047

Muir, R. S., Flores, H., Zinder, N. D., Model, P., Soberon, X., & Heitman, J. (1997). Temperature-sensitive mutants of the EcoRI endonuclease. *Journal of Molecular Biology, 274*(5), 722-737.

Nanassy, O. Z., Haydock, P. V., & Reed, M. W. (2007). Capture of genomic DNA on glass microscope slides. *Analytical Biochemistry, 365*(2), 240-245. doi: Doi 10.1016/J.Ab.2007.03.017

Nasri, N. W. M., Jamal, A. R. A., Abdullah, N. C., Razi, Z. R. M., & Mokhtar, N.

M. (2009). Preimplantation Genetic Diagnosis for beta-Thalassemia Using Single-cell DNA Analysis for Codons 17 and 26 of beta-globin Gene. *Archives of Medical Research, 40*(1), 1-9. doi: 10.1016/j.arcmed.2008.10.008

Nelson, J. R., Cai, Y. C., Giesler, T. L., Farchaus, J. W., Sundaram, S. T., Ortiz-Rivera, M., et al. (2002). TempliPhi, phi 29 DNA polymerase based rolling circle amplification of templates for DNA sequencing. *Biotechniques*, 44-47.

O'Sullivan, J. M., Sontam, D. M., Grierson, R., & Jones, B. (2009). Repeated elements coordinate the spatial organization of the yeast genome. *Yeast, 26*(2), 125-138. doi: 10.1002/yea.1657

Ohlsson, R., & Göndör, A. (2007). The 4C technique: the 'Rosetta stone' for genome biology in 3D? *Current Opinion in Cell Biology, 19*(3), 321-325. doi: 10.1016/j.ceb.2007.04.008

Panelli, S., Damiani, G., Espen, L., Micheli, G., & Sgaramella, V. (2006). Towards the analysis of the genomes of single cells: Further characterisation of the multiple displacement amplification. *Gene, 372*, 1-7. doi: 10.1016/j.gene.2006.01.032

Pingoud, A., Fuxreiter, M., Pingoud, V., & Wende, W. (2005). Type II restriction endonucleases: structure and mechanism. *Cellular and Molecular Life Sciences, 62*(6), 685-707. doi: DOI 10.1007/s00018-004-4513-1

Pingoud, A., & Jeltsch, A. (1997). Recognition and cleavage of DNA by type-II restriction endonucleases. *European Journal of Biochemistry, 246*(1), 1-22.

Pingoud, A., & Jeltsch, A. (2001). Structure and function of type II restriction endonucleases. *Nucleic Acids Research, 29*(18), 3705-3727.

Pingoud, V., Wende, W., Friedhoff, P., Reuter, M., Alves, J., Jeltsch, A., et al. (2009). On the Divalent Metal Ion Dependence of DNA Cleavage by Restriction Endonucleases of the EcoRI Family. *Journal of Molecular Biology, 393*(1), 140-160. doi: DOI 10.1016/j.jmb.2009.08.011

Ren, Z., Zhou, C. Q., Xu, Y. W., Deng, J., Zeng, H. T., & Zeng, Y. H. (2007). Mutation and haplotype analysis for Duchenne muscular dystrophy by single cell multiple displacement amplification. *Molecular Human Reproduction, 13*(5-6), 431-436. doi: 10.1093/molehr/gam020

Roberts, R. J., Belfort, M., Bestor, T., Bhagwat, A. S., Bickle, T. A., Bitinaite, J., et al. (2003). A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes. *Nucleic Acids Research, 31*(7), 1805-1812. doi: Doi 10.1093/Nar/Gkg274

Robinson, C. R., & Sligar, S. G. (1993). Molecular Recognition Mediated by Bound Water - a Mechanism for Star Activity of the

Restriction-Endonuclease EcoRI. *Journal of Molecular Biology, 234*(2), 302-306.

Robinson, C. R., & Sligar, S. G. (1998). Changes in solvation during DNA binding and cleavage are critical to altered specificity of the EcoRI endonuclease. *Proceedings of the National Academy of Sciences of the United States of America, 95*(5), 2186-2191.

