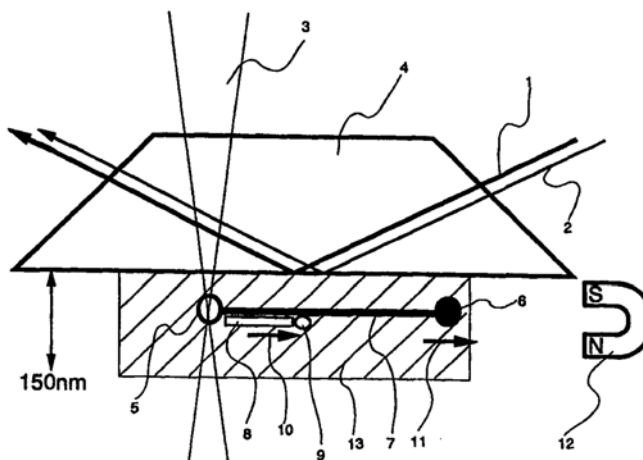


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<p>(54) Title of Invention: Method for Determining Nucleic Acid Base Sequences and Apparatus for Determining Nucleic Acid Base Sequences</p>		
<p>(57) Abstract</p> <p>A method for determining a DNA base sequence which comprises fixing in an extended state a molecule of a single-stranded sample DNA (7) carrying a bead (5) at one end thereof and a magnetic bead (6) at the other end within the visual field of a fluorescence microscope by means of a magnetic force (11) and a laser trap (3); binding a primer (8) thereto; effecting an extension reaction (10) with a polymerase to thereby cause the incorporation of a single chemically modified nucleotide (9) alone labeled with a fluorescent substance differing from base species to base species; measuring exclusively the fluorescent substance thus incorporated as a fluorescent microscopic image by the evanescent irradiation (13) with an excitation laser (1); determining the base species from the fluorescent substance; liberating the fluorescent substance with which the incorporated nucleotide has been labeled by the evanescent irradiation (13) with an ultraviolet laser (2); and then effecting the step of the incorporation of the next nucleotide followed by repeating these steps. Thus, the base sequence can be determined by using a single DNA molecule, and a DNA consisting of several hundred bases or more can be efficiently sequenced.</p>		



(57) Abstract

A single molecule of single-stranded sample DNA (7) having a bead (5) at one end thereof and a magnetic bead (6) at the other end thereof is elongated and fixed in the field of view of a fluorescence microscope by a magnetic force (11) and a laser trap (3), a primer (8) is bound thereto, and an elongation reaction (10) is conducted with polymerase. Only a single chemically modified nucleotide (9) that is labeled with a fluorophore that is different for each base type is incorporated. Only the single fluorophore incorporated is measured, by evanescent irradiation from an ultraviolet laser (2), as a fluorescence microscopic image, and the base type is determined from the fluorophore type. By the evanescent irradiation (13) from the excitation laser (1), the fluorophore that labels the incorporated nucleotide is released, and the next nucleotide is incorporated. DNA base sequences are determined by repeating this process. Base sequence determination can be performed using a single DNA molecule, and base sequences can be efficiently determined for DNA having several hundreds of thousands of bases or more.

Codes used for identifying PCT member nations listed on page 1 of the pamphlet for the international application laid open on the basis of the PCT (reference information)

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Specification

Method for Determining Nucleic Acid Base Sequences and Apparatus for Determining Nucleic Acid Base Sequences

Technical Field

This invention relates to an apparatus for analyzing DNA and RNA and the like, and more particularly to an apparatus that is effective for determining DNA and RNA base sequences or, alternatively, analyzing limited enzyme fragments or specific fragments.

Technical Background

Techniques for analyzing DNA, RNA, and the like are important in the fields of medicine and biology, inclusive of gene analysis and genetic diagnosis. The determination of DNA and RNA base sequences, and the analysis of limited enzyme fragments and specific fragments, are both based on separation by molecular weight by electrophoresis. A fragment or fragment group is subjected beforehand to radioactive labeling or fluorescent labeling, and, after conducting electrophoresis, or during electrophoresis, analysis is effected by measuring molecular weight-separation development patterns. Recently, in connection with genome analysis, the demand for DNA base sequence determining apparatuses, in particular, has grown, and the development of such devices is on-going. DNA base sequence determination using fluorescent labeling is now described. Prior to electrophoretic separation, a dideoxy reaction is carried out by the Sanger method. A nucleotide having a length of approximately 20 bases that is complementary to a known portion of the base sequence of the sample DNA to be analyzed is synthesized and *labeled with a fluorophore* [lit. *a fluorophore is labeled*]. This oligonucleotide is complementary-chain bound to approximately 10^{-12} mol of the sample DNA as a primer, and a complementary chain elongation reaction is conducted using polymerase. At this time, four deoxynucleotide triphosphates are added as substrates, namely deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), deoxythymidine (dTTP), and, in addition thereto, dideoxyadenosine triphosphate (ddATP). When ddATP is incorporated by complementary-chain elongation, because the complementary chain is not elongated beyond that, fragments of various lengths and terminated by adenine (A) are prepared. Reactions are conducted independently wherein, instead of ddATP in the reaction noted above, dideoxycytidine triphosphate (ddCTP), dideoxyguanosine triphosphate (ddGTP), and dideoxythymidine triphosphate (ddTTP), respectively, are added. However, while the primers used in each reaction have the same base sequence, four types of fluorophores, which can be mutually distinguished by separating fluorescence, are *used for labeling* [lit. *are labeled*].

