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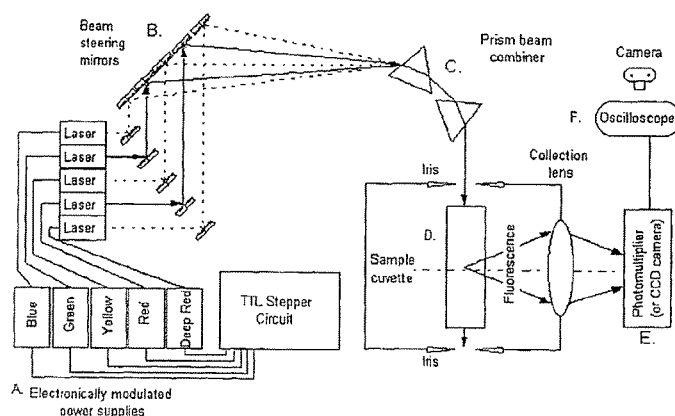
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(54) Title: PULSED-MULTILINE EXCITATION FOR COLOR-BLIND FLUORESCENCE DETECTION



(57) Abstract: The present invention provides a technology called Pulse-Multiline Excitation or PME. This technology provides a novel approach to fluorescence detection with application for high throughput identification of informative SNPs, which could lead to more accurate diagnosis of inherited disease, better prognosis of risk susceptibilities, or identification of sporadic mutations. The PME technology has two main advantages that significantly increase fluorescence sensitivity: (1) optimal excitation of *all* fluorophores in the genomic assay and (2) "color-blind" detection, which collects considerably more light than standard wavelength resolved detection. This technology differs significantly from the current state-of-the-art DNA sequencing instrumentation, which features single source excitation and color dispersion for DNA sequence identification. Successful implementation of the PME technology will have broad application for routine usage in clinical diagnostics, forensics, and general sequencing methodologies and will have the capability, flexibility, and portability of targeted sequence variation assays for a large majority of the population.



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PULSED-MULTILINE EXCITATION FOR COLOR-BLIND FLUORESCENCE**DETECTION****BACKGROUND OF THE INVENTION****I. Field of the Invention**

The present invention relates generally to the fields of high throughput genetic analysis applications and fluorescence spectroscopy. More particularly, it provides a variety of compositions and methods for use in high-throughput DNA sequence identification.

II. Description of Related Art

The Human Genome Project (HGP) holds tremendous promise for discoveries of the molecular mechanisms that trigger the onset of many common diseases over the next several decades. The initial HGP goals underway will provide or have provided the complete and accurate genome sequences of human and multiple well-studied genetic model organisms, such as mouse, rat, fruit fly, nematode, yeast and numerous bacteria. From this foundation of reference genome sequences, the elucidation of complete gene sets, coupled with comparative cross-species studies, are expected to assist significantly in the assignment to specific human genes of protein function and disease associations. Other technologies complement the assignment of biological functions: gene and protein expression profiling, mouse gene-knockouts, and techniques that measure protein-protein interactions. The elucidation of gene structure-protein function relationships are key to understanding how genomic sequence variation between individuals can cause increased risk or predisposition to certain complex diseases or are even the etiologic agents responsible for the onset of particular diseases. However, the use of genetic variation in clinical practice is only beginning and technology to facilitate its use is greatly needed.

The most commonly observed form of human sequence variation is single nucleotide polymorphisms (SNPs), which occur at a frequency of approximately 1-in-300 to 1-in-1000 base pairs. In general, 10%-to-15% of SNPs will affect either protein function by altering specific amino acid residues, or will affect the proper processing of genes by changing splicing mechanisms, or will affect the normal level of expression of the gene or protein by varying regulatory mechanisms. Several recent examples are the associations of mutations with the *NOTCH4* gene and schizophrenia (Wei *et al.*, 2000), peroxisome proliferator-activated receptor gamma (PPAR γ) gene and severe insulin resistance (Deeb *et al.*, 1998), and melanocortin-4 receptor (MC4R) gene and inherited obesity (Yeo *et al.*, 1998).

The identification of informative SNPs will lead to more accurate diagnosis of inherited diseases, better assessment of risk susceptibilities, and could be assayed in specific tissue biopsies for sporadic mutations. An individual's SNP profile could be used to offset and significantly delay the progression of disease by helping in the choice of prophylactic drug therapies. A SNP profile of drug metabolizing genes could be used to prescribe a specific drug regimen to provide safer and more efficacious results. To accomplish goals like these, genome sequencing will move into the resequencing phase of not just a handful of individuals, but potentially the partial sequencing of most of the population. Resequencing simply means sequencing in parallel specific regions or single nucleotides that are distributed throughout the human genome to obtain the SNP profile for a given complex disease.

For this technology to be applicable and practicable for routine usage in medical practice, it must be robust, easy-to-use, highly sensitive, flexible, portable, and the results should be accurate and rapidly obtained. While current technologies at large genome centers are robust and results are accurate, they are inadequate and inflexible for resequencing millions of individuals in routine clinical practice. It is therefore advantageous to develop a DNA sequencing instrument, which meets these needs. Miniaturization of this technology is also advantageous because smaller instruments potentially require less sample and reagents and can be more readily transported and located in areas such as clinics or doctors' offices.

Ideally, DNA sequencing technology would have the sensitivity for direct assays without DNA amplification, and be simple and portable for routine usage in basic, applied, and clinical laboratories. Currently, DNA sequencing technology for high-throughput analyses are specialized and centralized in large genome centers and require numerous molecular biology manipulations that take days or weeks of preparation before DNA sequence analysis can be performed. Thereafter, the state-of-the-art technology involves the attachment of four different fluorescent dyes or fluorophores to the four bases of DNA (*i.e.*, A, C, G, and T) that can be discriminated by their respective emission wavelengths, the electrophoretic separation of the nested set of dye-labeled DNA fragments into base-pair increments, and the detection of the dye fluorescence following irradiation by a single argon-ion laser source. Current instrumentation for electrophoretic separation comprises a 96-capillary array that disperses the different fluorescent signals using a prism, diffraction grating, spectrograph, or other dispersing element and images the four colors onto a charged-coupled device (CCD) camera. The throughput of each 96-capillary instrument is approximately 800 DNA samples per day, and the success of the HGP in large-scale genomic sequencing has been attributed to the use of hundreds of these machines throughout the world. The main disadvantages of the current technology are the

laborious cloning or amplification steps needed to provide sufficient DNA material for analyses, the relatively large size of the instruments (roughly the size of a 4-foot refrigerator), and the inadequate sensitivity of detection (*i.e.*, inefficient excitation of fluorescent dyes with absorption maxima far from the laser excitation wavelength).