Rodley, C. D. M., Bertels, F., Jones, B., & O'Sullivan, J. M. (2009). Global identification of yeast chromosome interactions using Genome conformation capture. *Fungal Genetics and Biology, 46*(11), 879-886. doi: 10.1016/j.fgb.2009.07.006

Rodrigue, S., Malmstrom, R. R., Berlin, A. M., Birren, B. W., Henn, M. R., & Chisholm, S. W. (2009). Whole Genome Amplification and De novo Assembly of Single Bacterial Cells. [Article]. *Plos One, 4*(9), 10. doi: e6864 10.1371/journal.pone.0006864

Ros, A., Hellmich, W., Regtmeier, J., Duong, T. T., & Anselmetti, D. (2006). Bioanalysis in structured microfluidic systems. *Electrophoresis, 27*(13), 2651-2658. doi: DOI 10.1002/elps.200500923

Schowalter, K. V., Fredrickson, J., & Thornhill, A. R. (2007). Efficient isothermal amplification of the entire genome from single cells. *Methods Mol Med, 132*, 87-99.

Simonis, M., Klous, P., Splinter, E., Moshkin, Y., Willemsen, R., de Wit, E., et al. (2006). Nuclear organization of active and inactive chromatin domains uncovered by chromosome conformation capture-on-chip (4C). *Nature Genetics, 38*, 1348-1354. doi: 10.1038/ng1896

Spits, C., Le Caignec, C., De Rycke, M., Van Haute, L., Van Steirteghem, A., Liebaers, I., et al. (2006a). Optimization and evaluation of single-cell whole, genome multiple displacement amplification. *Human Mutation, 27*(5), 496-503. doi: 10.1002/humu.20324

Spits, C., Le Caignec, C., De Rycke, M., Van Haute, L., Van Steirteghem, A., Liebaers, I., et al. (2006b). Whole-genome multiple displacement amplification from single cells. *Nature Protocols, 1*(4), 1965-1970. doi: 10.1038/nprot.2006.326

Stahl, F., Wende, W., Jeltsch, A., & Pingoud, A. (1996). Introduction of asymmetry in the naturally symmetric restriction endonuclease EcoRV to investigate intersubunit communication in the homodimeric protein. *Proceedings of the National Academy of Sciences of the United States of America, 93*(12), 6175-6180.

Stepanauskas, R., & Sieracki, M. E. (2007). Matching phylogeny and metabolism

in the uncultured marine bacteria, one cell at a time. *Proceedings of the National Academy of Sciences of the United States of America, 104*(21), 9052-9057. doi: 10.1073/pnas.0700496104

Takizawa, T., Meaburn, K. J., & Misteli, T. (2008). The meaning of gene positioning. *Cell, 135*(1), 9-13. doi: DOI 10.1016/j.cell.2008.09.026

Tang, F. C., Barbacioru, C., Wang, Y. Z., Nordman, E., Lee, C., Xu, N. L., et al. (2009). mRNA-Seq whole-transcriptome analysis of a single cell. *Nature Methods, 6*(5), 377-U386. doi: 10.1038/nmeth.1315

Taylor, R. J., Falconnet, D., Niemisto, A., Ramsey, S. A., Prinz, S., Shmulevich, I., et al. (2009). Dynamic analysis of MAPK signaling using a high-throughput microfluidic single-cell imaging platform. *Proceedings of the National Academy of Sciences of the United States of America, 106*(10), 3758-3763. doi: DOI 10.1073/pnas.0813416106

Venclovas, C., Timinskas, A., & Siksnys, V. (1994). 5-Stranded Beta-Sheet Sandwiched with 2 Alpha-Helices - a Structural Link between Restriction Endonucleases EcoRI and EcoRV. *Proteins-Structure Function and Genetics, 20*(3), 279-282.

