DNA FRAGMENT SIZING AND SORTING
BY LASER-INDUCED FLUORESCENCE

Inventor:     James H. Jett
               545 Navajo Road
               Los Alamos, New Mexico 87544

               Mark L. Hammond
               64 Isleta Drive
               Los Alamos, New Mexico 87544

               Richard A. Keller
               4 La Rosa Court
               Los Alamos, New Mexico 87544

               Babetta L. Marrone
               690 Totavi Street
               Los Alamos, New Mexico 87544

               John C. Martin
               4987 Trinity Drive
               Los Alamos, New Mexico 87544

CITIZENS OF THE UNITED STATES OF AMERICA

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED
BACKGROUND OF THE INVENTION

This invention relates to DNA analysis and, more particularly, to DNA fragment size distribution analysis and sorting. This invention is the result of a contract with the Department of Energy (Contract No. W-7405-ENG-36).

The human genome is comprised of some three billion nucleotides forming the 22 pairs of chromosomes plus 2 autosomes, each with continuous DNA pieces of 50-500 million nucleotides. The organization and sequence of DNA forming the human genome contains unique information about the source that provides the DNA. One method for accessing this information is to fragment the DNA at sites with known characteristics and then to analyze the distribution of fragment sizes, i.e., the number of nucleotides in each fragment between each of the sites. Polymorphisms in the genome structure lead to substantial variation in the fragment sizes obtained from fragmentation of DNA pieces and allow one to differentiate one person from another or to form a basis for assessing a person's susceptibility to genetic diseases. Analysis of these polymorphisms is often referred to as DNA fingerprinting.
DNA fingerprinting is an important medical diagnostic tool, with additional applications to forensic identification, medical genetics, monitoring the effects of environmental mutagens, and basic molecular biology research. One form of DNA fingerprinting involves "restriction fragment length polymorphism" (RFLP) where restriction enzymes are used to cut a DNA piece from a specific source into shorter pieces, or fragments, of DNA. RFLP provides a unique pattern of DNA fragments containing a unique DNA sequence ordered by fragment size (the DNA fingerprint) when a DNA specimen is digested with restriction enzymes. There are many known restriction enzymes and each recognizes a specific DNA sequence of four to twelve base pairs at which it cuts the DNA, resulting in smaller fragments of DNA.

Once the DNA piece has been cut into many fragments, electrophoresis is conventionally used to separate the fragments by size. An electric field is placed across a gel containing the fragments causing the smaller fragments to move faster than the larger ones. Gel electrophoresis is a well known technique and has been used to produce band patterns of DNA fragments that form a fingerprint to identify the individual source of the DNA piece under analysis. The band patterns of specific DNA sequences are conventionally visualized by binding radioactive DNA probes to the separated DNA fragments and exposing suitable film to the radioactive labeled fragments. See, e.g., J.I. Thornton, "DNA Profiling," C&EN, pp. 18-30 (November 20, 1989); K. Heine, "DNA on Trial," Outlook 26:4, pp. 8-14 (1989). In one variation, the fragment ends are tagged with a fluorescent dye so that the fragment migration time along a known path length in an electrophoretic gel can be
determined by automated fluorescence detection. See, e.g.,
A.V. Carrano, "A High-Resolution, Fluorescence-Based,
Semiautomated Method for DNA Fingerprinting," 4 Genomics,

There are, however, several limitations on the use of
gel electrophoresis, particularly where large fragment
sizes and radioactive labeling are involved. In both
instances, the electrophoretic separation process takes
considerable time to provide resolution for large size
fragments. The development of images from radioactive
probes is an additional time consuming step and has health
hazards and environmental concerns associated with
radioactive materials. Additionally, the distribution of
fragment sizes is logarithmic so that the separation, i.e.,
resolution, between large fragments is less than for small
fragments. Electrophoresis also requires relatively large
amounts of DNA to obtain a recognizable pattern.

It is desirable to provide a DNA fragment size analysis
technique that uses only small quantities of DNA (maybe
only a single strand), provides size information within a
short time, and has a high resolution between fragment
sizes. These and other problems of the prior art are
addressed by the present invention wherein flow
cytometry-based techniques are used to obtain a
distribution of DNA fragment sizes from a DNA piece.

