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Analysis of mitochondrial DNA in microfluidic systems

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Abstract

Abnormalities in mitochondrial function play a major role in many human diseases. It is often of critical importance to ascertain what proportion of the mitochondria within a cell, or cells, bear a given mutation (the mitochondrial "demographics"). In this work, a rapid, novel, on-chip procedure was used, in which a restriction enzyme was employed to excise a mitochondrial DNA (mtDNA) sequence from plasmid DNA that acted as a prototypical mitochondrial genome. The DNA was then denatured, reassembled to form duplexes, fluorescently labelled and analysed. This method was able to differentiate between a homogeneous population and a heterogeneous population. Using a microfluidic chip, the method could be performed in about 45 min, even without robotics or multiplexed operation, whereas conventional methods of analysis require days to perform. This method may ultimately form the basis for a means of characterizing the mitochondrial demographics of a single cell.

Keywords: Microfluidic; Lab-on-a-chip; Electrophoresis; Heteroduplex analysis; Restriction enzyme digest

1. Introduction

Methods for controlling DNA self-assembly play a significant role in the life sciences, but macroscopic processes tend to be slow (ranging from hours to days), labour intensive, and expensive. There is thus a need to develop a rapid and inexpensive method of excising and analyzing specific DNA segments from a small sample. This work is a preliminary demonstration of such a method, one that is based on the control of the self-assembly of DNA within microfluidic devices.

An area of significant interest in the life sciences is the analysis of mitochondrial DNA (mtDNA). The mitochondria are sub-cellular organelles responsible for the bulk of cellular energy production and their DNA is circular (approximately 16.5 kb), containing several dozen genes, as well as a regulatory region called the 'D-loop'. With each mitochondrion containing up to 10 copies of the mitochondrial genome, and each cell having hundreds or thousands of mitochondria, each cell may thus house many differing mitochondrial genomes. A cell having two or more variants of the mtDNA is referred to as being heteroplasmic, whereas one having a single type of mtDNA is homoplasmic.

Mitochondrial DNA mutations are associated with a wide range of human diseases, with reports documenting their connection to cardiomyopathies, neurodegenerative disorders, Alzheimer disease, aging and cancer [1]. A common characteristic of mitochondrial diseases is a threshold effect wherein a person presents with no or minor symptoms when the mutant population is below a threshold (typically about 80%) and very severe symptoms when the population exceeds that threshold [2]. For reasons not completely understood, the mitochondrial demographics vary with cell type, thus often requiring that samples be taken from various tissues [2]. In

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order to investigate such diseases effectively, a tool is needed that can analyse mitochondrial demographics and determine the proportion of mutant mitochondrial DNA.

Using conventional methods, the analysis of mitochondrial DNA is problematic. The preparation of mtDNA is very time consuming and labour intensive, and PCR amplification of mtDNA sequences present challenges due to the many copies of the mtDNA that are present in the nuclear genome [3]. Given the large tissue samples often used, the effectiveness of many studies is further reduced by the false assumption that all cells within the sample are identical. Even with sufficient DNA and purification, the task of directly sequencing the region of interest is expensive and unlikely to detect variations that account for less than 30% of the population [4]. This is problematic, as such minority populations are very important in the study of mitochondrial diseases. This inability to resolve low levels of heteroplasmy has also led to controversy over whether the mtDNA in a normal cell is homoplasmic or heteroplasmic. Recent reports suggest that heteroplasmy is the normal state in a variety of human cell types [5,6; Helmle and Glerum, unpublished]. Clearly, a method that can ease the task of analyzing mtDNA within a single cell is required in order to determine the role for mtDNA variants in human disease. Along with reducing sample size, such an analysis could underpin routine medical diagnostics that would ascertain the degree of mtDNA heteroplasmy in a patient and its relationship to the patient's health.