Vipond, I. B., Baldwin, G. S., & Halford, S. E. (1995). Divalent Metal Ions at the Active Sites of the EcoRV and EcoRI Restriction Endonucleases. *Biochemistry, 34*(2), 697-704. doi: 10.1021/bi00002a037

Wah, D. A., Bitinaite, J., Schildkraut, I., & Aggarwal, A. K. (1998). Structure of FokI has implications for DNA cleavage. *Proceedings of the National Academy of Sciences of the United States of America, 95*(18), 10564-10569.

Wah, D. A., Hirsch, J. A., Dorner, L. F., Schildkraut, I., & Aggarwal, A. K. (1997). Structure of the multimodular endonuclease FokI bound to DNA. *Nature, 388*(6637), 97-100.

Wang, H. Y., & Lu, C. (2006). Electroporation of mammalian cells in a microfluidic channel with geometric variation. *Analytical Chemistry, 78*(14), 5158-5164. doi: Doi 10.1021/Ac060733n

Watrob, H., Liu, W., Chen, Y., Bartlett, S. G., Jen-Jacobson, L., & Barkley, M. D. (2001). Solution conformation of EcoRI restriction endonuclease changes upon binding of cognate DNA and Mg2+ cofactor. *Biochemistry, 40*(3), 683-692.

Wei, H., Therrien, C., Blanchard, A., Guan, S. X., & Zhu, Z. Y. (2008). The Fidelity Index provides a systematic quantitation of star activity of DNA restriction endonucleases. *Nucleic Acids Research, 36*(9), -. doi: ARTN e50

DOI 10.1093/nar/gkn182

Wei, J., Wang, L.-M., Zheng, J.-F., Zheng, L., & Huang, Y.-R. (2007). The

detection of mutations in VHL gene from a single cell in a patient with von Hippel-Lindau disease. *Zhonghua Yixue Yichuanxue Zazhi, 24*(1), 67-71.

Woodhead, J. L., Bhave, N., & Malcolm, A. D. B. (1981). Cation Dependence of Restriction Endonuclease EcoRI Activity. *European Journal of Biochemistry, 115*(2), 293-296.

Wurtele, H., & Chartrand, P. (2006). Genome-wide scanning of HoxB1-associated loci in mouse ES cells using an open-ended Chromosome Conformation Capture methodology. *Chromosome Research, 14*(5), 477-495. doi: 10.1007/s10577-006-1075-0

Xu, Q. S., Kucera, R. B., Roberts, R. J., & Guo, H.-C. (2004). An Asymmetric Complex of Restriction Endonuclease MspI on Its Palindromic DNA Recognition Site. *Structure, 12*(9), 1741-1747. doi: DOI: 10.1016/j.str.2004.07.014

Yamaguchi, Y., Arakawa, T., Takeda, N., Edagawa, Y., & Shoji, S. (2009). Development of a poly-dimethylsiloxane microfluidic device for single cell isolation and incubation. *Sensors and Actuators B-Chemical, 136*(2), 555-561. doi: DOI 10.1016/j.snb.2008.11.052

Yang, C. G., Xu, Z. R., & Wang, J. H. (2010). Manipulation of droplets in microfluidic systems. *Trac-Trends in Analytical Chemistry, 29*(2), 141-157. doi: DOI 10.1016/j.trac.2009.11.002

Yin, H. B., Zhang, X. L., Pattrick, N., Klauke, N., Cordingley, H. C., Haswell, S. J., et al. (2007). Influence of hydrodynamic conditions on quantitative cellular assays in microfluidic systems. *Analytical Chemistry, 79*(18), 7139-7144. doi: Doi 10.1021/Ac071146k

Zhang, K., Martiny, A. C., Reppas, N. B., Barry, K. W., Malek, J., Chisholm, S. W., et al. (2006). Sequencing genomes from single cells by polymerase cloning. *Nature Biotechnology, 24*(6), 680-686. doi: 10.1038/nbt1214

Zhao, Z., Tavoosidana, G., Sjolinder, M., Gondor, A., Mariano, P., Wang, S., et al. (2006). Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions. *Nature Genetics, 38*(11), 1341-1347. doi: 10.1038/ng1891