Although the resolution of spectral emission wavelengths is the mainstream technology used in commercial and academic prototype instruments, several groups have explored other physical properties of fluorescence as a method for discriminating multicolor systems for DNA sequence determination. Recently, Lieberwirth *et al.* (1998) described a diode-laser based time-resolved fluorescence confocal detection system for DNA sequencing by capillary electrophoresis. In this system, a semiconductor laser (630 nm) was modulated using a tunable pulse generator at a repetition rate of 22 MHz (454 psec pulses) and focused by a microscope objective. The fluorescence was collected by the same objective and imaged on a single photon counting module APD (Lieberwirth *et al.*, 1998).

The Luryi group at SUNY Stony Brook have proposed a multiple laser excitation approach using different radio frequency (RF) modulations and demodulations to discriminate a mixture of fluorophores (U.S. Patents 5,784,157 and 6,038,023). U.S. Patent 5,784,157 describes a 4-laser based fiber optic single capillary monitoring device, which initially has a non-wavelength component, but later the invention discusses the coupling of spectral resolution for fluorophore discrimination. There are three significant flaws apparent in this system relating to the enhanced fluorescence cross-talk and laser scattered light, low sensitivity detection, and a system that does not appear to scale beyond one capillary.

As described, the target capillary is illuminated simultaneously by all four lasers, which are modulated by different RF signals. The different RF signals for all of the dyes are summed together and the detector photodiodes are demodulated by additional heterodyne RF signals. Interestingly, Gorfinkel and Luryi describe the creation of Bragg reflectors to eliminate cross-talk modulation for a given dye set. Fluorescence cross-talk, however, will not be eliminated using this technique. Signal from the "wrong" dye, which is weakly excited off-resonance by a particular laser, will be encoded with the corresponding "wrong" frequency, decoded, and added to the signal for the target dye. Moreover, scattered laser light will also be modulated, and is likewise not rejected by the heterodyne detection.

The simultaneous multi-modulation method also has a serious shortcoming for the detection of low light levels, which is a specific aim of the current invention. All the lasers are proposed to operate simultaneously, followed by detection of substantially all of the entire

fluorescence, and conversion of the collected fluorescence to an electrical signal. This design potentially creates a correspondingly high quantum statistical noise level, which should be distributed to all the detectors. The demultiplexing process of RFs does not remove this excessive random noise, even if the corresponding signal is small (Meaburn, 1976). In comparison, the Pulse-Multiline Excitation (PME) system described in the current invention exhibits noise levels in proper proportion, so that a weak signal originating from a particular laser pulse has a correspondingly low detected noise level during that laser's sub-cycle. Optimizing the optical system for producing low noise levels is essential in establishing the optimum contrast between the presence and absence of a given dye.

Finally, US patent 5,784,157 describes a rather complicated array of optical fibers, combiners, splitters, and 4 heterodyne detectors with their associated spectral filters for a single capillary channel. Scaling this system to a 2-capillary system would entail doubling the mentioned detector components. Unfortunately a CCD camera is not readily adapted for high frequency RF modulation, as it is an "inherently discrete-time" device. In a more recent document, US patent 6,038,023, the multiplicity of spectral filters has been replaced with a dispersing prism spectrometer and a high speed one dimensional array detector for use with a single capillary channel device; the potential to scale up to a capillary array system is more feasible as discussed by the Luryi group, but may require a multiplicity of such spectrometer units.

The current invention comprises a novel fluorescence device, which is capable of significant improvements in the limit of detection of multi-color fluorescence reactions and may be applied to direct measurement of such reactions from biological sources (*i.e.*, without the need for PCR or cloning amplifications). Moreover, this technology, called Pulse-Multiline Excitation or "PME" can be configured on a small work surface or in a small instrument, compared to the current DNA sequencing instruments. Thus, a DNA sequencer the size of a suitcase or smaller is described.

The development of improved DNA sequencing chemistries will likely improve the number of independent assays that can be run in parallel. This technology will have broad application in both general sequencing and forensic applications.

SUMMARY OF THE INVENTION

Thus, the present invention contemplates an apparatus and method for use in high-throughput DNA sequence identification. An aspect of the invention is a pulse-multiline

excitation apparatus for analyzing a sample containing one or more fluorescent species, comprising: one or more lasers configured to emit two or more excitation lines, each excitation line having a different wavelength; a timing circuit coupled to the one or more lasers and configured to generate the two or more excitation lines sequentially according to a timing program to produce time-correlated fluorescence emission signals from the sample; a non-dispersive detector positioned to collect the time-correlated fluorescence emission signals emanating from the sample; and an analyzer coupled to the detector and configured to associate the time-correlated fluorescence emission signals with the timing program to identify constituents of the sample.

The detector and the analyzer may be integral. In one embodiment, the two or more excitation lines intersect at the sample, or the two or more excitation lines may be configured so that they do not intersect in the sample. The two or more excitation lines may be coaxial.

In one embodiment of the invention, the apparatus may further comprise an assembly of one or more prisms in operative relation with the one or more lasers and configured to render radiation of the two or more excitation lines substantially colinear and/or coaxial.

The apparatus may have 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 or more excitation lines having 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 or more excitation wavelengths, respectively. The sample may be comprised in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, up to 20, up to 24, up to 28, up to 36, up to 48, up to 64, up to 96, up to 384 or more capillaries. A sheath flow cuvette may be used.

The timing program may comprise a delay between the firing of each laser of between about 10 fs and about 5 s, between about 1 ms and about 100 ms, or between about 50 ps and about 500 ps. One or more of the excitation lines is pulsed. The pulsed excitation line may be controlled by TTL logic or by mechanical or electronic means. In one embodiment, the apparatus may generate a sequence of discrete excitation lines that are time-correlated with the fluorescence emission signals from the sample.

The lasers may independently comprise a diode laser, a semiconductor laser, a gas laser, such as an argon ion, krypton, or helium-neon laser, a diode laser, a solid-state laser such as a Neodymium laser which will include an ion-gain medium, such as YAG and yttrium vanadate (YVO₄), or a diode pumped solid state laser. Other devices, which produce light at one or more discrete excitation wavelengths, may also be used in place of the laser. The laser may further comprise a Raman shifter in operable relation with at least one laser beam. In one embodiment

of the invention, the excitation wavelength provided by each laser is optically matched to the absorption wavelength of each fluorophore.

The detector may comprise a charged couple device, a photomultiplier tube, a silicon avalanche photodiode or a silicon PIN detector. The footprint of the device is preferably small, such as less than 4 ft x 4 ft x 2ft, less than 1ft x 1ft x 2ft, and could be made as small as 1 in x 3 in x 6 in.