Medintz et al. [7] have amplified and sequenced samples of the mtDNA D-loop using a high-throughput system based, in part, upon a microchip. In a further improvement of this approach, Blazej et al. [8] recently applied the technique of polymorphism ratio sequencing (PRS) to characterize mitochondrial DNA. The use of such microchip-based methods has the potential to greatly increase throughputs over those presently achieved. Those methods were based upon off-chip amplifications and on-chip electrophoretic analysis. However, it is well recognized that such sequencing methods are a very costly means of determining the mutation status of a sample [9]. In an effort to reduce these costs, alternatives such as heteroduplex analysis (HA) and single-strand conformation polymorphism (SSCP) analysis have been developed. An additional handicap of sequencing methods is that, as mentioned above, they can be ineffective if the sample is not uniform, missing differing sequences that have an abundance of less than 30% [4]. By contrast, HA has been found to be capable of detecting mutated components that comprise as little as 1% of the overall sample [10].

In HA, DNA is first placed in denaturing conditions, where double-stranded DNA (dsDNA) melts into two complementary single strands of DNA (ssDNA). The conditions are then altered so that the single strands are able to renature and recombine, either with a perfectly complementary sequence to form a homoduplex, or with a nearly perfect complementary sequence to form a heteroduplex. During electrophoresis, the imperfect fit of the heteroduplexes typically causes them to migrate more slowly than the homoduplexes. Often, however, molecules of very similar size and shape, such as two homoduplexes, co-migrate such that separate peaks are not resolved. Electrophoresis under conditions that enhance mobility differences can be used to determine the presence of such a heterogeneous state, and thus the presence of DNA mutations (e.g. [10,11]).

The present study was part of an exploration of the potential link between mutations in mtDNA and the onset of cancer, with the purpose of developing a method that ultimately will be capable of analyzing the mtDNA within a single cell and tracking the abundance of a mutant sub-population. The purpose of this study was to move toward the development of a powerful microchip-based method for the analysis of heteroplasmy in a small sample of mtDNA. At this interim stage of development we have used cloned fragments of mtDNA in a plasmid vector as a prototype for the five-fold larger mitochondrial genome.

In previous work we have demonstrated on-chip labelling, HA [12] and SSCP [13] on PCR products in order to rapidly detect DNA mutations. Although this was an effective approach for detecting mutations in nuclear DNA (nDNA), the use of PCR can obscure the presence of subpopulations in the sample (due to selective amplification). The present work, therefore, focused on the development of a quantitative method without the PCR amplification step. For this study, we started with plasmid DNA that contained a mtDNA sequence (referred to as the insert). We then digested on-chip to excise the mtDNA insert. In addition, we denatured, re-annealed and labelled the DNA resulting from the plasmid digestion in order to perform HA.

2. Materials and methods

The mtDNA fragments studied here were derived from total cellular DNA isolated from either normal fibroblasts or normal blood samples. All samples were obtained from volunteers with informed consent. PCR was carried out using 150–300 ng of template DNA in a 25 μL reaction volume (10 ng/μL primer, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 1 U Taq Polymerase (Invitrogen)). The Hyper Variable Region I (HVRI) PCR primers encompassed nucleotides 16049–16402, and consisted of the forward primer, 5′-GTACCACCCAAGTATTGA-3′ with 5′-CGGAGGATGGTGGTCAA-3′ as the reverse primer. Reactions were cycled 32 times for 30 s at 92 °C, 40 s at 55 °C and 50 s at 72 °C. The 334 bp PCR samples were then purified (QiaQuick PCR purification kit, Qiagen), ligated into a 3 kb plasmid vector (pGEM-T Easy, Invitrogen), and cultured in *E. coli*. Plasmid DNA isolation from *E. coli* was performed using the GenElute Plasmid Miniprep Kit (Sigma). *Eco*RI (New England Biolabs, Mississauga, Ont., Canada) enzymatic digestion of a small portion of plasmid DNA was performed in order to confirm the presence of the mtDNA D-loop insert. Clones were sequenced with M13 fluorescently