Accordingly, it is an object of the present invention
to provide rapid determination of DNA fragment sizes.

It is another object of the present invention to obtain
a high resolution of DNA fragments, particularly long
fragments.
One other object of the present invention is to require only a small DNA sample to provide accurate DNA fragment size fingerprints.

Yet another object of the present invention is to enable fragment length detection without the use of radioactive labels.

A further object of the present invention is to use fluorescent intensities to determine the length of DNA fragments.

Still another object of the present invention is to use the sorting capabilities associated with flow cytometry to sort the fragments by size, i.e., length, for further study.

Additional objects, advantages and novel features of the invention will be set forth in part in the description which follows, and in part will become apparent to those skilled in the art upon examination of the following or may be learned by practice of the invention. The objects and advantages of the invention may be realized and attained by means of the instrumentalities and combinations particularly pointed out in the appended claims.

SUMMARY OF THE INVENTION

To achieve the foregoing and other objects, and in accordance with the purposes of the present invention, as embodied and broadly described herein, the method of this invention may comprise the use of an induced fluorescence to quantitate the length of DNA fragments. A piece of DNA is fragmented at preselected sites to produce a plurality of DNA fragments. All of the DNA fragments are treated with a dye effective to stoichiometrically stain the nucleotides along the DNA fragments. The stained DNA
fragments are then fluorescently examined to generate an output functionally related to the number of nucleotides in each one of the DNA fragments. In one output, the intensity of the fluorescence emissions from each fragment is directly proportional to the fragment length.

**DETAILED DESCRIPTION OF THE INVENTION**

In accordance with the present invention, DNA polymorphisms are characterized, i.e., "fingerprinted," using flow cytometry-based techniques to provide a rapid analysis of DNA fragment sizes obtained by fragmenting a selected DNA piece with one or more enzymes selected to cleave DNA at known sequence sites. One exemplary procedure for sizing is the following:

1. A DNA piece from a selected source is fragmented by enzyme digestion to provide a solution of DNA fragments. The nucleotides comprising the DNA piece may be stained, i.e., labeled, with an appropriate fluorescent dye either before or after the DNA piece is fragmented.

2. The stained DNA fragments are passed through a detection apparatus at a concentration and rate effective to provide only one fragment in the fluorescence excitation volume at any one time.

3. Each stained DNA fragment is excited, e.g., with laser irradiation, in the excitation volume and the resulting fluorescence intensity is measured, wherein the intensity of the induced fluorescence is a measure of the amount of stain on the fragment and concomitant fragment length.

4. The number of fragments at each different intensity provides an analysis of the number of fragments of each length produced from the DNA piece by the selected enzyme or enzymes.
A DNA piece may be first selected from any suitable source, e.g., blood, tissue samples, semen, laboratory research specimens, etc. The DNA piece is then fragmented using an enzyme chosen for a particular application of the analysis. One particularly useful type of enzyme is a restriction endonuclease that recognizes specific sites, i.e., specific nucleotide sequences, and cleaves the DNA piece within the identified sequence. For example, the enzyme Eco RI cuts at the double piece recognition site

```
'GAATTC'
```

```
'CTTAAG'
```

Hundreds of different restriction enzymes and their respective cleavage sites are known. It will be appreciated that identical DNA pieces from a single source might be digested with different enzymes to yield a family of fingerprints. Alternatively, a DNA piece may be digested with multiple enzymes to further particularize the fragment size distribution analysis.

Fragmentation, i.e., digestion, of a DNA piece with a selected enzyme is a well-known process, where the optimum digestion conditions are specified by the enzyme manufacturer. A generic restriction enzyme process for use with 0.2-1 μg of DNA is given by J. Sambrook et al., Molecular Cloning, pp. 5.28-5.33, Cold Spring Harbor Laboratory Press (1989):

1. Place the DNA solution in a sterile microfuge tube and mix with sufficient water to give a volume of 18 μL.
2. Add 2 μL of an appropriate restriction enzyme digestion buffer and mix by tapping the tube. An exemplary buffer may be formed as follows:

200 mM potassium glutamate
50 mM Tris-acetate (pH 7.5)
20 mM magnesium acetate
100 μg/mL bovine serum albumin (Fraction V; Sigma)
1 mM β-mercaptoethanol.