When the four types of reactant noted above are mixed, fragments complementary to the sample DNA, having a length of up to approximately 1000 bases, and with lengths differing one base at a time, are prepared, with four types of fluorophores being *used in labeling* [lit. *labeled*], according to the terminal base type. The number of fragments of each base length, respectively, is approximately 10^{-15} mol. Next, the samples prepared are

separated with a resolving power of 1 base by electrophoresis. In electrophoresis, wide use is made of a slab gel wherein acrylamide is polymerized between two glass plates separated by an interval of approximately 0.3 mm. When the sample is injected at the upper end of the slab gel, and an electric field is applied to the upper and lower ends of the slab gel, the sample migrates toward the lower end while separating. When a position approximately 30 cm from the upper end is irradiated by a laser while conducting electrophoresis, separated fluorescently labeled fragments are excited as they pass the position of laser irradiation, in order from the shorter to the longer. When the emitted fluorescence is measured while being spectrally separated with the use of multiple filters, the terminal base types of all of the fragments can be determined, in order, from the shorter fragments to the longer, based on the change over time in the fluorescent intensity of the four types of fluorophore. With the order of these base types being in a complementary relationship with the sample DNA, the base sequence of the sample DNA can be determined.

A number of new DNA base sequence determination methods have been proposed which do not employ electrophoresis. With a first prior art, when a complementary-chain elongation reaction is conducted by polymerase using the sample DNA as a template, four types of base are added, in order, one at a time, and the base quantity incorporated into the complementary chain at each step is quantified by photoabsorption or fluorescence, whereupon the base sequence of the sample DNA is determined (TOKKAI [Unexamined Patent Application] No. H4-505251/1992, gazette). With a second prior art, using the sample DNA as a template, and employing four types of base labeled mutually differently, a complementary-chain elongation reaction is conducted by polymerase, after which one base at a time is released from the 3' end of the complementary chain synthesized by exonuclease, and the labels of the released bases are measured, in order, to determine the base sequence of the sample DNA (Journal of Biomolecular Structure & Dynamics 7, 301 – 309 (1989)). With a third prior art, the base sequence of sample DNA is determined by repeating a cycle of steps, namely a step for conducting a DNA polymerase reaction using four types of dNTP derivative (MdNTP) having labels that can be detected, and that, incorporated into a template DNA as DNA polymerase substrate, can stop a DNA chain elongation reaction by the presence of a protective group, a step for then detecting the MdNTP incorporated, and a step for returning the MdNTP to an elongatable state. With this prior art, DNA chain elongation is stopped at the point in time when one base is elongated, oxygen and the substrate are removed from the system (solution) wherein the template, primer, and MdNTP are present, the MdNTP incorporated is detected, the protective group (and label) of the MdNTP incorporated into the template are released, and a condition is induced wherein DNA chain elongation is possible (Pat. Applic. No. H2-57978/1990). However, these proposals are at present in the idea stage, and there are no reports of them having been made practicable.

Disclosure of Invention

Currently, insofar as practical methods of determining DNA base sequences are concerned, separation by molecular weight is conducted by electrophoresis. For DNA base sequence determination, a resolving power of 1 base length is required for separation by