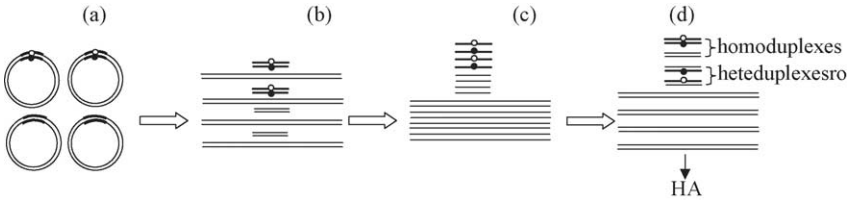

Fig. 1. Processing steps of the plasmid DNA: (a) four plasmids of 3 kb containing a 334 bp insert of mtDNA, two with wildtype inserts and two with inserts containing a mutation; (b) enzymatic digestion to form four 3 kb linear strands (referred to as vector DNA) and four homoduplexes of insert DNA; (c) denaturation to form eight single stranded 3 kb linear strands of vector DNA, four mutant and four wildtype single stranded 334 bp strands of insert DNA; and (d) renaturation to form four double stranded 3 kb strands of vector DNA, one homoduplex wildtype, one homoduplex mutant and two heteroduplexes double stranded 334 bp strands of inserted DNA.

labelled primers in order to determine the position and frequency of polymorphisms for each sample.

Plasmids were cultured within *E. coli*, which maintain only a single type of plasmid [14], in order to produce homoplasmic sources of the mtDNA sequence. Extraction of the plasmid DNA from these colonies thus provides a stable source of homoplasmic DNA. Using extracted plasmids with known sequence variations, homoplasmic or heteroplasmic DNA populations were produced by mixing plasmids in varying ratios prior to introducing the samples onto the microchip. Because of handling limitations, the smallest extracted DNA samples mixed were on the order of 1 μL.

We started with plasmid DNA containing mtDNA sequence, which was excised by performing enzymatic digestion on chip. We then performed HA on the chip as explained below. Fig. 1 illustrates this procedure: Starting with a mixture of plasmids containing two different (mitochondrial) insert sequences, duplexes could be generated by excising, denaturing and renaturing to form four different duplexes, two homoduplexes and two heteroduplexes.

2.1. Reagents

The samples ("#1" and "#2") used for this work had multiple sequence differences compared to the wild-type mtDNA sequence (www.mitomap.org). Sample #1 has the polymorphisms C16292T, C16294T and T16126C, while sample #2 has T16093C, T16189C, G16213A, C16223T, C16278T and insC16184 (Helmle and Glerum, unpublished). The PCR products were both from HVRI of the D-loop, with one being a homoplasmic wild-type and the other a heteroplasmic sample with seven sequence variations when compared to wild-type.

PCR reagents (polymerases and buffers) were from Invitrogen (Burlington, ON, Canada). GeneScan polymer was from Applied Biosystems (Foster City, CA). The RNase A, *Eco*RI buffer, and *Eco*RI enzyme were obtained from Roche Diagnostics (Laval, QB, Canada).

A standard Tris Borate with EDTA buffer (1 × TBE) was made using Tris-base and boric acid from Fisher Scientific (Fair Lawn, NJ), and EDTA from Merck (Darmstadt, Germany). A dilution of 5% GeneScan polymer (Applied Biosystems, Foster City, CA) in 1 × TBE was mixed with 10% glycerol (w/w) (Sigma, Saint Louis, MO). This dilution, referred to as "5GS10G" was used as the sieving medium for electrophoresis. The running buffer, referred to as "1 × TBE10G", was prepared by adding 10% glycerol to the 1 × TBE buffer solution (w/w). Formamide (minimum 99.5%) was purchased from Sigma (Saint Louis, MO) and deionized as per Sambrook and Russell [14]. Sytox Orange (SO), an intercalator suitable for labelling dsDNA, was obtained as a 5 mM stock solution in DMSO from Molecular Probes (Eugene, OR).

2.1.1. Off-chip labelling of DNA

In the case of PCR products analysed on-chip, the products were pre-labelled with SO by adding 3 μL of doubly deionized water, 0.2 μL of 0.5 μM SO, 0.45 μL 1 × TBE10G and 0.9 μL of PCR sample. All other samples were labelled on-chip.

2.1.2. Off-chip enzymatic digestion

For the purposes of comparison, some samples were digested off-chip. One microliters of plasmid DNA was digested with 1 μL *Eco*RI, according to the suggestions of the manufacturer. Once digested, some of the samples were mixed and reannealed thermally so as to produce heteroduplexes.