3. Add 1-2 units of restriction enzyme and mix by tapping the tube, where 1 unit of enzyme is defined as the amount required to digest 1 μg of DNA to completion in 1 hour in the recommended buffer and at the recommended temperature in a 20-μL reaction.

4. Incubate the mixture at the appropriate temperature for the required period of time.

5. Stop the reaction by adding 0.5 M EDTA (pH 8.0) to a final concentration of 10 mM.

DNA can be fragmented by a variety of other techniques in addition to restriction enzyme digest:

1. DNase hypersensitivity sites (DNase footprinting). Chromatin digestion by DNase will produce fragments of various lengths due to differences in proteins that bind to the DNA and prevent cutting of the DNA by a DNase at sites where protein is bound (D.J. Galas et al., Nucleic Acids Res., 5:3157 (1987)).

2. RNase cleavage of single-base pair mismatches. A fluorescent RNA probe is synthesized complementary to a normal, or wild type, DNA sequence of interest. This complementary probe is then annealed to the target DNA that is to be analyzed. To determine if a single nucleotide mismatch exists between the fluorescent probe strand and the target DNA strand, the RNA:DNA hybrid is treated with RNase A. RNase A specifically cleaves single stranded regions of RNA, thus cleaving the single base pair mismatch region in the fluorescent RNA strand of the RNA:DNA hybrid. (See R.M. Myers et al., Science 230:1242 (1985) and E.F. Winter et al., Proc. Natl. Acad. Sci. 82:7525 (1985)).
3. RecA-assisted restriction endonuclease cleavage. Short oligonucleotides coated with RecA protein are annealed to the complementary target DNA sequence. The DNA:oligonucleotide hybrid is treated with Eco RI methylase enzyme. Eco RI sites that are not protected by the oligonucleotide are methylated while oligonucleotide protected Eco RI sites remain unaffected. Eco RI restriction endonuclease will cleave only at protected sites (i.e., unmethylated). This method has been used to generate fragments>500,000 base pairs. (L.J. Ferrin, Science, 254:1494 (1991).

4. DNA fragmentation can also be accomplished by techniques other than enzyme digestion. For example, ultrasonic excitation at different frequencies might be used to produce a family of size distributions. Various chemicals also react with the nucleotides and may be used to fragment DNA pieces. The DNA fragments must be stained with a fluorescent dye for flow cytometric analysis. A fluorescent dye is selected to bind stoichiometrically to the DNA fragments. The complex may be formed in different ways, i.e., single stranded DNA, double stranded DNA, specific base pairs, etc. Well known dyes include ethidium bromide, acridine orange, propidium iodide, DAPI, Hoechst, chromomycin, mithramycin, 9 amino acridine, ethidium bromide heterodyne, asymmetric cyanine dyes, or combinations of these dyes for energy transfer. The selected dye or dyes bind to the oligonucleotides stoichiometrically along the DNA sequence, i.e., the binding sites along the fragments are such that the total number of dye molecules along any length of DNA is proportional to the number of base pairs (bp’s) forming the DNA. For example, under the staining protocol set
forth below, the number of ethidium bromide molecules bound to a DNA fragment is stoichiometric and can be as high as one-half the number of bp's. See, e.g., C.R. Cantor et al., "Binding of Smaller Molecules to Nucleic Acids," in Biophysical Chemistry, Part III: The Behavior of Biological Macromolecules, p. 1251, W.H. Freeman and Company, 1980.

One exemplary procedure for staining with ethidium bromide is:

Add DNA sample to a solution containing 1-5 µg of ethidium bromide per mL of solution and TE 8.0 buffer. A suitable buffer is available from GIBCO and is 10 mM Tris-HCL and 1 mM EDTA, pH 8.0. The reaction is complete in 5-10 minutes at room temperature.

