行政院國家科學委員會補助專題研究計畫成果報告

微晶片式生化電泳分析系統之研發(II) - 子計劃二：

微晶片式電泳光學偵測之模擬與實作

計畫類別：□個別型計畫 □整合型計畫
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執行單位：國立成功大學化學系

中華民國 89年 7月 31日
一、中文摘要

对于具易脆性 X 症状异常染色体的快速临床分析主要是利用 PCR 将基因大量複製後再藉由基因大小進行篩選分離。为建立一个能快速篩選易脆性 X 症状具(CGG)n 重複單元的突变因子进行電泳分析。於實驗中藉由 betaine-PCR 將 FMR1 基因中含(CGG)n 重複單元的基因序列予以複製然後進行去鹽的步驟再由 PMMA 材質的微晶片進行電泳分析。微晶片的分析程序长度为 6 公分，可在 3 分鐘內分辨出(CGG)n 重複單元相差 6 以上的不同基因序列。實驗中發現，同時加入 Cy-5 及 Topro-3 兩種染料可增加訊號的強度。本實驗共針對 12 個男生及 6 個女生的樣品進行分析。女生樣品的基因序列相差小於 6 個重複單元的樣品，則可藉由平板膠電泳做進一步確認。將男生與女生的樣品經由微晶片與平板膠電泳的測試結果進行比對，測試結果完全吻合。

關鍵詞：易脆性 X 症状異常染色體、微晶片電泳、平板膠電泳

二、Backgrounds and Significance

Microchip electrophoresis is emerging as a highly promising method for fast analysis with minimum amount of analytes, which is well-suited for clinical analysis. While most of the reports on microfabricated electrophoretic devices have utilized glass or silica as substrates [1-3], recent attention has been given to the use of polymeric microstructures. The polymer substrates are viewed as promising alternatives for the production of microfluidic systems [4-6] due to the fact that these materials are less expensive and easier to manipulate than silica based substrates. Moreover, the inherent neutral hydrophilic nature of the polymer substrate allows a direct use of the channel for clinical analysis of biomolecules without the need of surface modifications to reduce the wall adsorption [7]. We have previously demonstrated that the hepatitis C virus amplicon could be resolved by microchip electrophoresis in less than 1.5 minutes with the confidence interval of the migration time less than 1.3% [8]. Although the advantages of using microchip electrophoresis for fast analysis of PCR products with minimum sample preparation and consumption have been recognized, the real-time PCR device on microchip is also capable of achieving a rapid in-situ detection of PCR products. Microchip electrophoresis will be more useful for assays which require sizing in addition to the detection after the amplification, particularly for clinical applications in which a disposable kit can be developed using the polymer substrate.

This study is to explore the use of polymer microchip electrophoresis for the screening of abnormal chromosomes associated with Fragile X syndrome (FXS) in which the diagnosis is based on the repeating size of the gene. Fragile X syndrome (FXS) is caused by a CGG-repeat expansion located in the 5’ untranslated region of the familial MR type 1 (FMR1) gene [9] and the affected boys usually exhibit mental retardation, autisticlike behavior, protruding ears, and macroorchidism. However, individuals with premutation are generally asymptomatic, but their between the microchip electrophoresis and the existing methods.