Another aspect of the current invention comprises a method of identifying sample components comprising: (a) preparing a sample comprising sample components, a first dye and a second dye; (b) placing the sample in the beam path of a first excitation line and a second excitation line; (c) sequentially firing the first excitation line and the second excitation line; (d) collecting fluorescence signals from the samples as a function of time; and (e) sorting the fluorescence by each excitation line's on-time window, wherein the sample components are identified. It is an aspect of the invention that the fluorescence signals are collected from discrete time periods in which no excitation line is incident on the sample, the time periods occurring between the firing of the two excitation lines. This technique is known as "looking in the dark." Yet another aspect of the present invention is that the absorption maximum of the first dye substantially corresponds to the excitation wavelength of the first excitation line. The absorption maximum of the second dye may also substantially corresponds to the excitation wavelength of the second excitation line. In yet another aspect of the current invention there is a third and fourth dye and a third and fourth excitation line, wherein the absorption maxima of the third and fourth dyes substantially correspond to the excitation wavelengths of the third and fourth excitation lines, respectively. Similarly, there may be 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 or more dyes wherein the absorption maxima of the dyes substantially corresponds to excitation wavelengths of a 5th, 6th, 7th, 8th, 9th, 10th, 11th, 12th, 13th, 14th, 15th, 16th, or more excitation lines, respectively. The dyes may be a xanthene, fluorescein, rhodamine, BODIPY, cyanine, coumarin, pyrene, phthalocyanine, phycobiliprotein, Alexa, squaraine dyes, or some other suitable dye.

In one embodiment of the current invention, the sample components enable the determination of SNPs. The method may be for the high-throughput identification of informative SNPs. The SNPs may be obtained directly from genomic DNA material, from PCR amplified material, or from cloned DNA material and may be assayed using a single nucleotide primer extension method. The single nucleotide primer extension method may comprise using single unlabeled dNTPs, single labeled dNTPs, single 3'-modified dNTPs, single base-modified 3'-dNTPs, single alpha-thio-dNTPs or single labeled 2',3'-dideoxynucleotides. The mini-

sequencing method may comprise using single unlabeled dNTPs, single labeled dNTPs, single 3'-modified dNTPs, single base-modified 3'-dNTPs, single alpha-thio-dNTPs or single labeled 2',3'-dideoxynucleotides. The SNPs may be obtained directly from genomic DNA material, from PCR amplified material, or from cloned DNA materials and may be assayed using Sanger sequencing.

In another embodiment of the current invention, analyzing the signals is adapted for the accurate diagnosis of inherited disease, better prognosis of risk susceptibilities, identification of sporadic mutations, or prescribing tailor-made daily drug regimens for individual patients. Analyzing the signals may be adapted for routine usage in clinical diagnostics, forensics applications or determining general sequencing methodologies.

Yet another aspect of the current invention is a method of identifying sample components comprising: (a) obtaining a biological sample; (b) labeling said sample with one or more fluorophores; (c) separating components of said sample; and (d) detecting said sample components with a device wherein said device may comprise: one or more lasers configured to emit two or more excitation lines, each excitation line having a different excitation wavelength; a timing circuit coupled to the one or more lasers and configured to fire the two or more excitation lines sequentially according to a timing program to produce time-correlated fluorescence emission signals from the sample; and a non-dispersive detector positioned to collect the time-correlated fluorescence emission signals; wherein said detector collects time correlated data from said sample comprising fluorescent emissions of the sample as a result of irradiation by the one or more excitation lines.

The sample components may be nucleic acids, amino acids or proteins. The separation may be by electrophoresis, chromatography or mass spectrometry (MS) such as MALDI-TOF, quadrupole mass filter or magnetic sector MS. The sample components may be addressed on high density chip arrays.

In one embodiment, the method may further comprise: (e) contacting said sample components on a surface comprising immobilized oligonucleotides at known locations on said surface; and (f) performing a single nucleotide incorporation assay or a mini-sequencing assay. In yet another embodiment, the method may further comprise rastering said surface or said excitation lines such that said excitation lines contact said surface at multiple locations.

Another aspect of the current invention is a device comprising: (a) one or more lasers having two or more excitation lines; (b) one or more beam steering mirrors wherein said excitation lines each strike said mirrors; (c) a first prism, wherein said two or more excitation

lines strike one surface and exit from a second surface of said first prism; and (d) a second prism at an angle relative to said first prism, wherein said two or more excitation lines strike one surface of said second prism after exiting said first prism and exit said second prism, wherein said two or more excitation lines are substantially colinear and/or substantially coaxial after exiting said second prism. The angle of the second prism relative to the first prism is dependent on the optical material used. For example, for high dispersion flint glass, the two prisms will be arranged such that the second prism is angled at 45° relative to the first prism. For quartz, the angular displacement ranges from 30° to 50° .

Another aspect of the current invention comprises a method of illuminating a sample comprising: (a) steering two or more excitation lines onto a first surface of a first prism; (b) steering two or more excitation lines from the second surface of said first prism to a first surface of a second prism; wherein said second prism is angled about 45° from said first prism; (c) steering said two or more excitation lines onto a sample after exiting second surface of said second prism, wherein said two or more excitation lines are substantially colinear and/or substantially coaxial after exiting said second prism.

Yet another aspect of the current invention comprises a method of controlling a sequence of excitation lines comprising: (a) obtaining a TTL circuit comprising an electronic stepper wherein said circuit is operationally connected to one or more lasers having two or more excitation lines; (b) and controlling the sequential firing of the one or more lasers having two or more excitation lines with a clock pulse from the circuit, wherein the frequency of firing one laser is equivalent to the frequency of firing a second laser, but phased shifted so that one or more lasers having two or more excitation lines can be sequentially pulsed. The cycle time of one clock pulse may be from 1 μ second to 5 seconds, or from 100 μ second to 1 second. The length of time a first laser produces an excitation line may be similar to the length of time a second laser produces an excitation line. As used herein, similar means within 20%, within 10%, or more preferably within 5% of the time length. Between 2-to-16, or 2-to-8 excitation lines are sequentially pulsed.

Yet another method of the current invention comprises a method of controlling a sequence of excitation lines comprising: (a) obtaining a TTL circuit comprising an electronic stepper wherein said circuit is operationally connected to one or more lasers having two or more excitation lines; (b) and controlling the sequential firing of the one or more lasers having two or more excitation lines with a clock pulse from the circuit, wherein the frequency of firing a first

laser is different from the frequency of firing a second laser. This method may be used to control 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 lasers.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. An example of a PME device where each laser is regulated individually by its own power supply (A). The TTL stepper or clock chip circuit (FIG. 3) chooses which power supply is turned on at a specific time as it cycles through the five lasers. The beam steering mirrors (B) allow various degrees of adjustment to align the excitation lines so that once they go through a dual prism assembly (FIG. 2) (C), they will become colinear and/or coaxial. The beams enter the dark box (D) where scattered light is reduced by the use of irises and a long cell. The dyes are detected by the photomultiplier tube (E) through a collecting lens. The signals are recorded by the oscilloscope (F) where digital signals can be analyzed.