The stained DNA fragments are now analyzed using sensitive fluorescence detection techniques to determine the fluorescence intensity from each fragment passing through a detection region and having a high resolution to distinguish adjacent fragment sizes. Theoretically, while only a single DNA piece is needed to obtain the desired distribution analysis, a typical solution will be formed from many DNA pieces and a relative DNA fragment size distribution is obtained. The DNA fragments will typically range in size from 100 bp to 500,000 bp.

It is well-known how to form a sequential flow stream of particles for use in a flow cytometer or similar sensitive fluorescence detection apparatus. See, e.g., U.S. Patent 3,710,933, issued January 16, 1973, to Fulwyler et al. and Flow Cytometry and Sorting, 2nd Ed., ed. M.R. Melamed et al., Wiley-Liss, New York, 1990, incorporated herein by reference. A dilute solution of the DNA fragments is formed to a low concentration effective to provide the fragments spaced apart in the flow stream so
that only a single fragment is present in the excitation volume. The solution of DNA fragments is then injected within a laminar sheath flow stream for passage through the detection chamber for laser excitation of one fragment at a time. The flow rates of the sample and the sheath are adjusted to maintain separation between particles and to provide the optimum time for each particle in the excitation source. An optimum time is determined from a consideration of sizing rate, detection sensitivity, and photostability of the dye tags. A suitable excitation source is selected to initiate fluoresce in the dye used to stain the DNA fragments. For example, an argon laser at 488 nm is effective to cause ethidium bromide to fluoresce in a band around 600 nm.

The sensitivity of conventional flow cytometry system is improved by providing a small excitation volume, e.g., 10-20 μm diameter and 100 μm length, with a tightly focused laser beam. See, e.g., J.H. Hahn et al., "Laser-Induced Fluorescence Detection of Rhodamine-6G at 6x10^{-15}M," Appl. Spectrosc. 45:743 (1991), describing a probe volume of 11 pL, incorporated herein by reference. The small probe volume greatly reduces the amount of background emission, i.e., noise, in the output signal.

The laser excitation may be a pulsed laser with a pulse of e.g., about 70ps full width, with time gating to differentiate between dye emission photons (delayed) and Raman scattering photons (prompt). See, e.g., E.B. Shera et al., "Detection of Single fluorescent Molecules," Chem. Phys. Lett. 174:553 (November 1990), The prompt scattered photons occur within the laser pulse time while the dye emissions decay with a several nanosecond lifetime so that a delayed window is effective for discrimination of
fluorescence photons from Raman scatter photons.

Alternatively, the laser may be a cw laser. See, e.g., S.A. Soper, "Single-Molecule Detection of Rhodamine-6G in Ethanolic Solutions Using Continuous Wave Laser Excitation," Anal. Chem. 63:432 (1991). The number of emitted photons can be increased by increasing the DNA fragment transit time through the laser beam and by selecting a dye and solvent with high photostability for the dye. The number of detected photons (photoelectrons) is also increased by increasing the sensitivity of the detection apparatus. Furthermore, the present invention involves DNA fragments rather than single molecules so that the longer fragment length so that a larger output fluorescence intensity is obtained.

It will also be appreciated that the solution may contain some dye that was not bound to the DNA fragments. This dye will be excited along with bound dye and the system must discriminate between the fluorescence from the unbound and the bound dye. In one embodiment, a pulsed laser and gated detection technique may be used to provide this discrimination. For example, the excited state lifetimes for the unbound and bound ethidium bromide are 2 ns and 23 ns, respectively. Thus, the detection system can be gated to detect only the fluorescence from the bound ethidium bromide and, hence, provide an output signal functionally related to the length of the DNA fragment. Alternatively, a dye might be selected that provides different fluorescence or absorption wavelengths in the bound and unbound states. For example, a series of asymmetric cyanine dyes are reported by I.D. Johnson et al., "Asymmetric Cyanine Dyes for Fluorescent Staining and Quantification of Nucleic Acids," Fluorescence

Polarized fluorescence emission also provides a means of discriminating bound from unbound dye molecules. Fluorescence polarization of unbound DNA dyes is \( \leq 0.05 \), whereas the fluorescence polarization of DNA bound fluorochromes can be between 0.20 and 0.30. See, e.g., L.S. Cram et al., "Fluorescence Polarization and Pulse Width Analysis of Chromosomes by a Flow System," J. Histochem. Cytochem. 27:445, No. 1 (1979); T.M. Jovin, "Fluorescence Polarization and Energy Transfer: Theory and Application," Flow Cytometry and Sorting, Ed. M.R. Melamed et al., pp. 156, John Wiley & Sons (1979). Discrimination is accomplished by using a polarized excitation source and detecting the emissions through a polarization filter placed in front of a fluorescence detector. The polarization filter is aligned with the polarization direction parallel to the polarization direction of the excitation source.