2.2. Microchip methods

The microfluidic devices (Micralyne, Edmonton, AB, Canada) are depicted in Fig. 2. The Microfluidic Tool Kit (μTK, Micralyne) was used to manipulate reagents and DNA in these microchips. For the present work, a laser induced fluorescence (LIF) system that provides excitation at a wavelength of 532 nm and detection at 578 nm was used. A compiled LABVIEW interface supplied by Micralyne was used to record the LIF signal at 200 Hz. LIF detection was performed 76 mm downstream from the intersection. A C^{++} program was used to analyze the fluorescence data. Using this program, spurious isolated spikes, peaks consisting of a single point due to electrical noise, were removed, and then a low-pass filter was applied to the data by performing a running average with a neighbourhood of 30 points. Because of the high rate of data acquisition, this low-pass filtering had

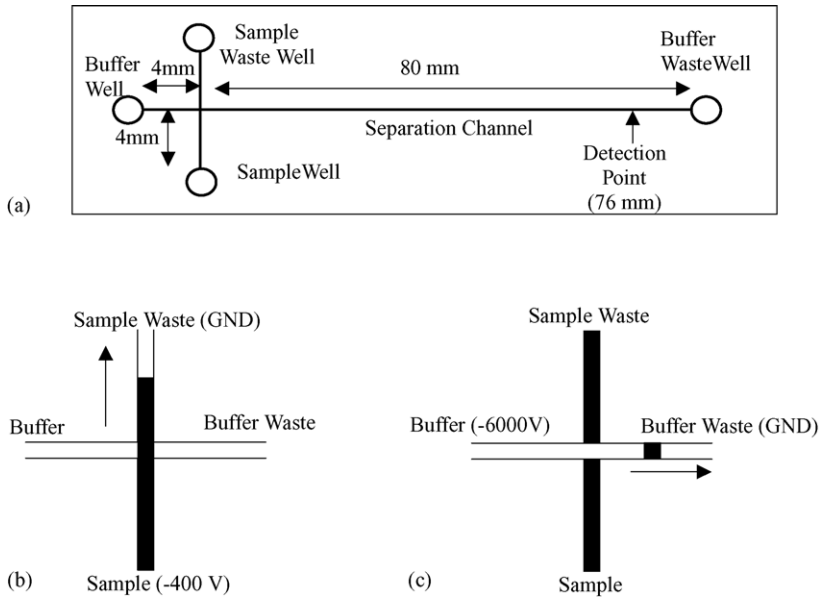

Fig. 2. The simple cross microfluidic chip: (a) a depiction of the simple cross microfluidic chip with reservoirs, 2 mm in diameter and 1.1 mm deep, linked by microchannels nominally 20 μm deep and 50 μm wide. The microchip is nominally 95 mm long and 16 mm wide; (b) a depiction of the microchannel intersection during the injection phase, showing the DNA (black) being drawn towards the sample waste well by applied potential; and (c) a depiction of the intersection of the microchannels during the separation phase, showing DNA (black) from the microchannel intersection being drawn towards the buffer waste well by applied potential. The contents of the injection arm (minus the intersection) are unaffected by this step.

no discernable effect upon the HA peaks. The analysis program created postscript files for all of the analysed data. More details can be found in previous work [11].

2.3. Chip preparation

The digestion of plasmids (sizing), denaturation, renaturation, labeling, and HA, all were performed on the microchip. The channels of the chip were filled with a sieving matrix—5GS10G polymer containing 0.2 μM of the intercalating dye SO, for on-column labeling of dsDNA. The plasmids and the reaction mix were placed in the sample well (Fig. 2). The remaining wells were filled with 1 × TBE10G running buffer, with 0.2 μM SO added to the buffer waste well to maintain intercalator levels in the microchannels. Subsequent runs of a sample were done without replacing the polymer matrix, but the polymer was replenished when changing samples. Further details are available in [11].

2.4. Sample injection and separation

The ionic contents of the wells were moved through the microchannels electrophoretically by applying high voltages. Fluorescently labeled molecules were then detected in the separation channel, 76 mm from the intersection (Fig. 2).