FIG. 2. An example of a PME device where each laser is regulated individually by its own power supply (A). The TTL stepper or clock chip circuit (FIG. 4) chooses which power supply is turned on at a specific time as it cycles through the five lasers. The beam steering mirrors (B) allow various degrees of adjustment to align the excitation lines so that once they go through a dual prism assembly (FIG. 3) (C), they will become colinear and/or coaxial. The beams pass through a spatial filter to improve beam quality and then enter the dark box housing a 10-cm sample cuvette (D) where scattered light is reduced by the use of iris diaphragms and a long cell. The PME induced fluorescence is collimated using a collection lens. Scattered laser light is further rejected *via* specific wavelength notch filters (only one shown). The dyes are detected by the photomultiplier tube (E) through a collecting lens. Scattered laser light is further rejected *via* specific wavelength notch filters (only one shown). Pulsed fluorescent signals are imaged onto the photomultiplier by a second lens. The signals are recorded by the oscilloscope (F) and the digital pictures are analyzed by a computer workstation.

FIG 3. Inversion dispersion scheme using a dual prism assembly to combine pulsed multiline excitation laser sources from discrete locations. The beams all enter from the left hitting the prism at varying angles and positions on the left side of the first prism (upper left). As they hit the first prism, the laser beams all bend approximately at a 45-degree angle. At this

point the beams are not yet colinear and/or coaxial but they are spatially closer together than before. As they hit the second prism, they once again hit at varying angles and positions, and become colinear and/or coaxial as they exit the prism.

FIG. 4. TTL Circuit Clock Chip (74174). MR, when low, resets the chip and sets all outputs to low; CP is the Clock Pulse Input. Q0 through Q7 are outputs that connect to and signal each of up to eight lasers to fire in sequence.

FIG. 5A, FIG. 5B and FIG. 5C. Photographic data from the oscilloscope output. Two channels from the oscilloscope were set to record the clock signal for firing the red laser (top line) and the PMT detector output (bottom line). Arrows correspond to the red and green laser pulses. Data on dilute aqueous solutions containing both BODIPY 523/547 and BODIPY 630/650 dyes (FIG. 5A), BODIPY 630/650 dye only (FIG. 5B), or BODIPY 523/547 dye only (FIG. 5C) were collected.

FIG. 6A and FIG. 6B. FIG. 6A shows overlapping excitation spectra of four PME dyes: Pacific Blue, 5-FAM, Texas Red, and Cy5.5. The arrows represent the spectral position of the matched lasers. FIG. 6B shows a comparison of the PME excitation cross-talk matrix with the ABI emission cross-talk matrix for V3 BigDye terminators. Lasers: Y-axes; Dyes: X-axes. The last row represents the percentage of off-resonant signal/total signal collected per detection window. For example, column one for the ABI matrix is $0.933/1.933 = 48.3\%$. The average percentages of all four detection windows are presented at the far right.

FIG. 7A and FIG 7B. A comparison of ABI spectral filtering of FAM, JOE, TAMRA, and ROX to PME color-blind detection of Pacific Blue, FAM, ROX, and Cy5.5 is shown. In FIG. 7A, transparent boxes represent the four 10 nm band-pass filters centered at 531 nm, 560 nm, 580 nm, and 610 nm. (FIG. 7B) The three inter-shaded boxes represent 20 nm notch filters, which are placed appropriately to block scattered laser light and Raman scattering from the upstream bluer laser. The outer two shaded boxes are long- and short-pass edge filters. See FIG. 2's legend for details of notch filter placement.

FIG. 8. Spectral overlap (shaded area) of 5-FAM emission (blue) and 6-ROX excitation (red).

FIG. 9. Raman spectra from ABI model 3700 DNA sequencer. The argon ion laser produces both 488 nm and 514.5 nm excitation lines, which in water produces Raman scattering at 529 and 561 nm (OH bending) and broader bands ranging from 577-592 nm and 614-631 nm (OH stretching), respectively. Reflected laser light from the tips of the capillaries can be observed above and below the Raman lines.

FIG. 10A and FIG. 10B. Raman scattering from the PME lasers. Because the 3400 cm^{-1} band is broad, this Raman band is listed as a range of the full-width half-maximum between 3150 cm^{-1} and 3590 cm^{-1} (FIG. 10A). Detection windows for the PME system (FIG. 10B) for simultaneous blocking of laser scattered light and the Raman 3400 cm^{-1} band.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

I. The Present Invention

The present invention describes a novel device and approach to fluorescence detection, which has general application for genetic analysis methodologies with particular emphasis on DNA sequencing technologies and high-throughput identification of single nucleotide polymorphisms (SNPs). The PME technology has two main advantages that significantly increase fluorescence sensitivity: optimal excitation of all fluorophores in the genomic assay and “color-blind” detection, which collects considerably more light than traditional dispersive detectors. The fluorescence detector can be designed to miniaturize DNA sequencing technology with a sensitivity enabling direct detection of fluorescent DNA assays from genomic DNA material. The PME is useful for clinical diagnostic, forensic, and general sequencing.

II. Pulsed Multi-line Excitation (PME) Detection

In the current invention, spectral dispersion or wavelength discrimination of fluorescent dyes is eliminated, which increases the amount of fluorescent signal detected. The sequential pulsed-laser excitation system using multiple lasers emitting specific wavelengths of light, which are matched for efficient excitation of a given set of fluorophores, can determine selectivity and sensitivity. By matching the absorption maximum of each fluorophore, the PME technology excites each dye with the highest quantum efficiency, thus considerably reducing the required sample size (*i.e.*, the number of fluorescent molecules required for detection). At first glance, replacing one laser with four lasers may appear counterintuitive to miniaturization. New solid state lasers, however, such as diode pumped Nd:YAG sources or diode lasers are much smaller (ca. 2” long) than standard argon ion lasers and are much more efficient requiring smaller power supplies for operation. For example, the footprint of four solid-state lasers together is approximately 20-fold smaller than a single argon-ion laser system. Simply replacing the argon-ion laser, which for a DNA sequencer relies on two excitation lines at 488 nm and 514.5 nm, with a equal power 532 nm Nd:YAG can reduce the laser size, but would reduce the excitation/emission intensities of shorter wavelength dyes, and still would not efficiently excite longer-wavelength dyes that have absorption maxima far from the laser wavelengths.