For continuous cw laser excitation, an energy transfer-type scheme may be used to distinguish bound and unbound dye molecules. If a second dye is also bound to the DNA, the bound first dye molecules used for fragment sizing will be in a close proximity to the second dye molecules so that excitation of the second dye molecules will result in energy transfer from the second dye molecules to the first dye molecules. The unbound first and second dye molecules in the surrounding fluid will not be in proximity effective for energy transfer. Thus, only the bound first dye molecules will fluoresce for fragment length determination when the second dye molecules are excited.
By way of example, the DNA specific dyes Hoechst and chromomycin can transfer energy. When Hoechst dye molecules are excited they can transfer energy to chromomycin molecules within a transfer radius of a few angstroms. This energy transfer pair is used in fluorescent analysis of chromosomes by flow cytometry. See, e.g., R.G. Langlois et al., "Cytochemical Studies of Metaphase Chromosomes by Flow Cytometry," Chromosa 77:239 (1980). Excitation by energy transfer may also be done by exciting the nucleotides at around 260 nm with subsequent energy transfer to the bound dye molecules. See, e.g., J.B. LePecq et al., "A Fluorescent Complex between Ethidium Bromide and Nucleic Acids," J. Mol. Biol. 27:87 (1967).

The flow cytometry-type apparatus also provides a high resolution to distinguish between adjacent fragment lengths. Indeed, the resolution is generally limited by shot noise in the photons arising from the fluorescense emissions and the percent resolution increases as the number of base pairs (bp's) forming the DNA fragments increases.

The percent resolution (R) is determined by the length of the fragment (L), the fraction of the fragment tagged (f), the number of photoelectrons collected per tag (b) (b is typically about 30), and the number of times a fragment is sized (N). The mean intensity is given by μ. The percent resolution R at a standard deviation of 3σ is given by

\[ R(\%) = 3 \times 100 \times N^{1/2} \left( \frac{L \times f \times b}{\mu} \right)^{1/2} / \left( \frac{L \times f \times b}{\mu} \right), \]

where

\[ \mu = L \times f \times b; \sigma = \mu^{1/2}. \]

For example, consider the case of a fragment 1000 bases long. L=1000, f=0.5 (for e.g., ethidium bromide), b=30. For N=1, \( \mu = 15000, \sigma = 122.474, \) and R=2.45%. The
resolution improves as $N^{1/2}$. For $N=1000$, $\sigma=3.87$ and $R=0.0775\%$. Sizing 10, 100, or even 10000 identical fragments is not a problem. There are many more fragments than 10000 in a typical electrophoresis band. Thus, it can be seen that the resolution can be much better than 1% on a 100,000 bp fragment, whereas a resolution of only 10-20% would be expected for separation of fragments in the 100,000 bp range by gel electrophoresis and the resolution degrades further as fragment length increases.

DNA fingerprinting according to the present invention can also be done very rapidly. A typical DNA fingerprint by electrophoresis has about 50 bands. At 1000 fragments per band, 50 bands would require only about 8.3 minutes to develop a fingerprint at a fragment analysis rate of 100 fragments/second. If only a single band is required for the desired resolution, the analysis time would be only about 1 second. If the desired resolution requires 100 fragments per band, then the 50 band analysis would take only about 50 seconds.

In an adaptation of the present invention, hybridization probes can be bound to the DNA restriction fragments and associated with fragment length sizes. Hybridization probes are conventionally formed containing a probe dye and hybridized to DNA fragments formed by base pair matching from the DNA piece being investigated. Excitation of the hybridized DNA fragments could then be designed to excite both the size-measuring dye and the probe dye so that correlation of the fluorescent outputs would associate the probe with various fragment lengths. In situ probe hybridization to DNA is discussed in Methods in Cell Biology, Vol. 33, Z. Darzynkiewicz et al. Ed., Academic Press, Inc. (New York 1990), Chapter 37,
"Fluorescence In Situ Hybridization with DNA Probes," pp. 383.