An electric field was used to draw DNA from the sample well in the direction of the sample waste well by applying −400 V at the sample well and grounding the sample waste well for 40 s, unless otherwise mentioned (Fig. 2b). During this long injection, the DNA has sufficient time to move directly from the sample well past the intersection. We also used shorter injections of 5 s, wherein the DNA reaching the intersection by the end of the injection period has come from within the injection channel, rather than the sample well.

After the injection step, the electrical connections between the sample and sample waste wells were removed and −6000 V was applied to the buffer well while the buffer waste well was grounded. These potentials were applied for 180 s, so that the DNA within the intersection (a volume of approximately 50 pL) was moved towards the buffer waste well (Fig. 2c). During this transport, DNA is separated according to size and shape, with smaller, more compact molecules moving more quickly through the gel matrix.

2.5. Enzymatic digestion and initial analysis

The restriction enzyme digestion reaction mix was made by adding 0.5 μL RNase A, 2.5 μL 10 × *Eco*RI buffer, 2.5 μL *Eco*RI enzyme, and 11.5 μL water in a microcentrifuge tube. The mixture was pipetted up and down to mix and stored on ice until used. An aliquot of 1.5 μL of the reaction mixture was placed in the sample well and 1.5 μL of a plasmid sample (either containing a single plasmid or an equal mixture of plasmids) was added and pipetted up and down 10 times to mix. This was then allowed to sit for 10 min in the chip to allow digestion to occur. During this time the chip was covered with paper towel to help prevent photobleaching of the dye. During this digestion, the enzyme cut the plasmids into linearized vector fragments and short D-loop inserts (i.e. converting the DNA loops of Fig. 1a. to the dsDNA strands of Fig. 1b.). After digestion, 1.5 μL of sample was removed from the sample well, and replaced by 2 μL of 0.2 × TBE2G.

The difference between the amount of digest mix removed and buffer added was to account for evaporation.

An electrophoretic run (as described in Section 2.4) was then performed to analyze the digested DNA, with separation times generally reflecting the size of linear DNA fragments.

2.6. On-chip heteroduplex analysis

Following the initial analysis, 1.5 μL of the sample well contents were removed, and 2 μL of formamide, a DNA denaturant, were added to the wells (approximately 67% of the total volume). This converts the dsDNA shown in Fig. 1b. to the ssDNA shown in Fig. 1c.). A second electrophoretic run was then performed, which served to manipulate the DNA and did not, itself, acquire any data. As described in Vahedi et al. this injects the ssDNA and clears the intersection while allowing the ssDNA to recombine in the injection channel [13]. Without the presence of the denaturant, the ssDNA (Fig. 1c.) reassembles to form dsDNA duplexes (Fig. 1d.). Finally, a third electrophoretic run was performed with a 5 s injection. With this short injection time, the separation step sampled dsDNA from within the injection arm. Data from this run was used to analyze the DNA sample for the presence of heteroduplexes, thus indicating whether or not the sample was heterogeneous.

3. Results and discussion

To verify that the electrophoretic conditions used in these experiments could demonstrate heteroduplex effects, we compared the electropherograms from samples of: (1) a PCR product known to be homoplasmic and (2) a PCR product of a sample known to be heteroplasmic. As expected for the homoplasmic sample, Fig. 3a shows an electropherogram with a single homoduplex peak. The heteroplasmic sample, Fig. 3b, shows a variety of peaks from the various homoduplexes and heteroduplexes. These PCR products were labelled off-chip and, although their signals were very weak, the electropherograms were reproducible. Further development of the procedure prompted us to use on-chip labelling methods, which gave stronger signals [13]. In future, we expect to use the SSCP method where each ssDNA species will give rise to one detected peak.