For the PME technology to discriminate four fluorescent dyes, four excitation lines are combined by inverse dispersion, which is illustrated but not limited to using a prism assembly or diffraction grating. The resultant beam would look on average like a "white light" laser beam. However, the solid-state lasers are electronically controlled and are pulsed or fired sequentially as discrete packages of wavelength specific light. Alternatively, laser sources can be pulsed or fired using a synchronized shutter system. Each dye brightly fluoresces when its matched laser source is turned on, while it responds only weakly, if at all, to the other three laser pulses. The fluorescence from each excitation event is collected using a non-dispersed or "color blind" method of detection. A non-dispersive detector is a detector in which the incident radiation is not separated based on the emission fluorescence wavelength of different fluorescent dyes. Thus, DNA sequence is determined by the PME technology based on the time correlation of detector response to specific wavelengths of excitation light, and not spectral resolution of emission wavelengths. Switching the solid-state lasers on a millisecond timescale is straightforward, hence thousands of 4-laser excitation cycles may be completed in the time scale for eluting a single base of DNA by capillary electrophoresis.

Moreover, an advantage of the non-dispersed system is that the detector (*i.e.*, CCD) collects significantly more light, since the fluorescent light is directly coupled to the detector. Typically for the current DNA sequencer, a dispersive element requires highly collimated light for effective wavelength separation. Moving the collection lens closer to the sample can increase the collected fluorescent light, but collimation is lessened, and spectral selectivity is reduced. Similarly, reducing the distance between the dispersing element and the detector results in reduced spectral selectivity. For the non-dispersed system, however, moving the collection lens much closer to the sample or to the detector increases the collected light, inverse to the square of the distance, but without sacrificing the selectivity that is provided by four laser cycling. Thus, the miniaturization process inherently delivers more fluorescent light to the detector.

Typically, miniaturizing a system incurs inevitable penalties in sensitivity and selectivity. For example, fluorescent signal is lost as the laser source becomes smaller in size and power, and selectivity is compromised because spectral dispersing elements need physical space to separate emission wavelengths and compressing the spectrometer portion of the detection sacrifices spectral resolution. Consequently, the sample size is increased to offset these losses, which tends to marginalize the benefits derived by shrinking the conventional dispersive optical system. The design described herein minimizes the losses in downsizing instrumentation, but increases the sensitivity considerably by the process of miniaturization. The current invention comprises a

novel detection system that allows the optical components to act synergistically when miniaturized.

An additional advantage of non-dispersed detection is enhanced signal-to-noise compared to the current 96-capillary DNA sequencer. To obtain the wavelength spectrum for each DNA sequence reaction, a large number of pixels are read out, and this electronic readout process adds noise for every pixel read. For the non-dispersed system, all pixels that receive light from a particular capillary are "binned" and read out as a single unit, considerably reducing the associated electronic noise.

III. Fluorophores

An advantage of using PME or any time-based detection as opposed to wavelength discriminating detection is the increase in the number of fluorophores, which can be used. At any given excitation wavelength, there are often only about two or three commercially available dyes that emit with narrow enough emission bands with sufficiently separated wavelength maxima that can be individually measured simultaneously (U.S. Patent 6,139,800). If three or more fluorophores can be found, there is still substantial cross-talk or overlap of the emission spectra that will require substantial deconvolution of the spectra with a corresponding increase in the likelihood of error in identifying the species.

One solution to this problem has been the addition of a second laser to allow for the simultaneous or sequential detection of up to approximately six dyes (U.S. Patent 6,139,800). However, this solution still has the problem of substantial overlap in the spectra and the need for signal intensities great enough to be detected after spectral dispersion of the signal.

Optimally, the way to obtain the highest emission signal possible is to optically match an excitation source with the absorption maxima of a dye with a high molar extinction coefficient. This is done for every fluorophore. However, the excitation source need not match the absorption maxima exactly, instead, it is important to obtain laser-dye combinations where each dye has an absorption maxima which substantially corresponds with one source wavelength with concomitant emission, coupled with minimal absorption/emission (cross-talk) from the non-matched laser sources used in the assay.

A system with four fluorophores used to detect the 4 DNA bases is preferred. However, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 different fluorophores may be used with the PME system.

A non-limiting list of dyes that may be used in the current invention include BODIPY dyes (BODIPY 630/650, BODIPY 650/665, BODIPY 589/616 or BODIPY-TR, BODIPY 581/591 BODIPY 523/547 or BODIPY-R6G, 5,7-dimethyl-BODIPY (503/512) or BODIPY-FL, 1,3,5,7-tetramethyl-BODIPY (495/503), BODIPY-TMR-X or BODIPY (564/570)-X, BODIPY-TR-X or BODIPY (589/616)-X, BODIPY (530/550), BODIPY (564/570), and BODIPY (558/568)), a xanthene dye, a rhodamine dye (rhodamine green, rhodamine red, tetraethylrhodamine, 5-carboxy rhodamine 6G (R6G), 6-carboxy R6G, tetramethylrhodamine (TMR), 5-carboxy TMR or 5-TAMRA, 6-carboxy TMR or 6-TAMRA, rhodamine B, X-rhodamine (ROX), 5-carboxy ROX, 6-carboxy ROX, lissamine rhodamine B, and Texas Red), a fluorescein dye (FITC, 5-carboxy fluorescein, 6-carboxy fluorescein, fluorescein diacetate, naphthofluorescein, HEX, TET, 5-carboxy JOE, 6-carboxy JOE, Oregon Green 488, Oregon Green 500, Oregon Green 514, erythrosin, eosin), a coumarin dye (7-hydroxycoumarin, 7-dimethylaminocoumarin, 7-methoxycoumarin, 7-amino-4-methylcoumarin-3-acetic acid or (AMCA), and Pacific Blue), a cyanine (Cy) dye (Cy3, Cy3.5, Cy5, Cy5.5, Cy7), a phthalocyanine dye, a phycobiliprotein dye, (B-phycoerythrin (B-PE), R-phycoerythrin (R-PE), and allophycocyanin (APC)), a pyrene and a sulfonated pyrene, (cascade blue), a squaraine dye, an Alexa dye (Alexa 350, Alexa 430, Alexa 488, Alexa 532, Alexa 546, Alexa 568, Alexa 594) and Lucifer yellow.

IV. Excitation Sources

A central principle of the PME technology is the discrimination of a mixture of different fluorophores by the time correlation of "colorblind" fluorescence emission triggered by serially pulsing different excitation lasers. This approach significantly contrasts that of the widely used method of wavelength discrimination of fluorescence emission, where a single excitation source, typically an argon ion laser (488 nm and 514.5 nm) excites four spectrally resolvable fluorescent dyes. The dye of this set, which emits at the longest emitting wavelength is usually the least optimally excited, which is due to poor spectral overlap between the excitation source and the dye's absorption maximum. This inefficient excitation has been partially overcome by the use of fluorescence resonance energy transfer (FRET) dye-primers (Ju *et al.*, 1995; Metzker *et al.*, 1996) and dye-terminators (Rosenblum *et al.*, 1997) to increase signal intensities. Obviously, the optimal method in obtaining the highest emission signal possible would be matching the excitation source with the absorption maxima for every fluorophore in DNA sequencing assays.