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(CGG)n is unstable and tends to expand when passing into the next generation, especially from a premutation carrier mother to their children. Therefore, prevention of these disorders through a genetic examination would be important. Southern blot analysis as well as 32P-included PCR followed by sequencing gel analysis to determine the exact allelic repeat number [10] are effective but labor/time consuming and difficult to be implemented for a large screening. In previous studies [11,12], a simple nonradioactive PCR followed by ethidium bromide-staining gel and UV detection was applied as a simple and sensitive exclusion test. In this method, the (CGG)n alleles with normal to premutation repeat range can be successfully amplified and the PCR bands with more than 10 CGG-repeats in difference could be clearly distinguished by gel electrophoresis [12]. With further optimizations for gel electrophoresis, a separation power up to 3 CGG-repeats in difference can be achieved. However, extremely high C+G component in full mutations or large premutation alleles will result in failure of the PCR amplification. These samples as well as female samples with less than 3 CGG-repeats difference from two chromosomes would need to be reevaluated by Southern blot analysis in order to eliminate or confirm the findings. Fortunately, the incidence of FXS is low and should be about 1 in 1369 in men [13]. The occurrence of two chromosomes with the same repeat-number is around 30% in females. Therefore, for large research or screening projects, the nonradioactive PCR is a reliable and quick exclusion test for a large majority of samples, especially for boys or men. The intention of the current work is to take this method a step further by replacing the ethidium bromide staining-gel electrophoresis and UV detection with polymer microchip electrophoresis and laser-induced fluorescence detection. In the literature, capillary electrophoresis (CE) has been demonstrated to allow a throughput of 144 samples in the normal and premutation range in 48 hours [14]. Although an intra-assay accuracy in size determination is reported to be around 0.2 - 1.8 bp, the differentiable size separation shown in the electropherogram appears to be around 0.2 - 1.8 bp, the differentiable size separation shown in the electropherogram appears to be around 10 repeat number (Figure 1 in reference 14). With the use of microchip electrophoresis, the throughput can be further extended due to the rapid separation and multichannel format. Moreover, a disposable plastic microchip will provide a user-friendly means for clinical applications.

Results and discussions

Microchip system. A schematic diagram of the device configuration is shown in Figure 1. The channels were fabricated on PMMA plexiglass pieces approximately 2 cm in width x 10 cm in length and 2.0 mm thick using either wire-imprinting [6] or heat-embossing method. For heat-embossing method, the micro channels on master templates are formed by the combination of metal etch mask and wet chemical etching. A commercial blank photo mask substrate (Nanofilm, Inc.) consisting of three layers (1 µm photoresist, 1 µm Cr, and 2.3 mm quartz, respectively) was used as a master template on which micro capillary channels were etched by buffered oxide etchant (BOE, 6:1) at room temperature. The resulting structure was inverse raised three-dimensional image of the channels on quartz substrate. Channels on plastic blanks were imprinted by placing quartz templates with inverse raised image on the top of PMMA blanks. The micro channels on PMMA substrates were formed by heating the entire devices at 103°C for 5 minutes. This temperature was chosen to
ensure the softening of the plastic such that channels can be imprinted with ease. The resulting PMMA plates with microfluidic channels were then clamped with another PMMA cover plate to form the sealed channels. The PMMA devices were heated at higher temperatures for at least 8 minutes for bonding. Prior to bonding, four through holes (1 mm in diameter) were drilled on the cover plate as analyte and sample reservoirs. These holes were aligned under microscope with the ends of the channels. The good sealing of micro channels were observed. The bonding strength of the chips was estimated to be about 1.0 MPa using homemade tensile test equipment.

Two identical power suppliers (CZE 1000R, Spellman, Hauppauge, NY, USA) were utilized to furnish the loading and separation voltages respectively and the power switching was controlled by a program written in LabVIEW (National Instruments, Austin, TX, USA) running on a Pentium 75 MHz computer. The amplicons were desalted via microdialysis membranes against de-ionized water prior to the injection. The sample loading was performed by applying -300 V (-150 V/cm) to the buffer channel (between reservoir III and IV) for 0.12 minute while keeping the separation channel (between reservoir I and II) floating. For the separation, -1.8 kV (-250 V/cm) was applied to the separation channel while keeping the buffer channel floating. The separation channel was composed of 1.3% HPMC in 100 mM TBE buffer and 10 µmol/L TOPRO-3. For female samples, the composition of HPMC was increased to 1.8% while keeping other conditions the same. The fabricated device was used directly for chip electrophoresis without further modifications.