Fig. 4 shows an electropherogram corresponding to the analysis of a sample that contained two populations of plasmids. The plasmids differ at nine positions in the human mtDNA fragments and were enzymatically digested in the sample well of the microchip (See Fig. 2) to selectively excise the 334 bp sequence of mtDNA, leaving a 2997 bp-length vector fragment. Due to the use of intercalators, the fluorescent signal is typically proportional to the strand length. Therefore, the single small peak near 180 s corresponds to the small, fast moving mitochondrial homoduplexes (334 bp). A stronger peak near 200 s, corresponding to the vector DNA, followed this peak. Uncut plasmids travel as a broad hump,

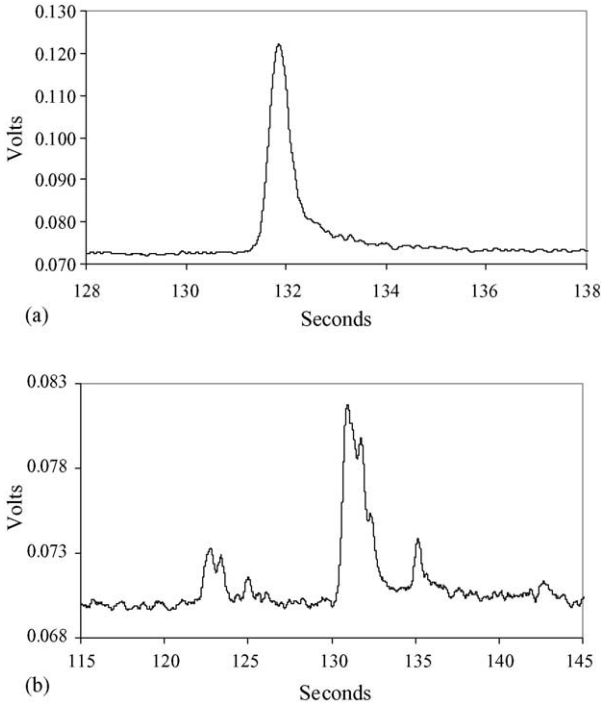

Fig. 3. Electropherograms (relative fluorescence units vs. time) of: a PCR product known to be homoplasmic (a) and a PCR product of a sample known to be heteroplasmic (b).

arriving near 130 s. The leading peak near 180 s is shown in more detail in the inset of Fig. 4. It is apparent that there is only a single peak present in the inset, indicating that we cannot distinguish between wildtype and mutant populations at this point (since both are homoduplex dsDNA).

It is clear from the results of Fig. 4 that the on-chip digest successfully excised enough of the human mtDNA to produce distinct peaks. However, in order to produce a larger signal, the digest could be improved to more completely excise the insert from the plasmid. The vector peak in Fig. 4, and subsequent figures, is a double peak. The reason for this was

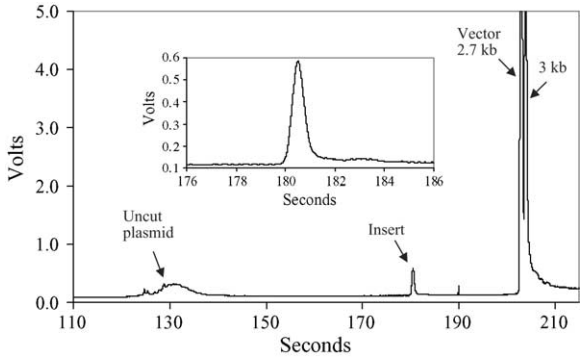

Fig. 4. An electropherogram of a mixure of sample #1 and #2 containing two types of mtDNA differing by 9 mutations following a 40 s injection after on-chip enzymatic excision. As expected, without denaturing and reannealling, only a single peak is seen. The small peak near 180 s represents the passage of the 334 bp segment of human mtDNA, which is shown in more detail in the figure inset.

determined in a separate experiment. In that experiment, an enzyme (NdeI) was used, which, unlike *Eco*RI, only cuts the plasmid once, thus linearizing it. The resulting electropherogram showed that the linearized vector peaks ran at the same time as the second of the two vector peaks seen in Fig. 4 (data not shown), thus suggesting that the second peak is the result of the enzyme cutting the plasmid DNA at only one position (partial digestion). Therefore, the faster peak is vector DNA from which the plasmid DNA was completely excised, as it runs more quickly, being the shorter of the two sequences (3 kb).