The invention may use at least one laser and is flexible to accommodate as many different lasers as is feasibly possible. There may be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or more lasers, depending on the system.

A single laser may produce 1, 2, 3, 4, 5, 6, 7, 8 or more different wavelengths for the excitation of fluorophores with different absorption maxima. This can be accomplished using the technique of Stimulated Raman Shifting (SRS). This technique may be employed for conversion to either shorter or longer wavelength(s). The Raman effect enables a laser frequency to be modified by discrete increments, (the Stokes and Anti-Stokes shifts). Frequency conversion is accomplished by passing laser light through a suitable crystal or a stainless steel cell containing gas at an elevated pressure, (*i.e.* several atmospheres). Conversion efficiency for the principal Stokes shift to longer wavelength can be as high as 35%. The nature of the crystal or gas determines the frequency output, for example, N₂, O₂, H₂, D₂, and CH₄ give shifts of 2330 cm⁻¹, 1550 cm⁻¹, 4155 cm⁻¹, 2987 cm⁻¹, and 2917 cm⁻¹ respectively, while Ba(NO₃)₂ gives a shift of 1047 cm⁻¹. A preferred Raman medium for this invention is molecular nitrogen, as 2330 cm⁻¹ is about the desired spacing between excitation frequencies.

Until recently, gas lasers have been widely used for the excitation of "blue" and "green" fluorophores with absorption maxima ranging between 488 nm and 543 nm for DNA sequencing applications. In general, these lasers include the argon ion, the krypton ion, and the helium-neon (He-Ne) lasers. These lasers are large in size, highly inefficient and relatively expensive devices. Moreover, the lifetime of gas laser is approximately 1,000-to-3,000 hours of use, which imposes high maintenance cost for these instruments. Despite these disadvantages, the argon ion laser has been widely used in automated DNA sequencing instrumentation for 15 years now (Smith *et al.*, 1986; Probe *et al.*, 1987) and is frequently described as the excitation source in many capillary electrophoresis systems, see below.

On the other hand, semiconductor lasers or laser diodes are much smaller, lighter, and more rugged than any other laser types and have been employed in a wide variety of applications such as CD players, laser printers, and telecommunication systems. These compact lasers typically produce monochromatic light between 630 nm and 1100 nm. These extremely compact, but durable lasers can produce power in the 10-100 mW range and have a useful lifetime of up to 100,000 hours.

The neodymium:YAG (Yttrium Aluminum Garnet) laser is the most common solid-state laser in use today with instruments being found in a variety of applications such as in industry welding of heavy metals, in surgical operating devices, in laboratory spectroscopic equipment, or

on unmanned space probes. A solid-state laser is a source in which the active medium is usually a transparent crystal containing a transition metal, (typically 1 % or less), such as neodymium, chromium or holmium. Transitions in the metal ion are responsible for the laser's action. These lasers are optically pumped by either broadband flash-lamp sources, or one or more diode laser sources. For blue and green excitation, solid-state lasers contain a frequency doubling or second harmonic generating (SHG) crystal such as lithium borate or potassium titanyl phosphate. For example, the frequency doubled Nd:YAG laser has a fundamental excitation line of 1064 nm, which is doubled by an SHG crystal to generate green 532 nm light.

Until recently, the application of the PME technology has been unrecognized by the lack of available and reliable solid-state lasers that produce monochromatic light at wavelengths between 400 nm and 630 nm. This emerging field, however, has recently produced solid-state lasers that generate monochromatic light at wavelengths of approximately 400 nm, 473 nm, and 488 nm, which becomes suitable for DNA sequencing applications. Thus, the development of PME is uniquely coupled to this emerging field of laser development and well positioned to incorporate new advances in laser technology, when available.

V. **Inverse Dispersion**

Because of the need for multiple laser beams incident on a single sample, the laser beams must be steered so that they all pass through or contact the sample. This can be accomplished by spatially combining the different laser beams into one overlapping "white" beam.

Other groups have developed devices to combine two or more laser beams. Conemac (U.S. Patent 6,226,126) describes a laser beam mixer having a beam combining element with a transmissive portion and a reflective portion. However, this technology requires that the cross sectional shape of the second, third and so forth laser beams be distorted. Another limitation is that for each laser beam added, the combined beam must pass through an additional optical element, which introduces loss into the system.

U.S. Patent 5,991,082 discloses a lens system that forms narrow superimposable focal lines from multiple focal lines. This system uses a prism with multiple longitudinally arranged facets bounded by parallel ridge lines and can be used to obtain a high energy radiation beam for use in pumping an X-ray laser.

U.S. Patent 6,215,598 discloses an apparatus for concentrating laser beams, which comprises collimating devices that converge the laser beams into a laser beam sheet. A digital optics device shapes and concentrates the laser beam sheet into a narrow overlapping laser beam.

In the present invention, an inverse dispersion system can be used to steer the light from multiple lasers onto a single sample. Inverse dispersion uses optical dispersion elements such as a prism assembly or a diffraction grating positioned such that light from discrete locations is steered to be substantially colinear and/or substantially coaxial upon exiting the system. The term "substantially colinear" means that the laser beams or excitation lines diverge from each other at angles of less than 5°. The term "substantially coaxial" means that the laser beams or excitation lines diverge from each other at angles of less than 5°.

The inverse dispersion system may be configured as shown in FIG.3 in which five excitation lines from discrete locations are combined into a single colinear and/or coaxial line.

High dispersion equilateral prisms constructed from high grade glass, quartz or silica are used in one preferred embodiment of the invention. The preferred orientation for the prisms relative to each other when using high dispersion flint glass prisms is 40° - 50°, or more preferred 45°. The preferred orientation for the prisms relative to each other when using quartz prisms is 25° - 55° or more preferred 30° - 50°. This angle allows efficient overlap of the multiple beams by inverse dispersion into a single beam. A two prism assembly is preferred, however, a single prism or an assembly with three or more prisms is also contemplated. Similarly, a diffraction grating such as a ruled or holographic grating can be used to combine the multiple beams. Multiple excitation lines can be steered onto a diffraction grating such that the diffraction of the grating causes the beams to combine.

In addition to being colinear, the beams may be coaxial, with all of the laser beams passing through the same columnar space in the sample. The same sample molecules will be exposed to each of the laser beams sequentially in turn as the lasers are fired.

The inverse dispersion approach uses the same principle first demonstrated by Sir Isaac Newton, but in reverse direction. In his experiment, collimated white light, from the sun, passed through an equilateral prism, and the various wavelengths became separated by angle. The beam of light passes into the prism, forming a non-zero angle with respect to the normal to the entrance surface. According to Snell's law, all of the rays will be bent towards the normal as the light passes into the more optically dense medium. Due to dispersion of the glass, the shorter wavelengths deviate more. When the rays exit the prism, all are bent a second time, but again, the shorter wavelengths bend more. The result is that the shorter wavelengths now have an angular separation from the longer wavelengths. Blue light is deviated more than green, which is deviated more than yellow, and that in turn more than red.