Signals were detected on-microchip via LIF. The detection system was constructed through modifications of a commercial reflection microscope (Model BX40, Olympus, Tokyo, Japan). Briefly, a Helium-Neon laser beam with a wavelength of 632.8 nm (10 mW, LHR-991, Melles Griot, Carlsbad, CA, USA) was focused at a position 3 cm downstream from the cross section within the channel using a 50x (NA = 0.5) long working distance objective. Fluorescence was collected by the objective and passed through a dichroic cube with band-pass filter, followed by spatial filtering prior to photomultiplier detection operated at -650 V (R928, Hamamatsu, Tokyo, Japan). Amplified photoelectron pulses were converted to an analog signal and acquired by a commercial interface (Model 9524, SISC, Taipei, Taiwan) running on the same computer as for voltage switching.

Non-radioactive PCR of FMR1 genes

Based on the copy number of CGG-repeat, (CGG)n, the FMR1 is classified as normal (below 54), premutation (55-200), or full mutation (above 200). The common problem associated with PCR analysis of FMR1 genes is the high CG content and the repetitive nature of the (CGG)n alleles. These physical properties lead to very high melting temperatures and the formation of secondary structures within the (CGG)n repeat may lead to severe shadow bands, caused by polymerase slippage during the PCR. In a previous study [11,12,15], it was demonstrated that the inclusion of betaine in PCR buffer allowed the CGG-repeat genes within the normal and premutation region to be successfully amplified without the need of 7'-deaza modification for dGTP. It was also found that the use of 7'-deaza dGTP lead to the failure of ethidium bromide staining that was used for gel electrophoresis detection. Therefore, the betaine PCR assay was directly adopted for this study.

Microchip electrophoresis

Size separation of the control samples with a repeat number of 29, 40, and 52 which have PCR bands in the range of 200 to 300 bp was first attempted. Because the commercial sieving buffer used as the separation medium in previous study via CE [14] cannot be found, a common polymer, HPMC, was used as the sieving medium for this study. With the use of 1.3% HPMC, these three samples can be clearly identified in less than 3 minutes on a 6-cm micro-channel with a confidence interval of the migration time around 4% (Figure 2). It was found that in addition to Cy5-labelled primers, the use of TOPRO-3 in the separation medium as the intercalating dye for fluorescence detection had greatly increased the detection sensitivity without much influences on the migration pattern (Figure 2). As shown in Figure 2, the addition of TOPRO3 in the separation medium caused the background noise to be slightly reduced and the signal for the amplicons to be increased by 4 times. Interestingly, the signals for primer and primer-dimer were not changed significantly, possibly due to their short length of the DNA molecules. The use of intercalating dye may cause errors for quantitation since the number of intercalated fluorophore increases with the number of base-pair for DNA molecules. However, it does provide an easy way for qualitative analysis of DNA using fluorescence detection. To our best knowledge, this is the first report using both covalent and intercalating dyes for DNA detection in improving the sensitivity. It was noticed that the Cy-5 labelled DNA molecules tend to have less secondary structures compared to the non-covalent labelled DNA molecules. The secondary structures would otherwise cause small shadowed bands at a longer migration time. It was also noticed that the desalting step was important for microchip electrophoresis to provide an adequate sensitivity for the assay via electrokinetic injection since the PCR buffer used for this study has a relatively high salt content.