In a separate experiment, we confirmed that the uncut plasmid DNA contributes the broad hump at 130 s. An experiment was run with no enzyme in the digestion mixture, so that the only peak present would be due to uncut plasmid. It was found that the undigested plasmid signal appeared as a hump, running at the same position as the hump in Fig. 4. We hypothesize that the reason for the timing and shape of the uncut plasmid DNA hump is that the plasmid DNA self-assembles into a compact form that migrates more quickly than linear insert and vector peaks. Since it is likely that the plasmids will take on a variety of conformations, this will lead to varying degrees of accessibility of the DNA to the intercalator, thus giving rise to a range of separation times—both from the variety of conformations and from the varying degrees of intercalator labelling. This causes the signal to take the shape of a hump, rather than a distinct peak.

To verify that the enzymatic digestion reagents did not interfere with the generation of the heteroduplex effects, we tested two different plasmid preparations by digesting them, thermally reannealing the excised DNA and performing an injection-separation run upon the re-annealed DNA (i.e. HA). At this stage, both digestion and re-annealing were done off the chip. Fig. 5a and b show the results of the HA run of samples #1 and #2, respectively. Since each plasmid sample consisted of homoplasmic DNA, each of these samples generated a single homoduplex peak. By contrast, Fig. 5c shows the results of a mixture of sample #1 and sample #2. Clearly, both homoduplexes and heteroduplexes have been formed.

Fig. 6 shows an electropherogram for a mixture of sample #1 and #2 after an on-chip enzymatic digestion and on-chip denaturation (with formamide). While the uncut plasmid peak and vector peaks remained the same in shape and time of arrival, the insert peak clearly changed from a single peak to one larger peak followed by two smaller ones. The insert peaks are shown in more detail in the inset of this figure. The appearance of this more complex peak structure reflects the presence of variations in the DNA. The first peak likely represents the two homoduplexes, which, being the same size and having no mismatched sequences, move down the separation arm more quickly than the two heteroduplex peaks. The second and third peaks are likely the two heteroduplex peaks. The shift in time between Figs. 5 and 6 reflects the difference in injection times. The peaks near 135 s were obtained reproducibly, whereas some weaker peaks appeared randomly and non-reproducibly, such as the peak at 116 s, possibly due

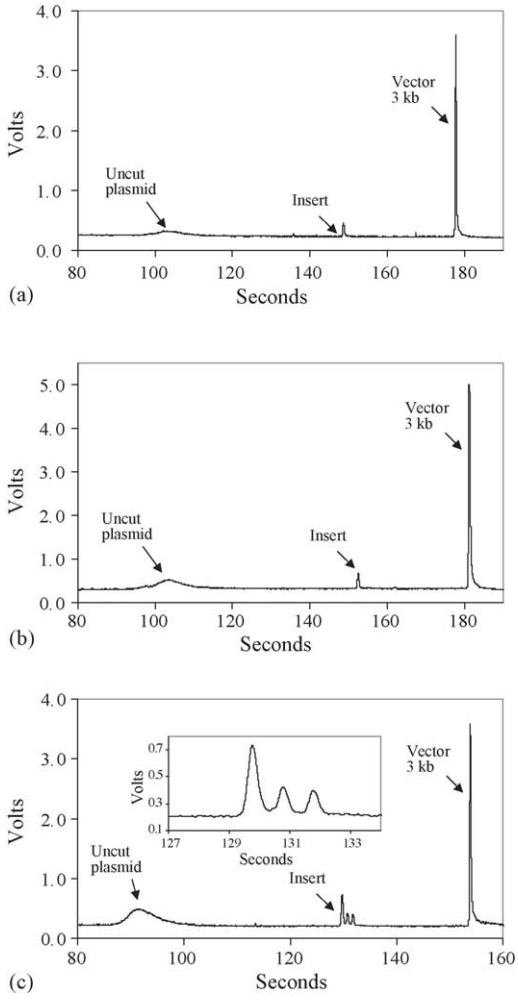

Fig. 5. Electropherograms of samples containing two populations of mtDNA: (a) sample #1, (b) sample #2 differing by 9 mutations, and a mixture of sample #1 and #2; and (c) using conventional heteroduplex analysis, i.e. off-chip enzymatic excision and reannealling.