This process may be reversed, and that is the principle utilized for inverse dispersion. If the separated rays are made to trace paths that are just the reverse of above, then the various wavelengths are combined into a single beam of "white" light. For example, light from lasers, light emitting diodes, arc lamps, incandescent lamps, etc. may be combined (after collimation and spectral filtering, if appropriate) by this inverse dispersion method. The shorter wavelength rays or beams enter the prism at the appropriate larger off-normal angle than the longer wavelength beams. When the correct angles are determined from Snell's law, the beams will all combine into a single coaxial beam. If desired, the beams may be spatially offset to provide colinear beams that, while parallel to each other, pass through the sample at slightly different positions. In the former case, the fluorescence from all of the beams may be imaged onto a single detector. In the latter case, the beams may be imaged onto four or more separate detectors, or separate regions of one detector, such as a CCD camera. If the light sources are pulsed in rapid succession, the combined beam appears to be white or nearly so. If the pulsing is slow enough for the eye to follow, the combined beam will exhibit a changing color pattern originating from the same spatial location.

The prism is typically used at the minimum deviation angle, whereby the entering and exiting angles for a given beam are equal, or as nearly so as practical. The apex bisector will also bisect the angle formed by the entering and exiting beam. If the prism is then rotated, then these two angles are no longer equal. This is advantageous in that it increases both the overall deviation angle and the amount of dispersion. However, this also causes anamorphic changes in the beam diameter. Such anamorphic expansion can be useful if it is desirable to change a round beam cross section to an oblong one, or an oblong beam shape to a round shape. If the inverse dispersion combining of beams is to be done with minimal distortion of the original beam shape, then the minimum deviation angle is preferred.

The angular separation of the beams is increased in proportion to the number of prisms the beam passes through. For example, the use of two identical prisms doubles the angular separation. The anamorphic beam changes can be nearly canceled by using a pair of prisms at non-minimum deviation angles. Use of high dispersion glass, such as flint glass also increases the angular separation of the incoming beams. This in turn reduces the distance needed to achieve spatial separation of the incoming beams, and provides for a more compact optical apparatus. As an example, the present apparatus utilizes two F2 flint glass prisms.

A diffraction grating may also be used as a suitable inverse dispersion element. For a grating exposed to a collimated beam of white light, the beam is diffracted in accordance with the grating equation. If it is a transmission grating, the beam is diffracted away from a straight line path, called the zero order, with the longer wavelengths deviating at a larger angle than the shorter wavelengths. The order of the dispersed wavelengths is the reverse of that for prisms. If a reflection grating is used, again the longer wavelength beam is deviated further from the zero order reflection than a shorter wavelength. As with the prism combiner above, beams of different wavelengths incident on the grating in the reverse direction will provide the same sort of inverse dispersion, leading to a colinearity of the beams. They may be made coaxial if the beams are incident on the same area on the grating but at the appropriate angles for each of the wavelengths. Conventionally ruled gratings are suitable for this purpose, however holographic gratings generally exhibit less scattered light. Gratings generally can be obtained that have considerably higher dispersion than prisms, and hence dispersion angles are larger and the spacing between the light sources can be reduced. They are generally less efficient, so that light losses are greater. Gratings and prisms are both sensitive to the polarization of the light. Since the fluorescence emission is also sensitive to the direction of the polarization, proper orientation of the electric vector of the light should be considered. Polarization rotation devices may be added to improve the transmission efficiency.

VI. Collection Devices

Of the possible choices for detection of fluorescence, the optimum one will depend upon the level of fluorescence intensity. For all detectors described herein, a photon striking the detector is converted into a charge carrier, which is then detected electronically. Charge carriers, however, are generated by extrinsic processes unrelated to the fluorescence signal from a variety of sources: thermal generation, cosmic rays, and natural radioactivity. All carriers generated both from fluorescence and from unrelated sources contribute to shot noise, a well-understood statistical phenomenon. Of the extrinsic sources of excess carrier noise, thermal generation of carriers is usually the dominant extrinsic noise source, which can be reduced by cooling the detector. Fundamentally all that is needed for satisfactory detection is that the number of charge carriers generated by fluorescence during an observation time window is much greater than the square root of the number of all carriers generated during the same time interval. However, once the carriers leave the detector, the amplifying electronics will introduce noise as well, and this electronic noise may dominate. To avoid this situation, devices have been invented that incorporate their own very low noise amplifiers. There are two such devices: the photomultiplier tube (PMT) and the silicon avalanche photodiode (APD). Another approach to reducing

amplifier noise is to collect carriers for a period of time and then rapidly read the collected charges out. This mode of operation is used for photodiode array charge-coupled device (CCD) detectors.

For light levels with high enough signals that the noise generated in the external amplifiers is negligible, the simplest device for fluorescence detection is the silicon PIN detector. This device consists of a thin hole rich region (p) and an electron rich region (n) separated by thick carrier deficient region (i). It is back-biased with a negative voltage applied to the p side and a positive voltage applied to the n side. Light striking it penetrates the p region and is absorbed in the i region generating an electron hole pair with the electron being attracted to the n side and the hole to the p side creating a current through the device. The quantum efficiency of this process is very high (~80%). Because it is the simplest, most compact, and cheapest detector, the silicon PIN is a preferred detector. Other, more sensitive detectors may also be used for detecting low levels of light emission for a multi-capillary electrophoresis system and direct detection assays.

The silicon PIN detector can be made suitable for the detection of very low light levels by introducing carrier gain into it. A photon strikes the detector creating an electron hole pair. The electron is accelerated by an electric field and creates additional carriers by ionization. The additional electrons are accelerated to produce more carriers resulting in an avalanche process and current gain. Silicon avalanche detectors operate in two modes analogous to that of the PMT: an analog current measurement mode and a digital counting mode. When operated in the analog mode, the current gain (~300) is less than that of a PMT ($\sim 10^5$ - 10^6), but with exception to the lowest light levels, the signal is still much larger than the noise in the external amplification circuit. In addition, the wavelength response range (300 to 1100 nm) of silicon detectors is much wider than any individual PMT photocathode, covering the fluorescent maximum of any dye that might be used for DNA sequencing instrumentation.

Alternatively, a silicon APD can be thermoelectrically cooled and operated in the Geiger counting mode, where individual fluorescence photons are counted. This provides a high quantum efficiency (~80%) with dark count levels approaching a cooled PMT (quantum efficiency typically 10%). The thermoelectrically cooled silicon APD provides a compact form combined with state-of-the-art sensitivity. While higher sensitivity may not be required for detecting fluorescent signals from standard sequencing reactions, silicon APDs in the counting mode can be ideal for detecting fluorescent signals directly from genomic DNA assays (i.e., without amplification).