The microchips were further applied for the analysis of the amplicons from clinical samples. For this application, the control samples tested above were used as the size marker and the results were also confirmed by spiking the n=52 control marker into each sample prior to the injection. We tested the method on clinical samples by reanalysis of 12 samples.
from boys or men and 6 samples from females. All these samples were previously analyzed by PCR/slab gel electrophoresis and Southern blotting methods. The reanalysis was done blind so that the person who performed it had no knowledge of the results of the previous analysis. After the analysis, the two results were compared and showed a 100% correlation (Figure 3 and 4). For male samples, as shown in Figure 3, sample number 1-4 have a repeat size between 29 and 52 and the repeat size of sample 5 is less than 29. It is particularly noticeable that sample 6 could be clearly identified to have a repeat size in the premutation range from microchip electrophoresis but the signal was rather smear from gel electrophoresis. The exact repeat size of sample 6 was determined to be n=98 based on a radioactive PCR followed by sequencing gel electrophoresis. It is indicated from Figure 3 that the size resolution is poorer for microchips compared to the slab gel electrophoresis and this is due to the short separation length. While the separation performance of microchips measured per unit length is similar to or exceeds that found with conventional CE, the absolute resolution is lower due primarily to shorter separation lengths [16]. We found that the resolution of a 3-cm chip is poorer than the current 6-cm chip. However, the current chip size and the instrument setting that we used have limited the separation length of the simple cross configuration. We are investigating several novel designs to increase the channel length in a compact footprint of the microchip [17,18] without adding geometrical contributions to analyte dispersion [19]. For female samples, two bands associated with two X chromosomes are expected for their (CGG)n alleles in the normal range. If one band is detected, it may be attributed to two possibilities. The first possibility is due to one allele in the pre- or full- mutation range such that only the allele in the normal range is amplified and females carrying such genes are likely to pass the abnormal gene into the next generation. The other possibility is due to the homozygous genes or the small difference (∆n<6) in their repeat size and females carrying such genes are considered to be normal. Therefore, further experiments using Southern blotting method is needed to exclude or confirm the findings. As shown in Figure 4, two bands were detected for samples containing two amplicons with a difference in repeat size greater than 6 (The top four electropherograms in Figure 4). For the bottom electropherogram in Figure 4, only one band was detected with a repeat number around 40 and further experiment using Southern blotting method indicated the existence of a premutation gene. Although the separation power can be improved to detect one repeat size difference by increasing the separation length as mentioned above, there are still about 30% of normal female genes being believed to be homozygous. Therefore, the electrophoretic method is best considered to serve as an exclusion test and the accuracy can be up to 100% for male and 70% for female.

In this study, the fast sizing capability of microchip electrophoresis was demonstrated to combine with a simple nonradioactive PCR as a fast exclusion test of FXS. Although the existing methods can differentiate 3 CGG-repeat difference compared to 6 repeat difference using microchips, the run time using microchip electrophoresis is less than 1 minute, which is about one to two order of magnitude less than that (70 minutes) for gel electrophoresis. For both methods, further experiments are required to confirm the positive findings. Since the instrumentation of microchip electrophoresis with automatic settings has become available recently [20], the reported method can be easily modified and adopted for high-throughput screening of FXS.

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Figure 1. Channel configuration of the microchip. The buffer, analyte, and two waste reservoirs were indicated as I, II, III, IV, respectively.

Figure 2. Microchip electropherograms of the amplified FMR1 genes used as the size marker with a repeat number of 29, 40 and 52. The separation buffer was composed of 1.3% HPMC in TBE buffer (100 mM Tris-borate, and 5 mM EDTA, pH 8.2) with (A) 1uM TOPRO-3 and (B) no TOPRO-3.

Figure 3. Comparisons between slab gel electrophoresis and microchip electrophoresis for FMR1 PCR bands obtained from male blood samples. A. Electropherogram obtained from slab gel composed of 5% acrylamide and 19:1 of acrylamide:bis in 10% glycol and 0.5 x TBE buffer (50 mM tris-borate, 2.5mM EDTA). Lane M was the size marker with n=20 and 52. Lane 1-6 were the amplified products from the male bloods. B. Microchip electrophogram of the corresponding samples in lanes M and 1 to 6 of A, respectively. The first two bands were due to primers and primer-dimers, the third band was due to the PCR product. The separation buffer was the same as in Figure 2.
Figure 4. Comparisons between slab gel electrophoresis and microchip electrophoresis for FMR1 PCR bands obtained from female blood samples. A. Electropherogram obtained from slab gel with the same composition as in Figure 3A. Lane M was the size marker with n=29, 40 and 52. Lane 1-4 were the amplified products from the female bloods. B. Microchip electropherogram of the corresponding samples (from the top to the bottom) in lanes M and 1 to 4 of A, respectively. The separation buffer is the same as in Figure 2 and 3B except that the HPMC composition was changed to 1.8%.