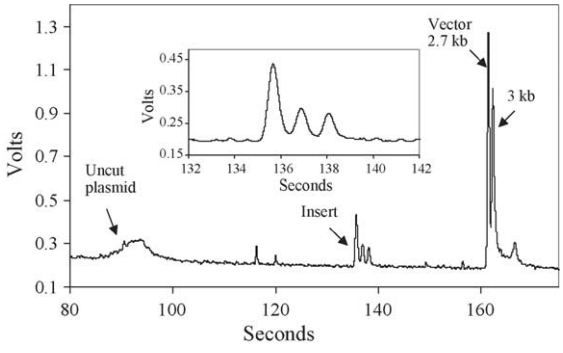

Fig. 6. An electropherogram of the same mtDNA sample shown in Fig. 4, directly following a 5 s injection after the on-chip enzymatic excision, denaturation and reannealling. The small peaks near 135 s represents the passage of the 334 bp segments of human mtDNA and are shown in more detail in the inset of the figure. (* peaks were sporadic and not reproducible—all others were reproduced in repeated runs).

to DNA-dye aggregates. As previously explained, it can be concluded that during the second electrophoretic run, the injection stage served to separate the ssDNA strands from the formamide, as the electrically charged ssDNA moved into the injection arm, while the electrically neutral formamide remained in the sample well. This allowed the ssDNA that remained in the injection arm during the second separation to reassemble into dsDNA, forming both homoduplexes and heteroduplexes within the microchip. SO does not bind to ssDNA so ssDNA peaks are not visible in this work. We did not analyze the second run, since it contained primarily ssDNA. The third electrophoretic run (and subsequent runs) served to move newly formed dsDNA from the injection arm to the intersection, and then down the separation arm, where the duplexes were detected as shown in Fig. 6.

It is clear from Fig. 6 that on-chip digestion and heteroduplex analysis in combination with on-column dsDNA labelling was successful in detecting the sequence variations present in the sample. Although we have previously applied on-chip HA and SSCP to (linear) PCR products [11], here we have introduced a novel integration of sample preparation, on-chip enzymatic digestion and denaturation, followed by on-chip re-assembly and fluorescent labelling. This allows us to excise and analyse DNA within circular loops of unlabelled DNA.

4. Conclusions

This study developed a means of using DNA self-assembly within a microfabricated chip to extract, prepare and compare human mitochondrial DNA (mtDNA) sequences and assess the degree of heteroplasmy in a sample. Through this work, an improved level of microchip integration has been reached. The process of DNA mutation analysis has been accelerated through its transfer to a microfluidic environment. The ability to work with very small samples, potentially including single molecules, will be particularly useful in exploring future medical applications. As well, the degree of control provided by microfluidics provides a path to the development of effective self-assembly nanotechnologies. The chip-based technologies presented here have allowed the detection and characterization of mtDNA sequence variations, a technique that will greatly improve our ability to study the effect of mtDNA mutations in various diseases.

Although this lab is now working with considerably more complex chips than the one used here, the present work represents a high level of integration, using a relatively simple chip with an advanced control system and well-adapted molecular biology protocols. The microchip-based procedure can be performed in under an hour, while its macroscopic forerunner often requires days.

This method was developed by using mtDNA sequences inserted into a plasmid vector that was then mass produced in *E. coli*. This allowed for extraction and purification of these plasmids and, more importantly, for the simulation of any degree of heteroplasmy by mixing plasmids in the microchip sample well. In future, this lab intends to apply this method directly to cell lysates containing mtDNA. It is likely that the protocol used here could also suffice to extract and analyse the demographics of the D-loop DNA within the mitochondrial genome, a 16.5 kb circular loop. To apply this method to the entire mtDNA molecule, a more complex chip would be required to enable the use of multiple enzymes to effectively analyze a mtDNA region of interest.

The present work illustrates the successful application of the precursor of a method that may ultimately be used to analyse the mtDNA demographics of a few cells or even a single cell, given the large number of mtDNA molecules in a cell [1] and the ability, with intercalators, to detect single molecules [15]. Combined with a more complete method of on-chip sample preparation, this would enable mitochondrial diagnostics to be performed with the small quantity of cells provided by a needle biopsy.

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