Detectors with the required characteristics are commercially available and include the simple silicon PIN detector, the silicon APD, or a photodiode array (CCD). The simple silicon detector is the cheapest, the silicon avalanche is the most sensitive, and the CCD is most useful for multiple capillary systems as it provides many detectors in a single unit.

VII. Coupled Systems

The PME technology has sufficient flexibility for coupling to a variety of formats, for example, the PME system can be coupled to conventional capillary electrophoresis (CE) or to separation and/or purification using high-density arrays/biochips. Ideally, a DNA sequencing system capable of direct detection of fluorescent assays for genomic DNA should (i) optimally excite all fluorescent dyes, (ii) be capable of efficiently collecting photons over a large part of the UV, visible and infrared spectrum, (iii) continuous monitoring of fluorescent signals in high-throughput array or high-density formats, (iv) maximize fluorescence emission signals for detection, (v) be configured to minimize background scattered light, and be automated using replaceable gel matrices.

a. Capillary Electrophoresis

The PME fluorescence detection system of the present invention can be coupled to conventional capillary electrophoresis (CE) as a preferred method for resolving DNA fragments.

Microcapillary array electrophoresis generally involves the use of a thin capillary or channel, which may or may not be filled with a particular separation medium. Electrophoresis of a sample through the capillary provides a size-based separation profile for the sample. The use of microcapillary electrophoresis in size separation of nucleic acids has been reported in, *e.g.*, Woolley and Mathies (1994). The high surface to volume ratio of these capillaries allows for the application of higher electric fields across the capillary without substantial thermal variation across the capillary, consequently allowing for more rapid separations. Furthermore, when combined with confocal imaging methods, these methods provide sensitivity in the range of attomoles, which is comparable to the sensitivity of radioactive sequencing methods. Microfabrication of microfluidic devices including microcapillary electrophoretic devices has been discussed previously (*e.g.*, Jacobsen *et al.*, 1994; Effenhauser *et al.*, 1994; Harrison *et al.*, 1993; Effenhauser *et al.*, 1993; Manz *et al.*, 1992; and U.S. Patent 5,904,824). Typically, these methods comprise photolithographic etching of micron scale channels on a silica, silicon or other crystalline substrate or chip, and can be readily adapted for use in the present invention. In some embodiments, the capillary arrays may be fabricated from the same polymeric materials

described for the fabrication of the body of the device, using the injection molding techniques described herein.

Tsuda *et al.*, (1990), describes rectangular capillaries, an alternative to the cylindrical capillary glass tubes. Some advantages of these systems are their efficient heat dissipation due to the large height-to-width ratio and, hence, their high surface-to-volume ratio and their high detection sensitivity for optical on-column detection modes. These flat separation channels have the ability to perform two-dimensional separations, with one force being applied across the separation channel, and with the sample zones detected by the use of a multi-channel array detector.

In many capillary electrophoresis methods, the capillaries, *e.g.*, fused silica capillaries or channels etched, machined or molded into planar substrates, are filled with an appropriate separation/sieving matrix. Typically, a variety of sieving matrices are known in the art, which may be used in the microcapillary arrays. Examples of such matrices include, *e.g.*, hydroxyethyl cellulose, polyacrylamide, agarose and the like. Generally, the specific gel matrix, running buffers and running conditions are selected to maximize the separation characteristics of the particular application, *e.g.*, the size of the nucleic acid fragments, the required resolution, and the presence of native or undenatured nucleic acid molecules. For example, running buffers may include denaturants, chaotropic agents such as urea or the like, to denature nucleic acids in the sample.

The use of replaceable gel matrices, which suppress electroosmotic flow and DNA-capillary wall interactions such as polydimethylacrylamide (Madabhushi, 1998), may be used for electrophoretic separations in the present invention.

b. Chromatographic Techniques

Alternatively, chromatographic techniques may be coupled to the PME fluorescence detection system of the present invention. There are many kinds of chromatography, which may be used including liquid chromatography, HPLC and many specialized techniques, such as reverse phase HPLC, normal phase HPLC, anion exchange, cation exchange, denaturing HPLC, size exclusion or gel permeation, and hydrophobic interaction.

c. Microfluidic Techniques

Microfluidic techniques can be used for fluid flow with the PME system, and includes the use of a platform such as microcapillaries, designed by ACLARA BioSciences Inc., or the LabChipTM "liquid integrated circuits" made by Caliper Technologies Inc. Miniaturizing some of

the processes involved in genetic analysis has been achieved using microfluidic devices. For example, published PCT Application No. WO 94/05414, by Northrup and White, incorporated herein by reference, reports an integrated micro-PCR™ apparatus for collection and amplification of nucleic acids from a specimen. U.S. Patents 5,304,487 to Wilding *et al.*, and 5,296,375 to Kricka *et al.*, discuss devices for collection of cell containing samples and are incorporated herein by reference. U.S. Patent 5,856,174 describes an apparatus, which combines the various processing and analytical operations involved in nucleic acid analysis and is incorporated herein by reference.

d. Chip Technologies

Specifically contemplated by the present inventors for combining with the PME system are chip-based DNA technologies. These techniques involve quantitative methods for analyzing large numbers of genes rapidly and accurately.

Chip based technologies that can be used in the current invention include the those described in U.S. Patent 6,153,379 and Shumaker *et al.* (1996) where a method of analyzing oligonucleotides is described in which oligonucleotides are extended with fluorescent dideoxynucleotides, and detected using an automated fluorescent DNA sequencer. The oligonucleotide length identifies the known mutation site, and the fluorescence emission of the ddNTP identifies the mutation. Another method of analyzing oligonucleotides involves using template DNA annealed to an oligonucleotide array. The analysis is done using a Phosphor Imager and alpha-³²P labels. Kurg *et al.*, (2000) describes an integrated system with DNA chip and template preparation, multiplex primer extension on the array, fluorescence imaging, and data analysis. The method includes annealing DNA to immobilized primers, which promote sites for template-dependent DNA polymerase extension reactions using four unique fluorescently labeled dideoxy nucleotides. A mutation is detected by a change in the color code of the primer sites.

Motorola BioChip Systems has the I-based SNP systems with array technology centered on a three-dimensional gel pad format consisting of flexible content architectures.

The MassARRAY system, developed by SEQUENOM (U.S. Patents 5,547,835, 6,238,871, and 6,235,478) has a platform capable of high throughput SNP analysis using enzymology, bioinformatics and miniaturized chip-based disposables with mass spectrometry detection. The MassARRAY technology can be used to distinguish genotypes using MALDI-TOF mass spectrometry. DNA fragments associated with genetic variants are simultaneously