In addition to length determination, it is possible to derive coarse base composition information from the DNA strands. For example, the DNA specific dye Hoechst preferentially binds to AT rich regions in DNA and chromomycin preferentially binds to GC rich regions. By exciting the fluorescence of these two dyes, as is done in bivariate chromosome analysis (see Langlois, supra), the AT:GC ratios for fragments can be determined. This ratio will provide further fingerprinting information in addition to fragment length. Other binding dyes with different sequence specificities can be used for fragment base characterization. Further, synthesis of a piece of DNA in the presence of fluorescently tagged nucleotide precursors will label the piece of DNA according to its base composition and this information can be subsequently associated with the fragment length fluorescence analysis. For example, replication of a piece of DNA using three normal nucleotides and one fluorescently tagged C nucleotide would then yield information about the number of G nucleotides in the original piece since the tagged C nucleotides would bind only to G nucleotides.

An additional capability of the system can be provided by using various sorting systems associated with flow cytometers. See, e.g., U.S. Patent 3,710,933, supra, and Flow Cytometry and Sorting, supra. A sorting capability would enable one or more fragment sizes to be sorted from the flow stream for additional processing or study. If hybridization probes are used, the sorting can separate the hybridized fragments from the flow stream.
Conventional sorting apparatus, as discussed in U.S. Patent 3,710,933 and in T. Lindmo, "Flow Sorters for Biological Cells," Flow Cytometry and Sorting, Second Edition, Ed. M. Melamed et al., pp. 145-169, John Wiley & Sons (1990), uses the fluorescence output signals discussed above. After the fragments have passed through the excitation volume for generating the output, the hydrodynamic flow stream is broken into droplets by, e.g., ultrasonic vibrations, where each drop contains no more than one fragment. Drops containing DNA fragments that have a selected fluorescence response to an excitation are charged by the application of a high voltage pulse across the drops, which then pass through charged plates that generate an electrostatic field to selectively deflect the charged drops. The charge applied to the selected drops is controlled by circuitry that is responsive to fluorescent emissions from the excitation volume within the flow cytometer, where the charging pulse is activated to produce a deflection of drops containing a material emitting fluorescence at a selected wavelength and intensity.

While the above description has been directed to DNA pieces, the process is equally applicable to RNA strands. Any reference to DNA in this case should be construed to include RNA. Likewise, the form of signal detected is taught to be fluorescence. However, any form of light emission may be obtained, depending on the specific dye, such that the term fluorescence should be interpreted to include phosphorescence and luminescence. Further, the DNA or RNA being fingerprinted may not necessarily be from humans, since all organisms have a genome that determines their specific characteristics.
The foregoing description of the preferred embodiments of the invention have been presented for purposes of illustration and description. It is not intended to be exhaustive or to limit the invention to the precise form disclosed, and obviously many modifications and variations are possible in light of the above teaching. The embodiments were chosen and described in order to best explain the principles of the invention and its practical application to thereby enable others skilled in the art to best utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated. It is intended that the scope of the invention be defined by the claims appended hereto.
ABSTRACT

A method is provided for obtaining DNA fingerprints using high speed detection systems, such as flow cytometry to determine unique characteristics of DNA pieces from a selected sample. In one characterization the DNA piece is fragmented at preselected sites to produce a plurality of DNA fragments. The DNA piece or the resulting DNA fragments are treated with a dye effective to stain stoichiometrically the DNA fragments. The fluorescence from the dye in the stained fragments is then examined to generate an output functionally related to the number of nucleotides in each one of the DNA fragments. In one embodiment, the intensity of the fluorescence emissions from each fragment is directly proportional to the fragment length. Additional dyes can be bound to the DNA piece and DNA fragments to provide information additional to length information. Oligonucleotide specific dyes and/or hybridization probes can be bound to the DNA fragments to provide information on oligonucleotide distribution or probe hybridization to DNA fragments of different sizes.