molecular weight. The resolving power of electrophoresis ordinarily declines as the lengths of the bases to be separated become longer. That is, even though one base length can be separated between a base length of 50 bases and one of 51 bases, that does not mean that one base length can be separated between a base length of 500 bases and one of 501 bases. How long of a base length can be resolved by 1 base length, that is, the limit of base length separation, is determined by the electrophoresis conditions, that is, the composition of the separation medium, the electrophoresis path length, and the electric field intensity. In order to increase the limit of base length separation, various optimizations have been implemented to date, but there are no reports of a limit exceeding a length of 1000 bases (Electrophoresis 13, 495 – 499 (1992), Electrophoresis 13, 616 – 619 (1992)). The maximum of 1000 bases for the limit of base length separation has been theoretically explained (Electrophoresis 13, 574 – 582 (1992)). That is, insofar as electrophoresis is employed, the maximum length at which the base sequence can be determined from one sample DNA is a length of 1000 bases. With large-scale base sequence determination typified by genome analysis, on the other hand, the limit of base length separation holds the key to analytical efficiency (Science 254, 59 – 67 (1991)). The YAC clone, which is a typical large clone, for example, has a base length of approximately 1M bases, wherefore, a minimum of 1000 samples must be analyzed in order to analyze each base length of 1000 bases. However, it is not possible to prepare fragments one by one having a base length of 1000 bases, in order, from the end of a long DNA, without either excess or insufficiency. In practice, random fragments are prepared, these are randomly analyzed, and the base sequence of the original long DNA is reconfigured using overlaps in the base sequences of the fragments. This method, called the shotgun method, is the most widely used method today in genome analysis. However, in order to impart overlaps to random fragments so as to enable reconfiguration, it is necessary to repeatedly analyze the same base sequence, over and over, which is a problem.

The degree of this overlapping, which is called redundancy, increases as the base length wherewith one-time base sequence determination is possible becomes smaller, that is, as the limit of base length separation in electrophoresis becomes smaller. For a limit of base length separation of 1000 bases, a redundancy of approximately 10 is required. That is, it is necessary to conduct base sequence determination for a length that is 10 times that of the DNA for which the base sequence is to be determined. As a consequence, one must analyze approximately 10,000 samples in order to determine the base sequence for a base length of 1M bases. The number of samples that can be analyzed in one day by one DNA base sequence determination device is 100 at most. To determine the base sequence of the entire base length of 1M bases with one apparatus would require 100 days. In order to practically conduct genome analysis, or the genetic diagnostics the importance whereof will increase in future, requires art whereby base sequences 1M bases long can be determined in a few days.

Another problem with the prior art is the necessity therewith of having many DNA samples in order to determine base sequences. In order to conduct the dideoxy reactions with the Sanger method, a DNA sample of approximately 10^{-12} mol is ordinarily needed for one sample. For that reason, it is necessary to refine and amplify sample DNA beforehand, using techniques such as cloning or PCR. Such procedures require time and effort, and

constitute a large rate-limiting process which impedes the progress of genome analysis. In order to advance genome analysis and genetic diagnostics efficiently, art is wanted whereby base sequences can be determined from a small quantity of sample DNA, and, ultimately, even from a single DNA molecule. The DNA base sequence determination methods proposed thus far which do not use electrophoresis are, however, subject to the following shortcomings.

In the first prior art example, four types of nucleotide substrate are added, in order, one at a time, making it necessary to repeatedly alter the composition of the solution wherein the complementary-chain elongation reaction is being conducted. When changing the base type in a solution, moreover, cleaning is necessary in order to prevent the admixture of another type of base. As a consequence, at least eight solution replacements are required for every cycle. Because, ordinarily, one base is determined each cycle, on average, eight solution replacements will considerably reduce the speed of base sequence determination. The most fundamental problem with the first prior art is that the quantity of signals to be quantified accumulates as the complementary chain lengthens, making it extremely difficult to measure the changes in signal quantity corresponding to the incorporation of one base. In other words, determining the base sequences of DNA having long base length is very difficult.

With the second prior art, four types of base labeled with four types of fluorophore are incorporated into the complementary chain, but this is technically very difficult. As will be discussed subsequently, because the fluorescence quantity is large, or because the molecular size of the fluorophores that can mutually be distinguished by fluorescent spectral separation, is large, it is very difficult, due to steric hindrance, for a plurality of fluorescently labeled nucleotides to be incorporated next to each other in the same complementary chain. Another problem with the second prior art is that it is very difficult to control the release of one base at a time using exonuclease. When a plurality of bases has been released continuously within a range equal to or less than the detection limitation time, the released bases are all detected simultaneously, so the order of base release, which is to say the base sequence, cannot be determined. In order to determine the base sequence, it is necessary for the release of each base to occur at some interval of time, and not continuously, but effecting such control is extremely difficult.

With the third prior art example, it is necessary, in the step for detecting the MdNTP incorporated into the template, and in the step for returning the MdNTP to an elongatable condition, to remove MdNTP not incorporated into the template from the solution, and also to add MdNTP into the solution at the point in time when the next, new, cycle starts. When this is neglected, the signals from the MdNTP incorporated into the template and the signals from the MdNTP not incorporated into the template become mixed, whereupon the signals from the MdNTP incorporated into the template targeted cannot be accurately measured. Furthermore, when the protective groups of MdNTP not incorporated into the template are also released, and dNTP from which the protective group has been released is incorporated into the template, the prescribed cycle ceases to advance. As a consequence, a minimum of two solution content changes will be necessary for each cycle, which causes the speed of base sequence determination to be retarded. Thus, when the change in solution content is incomplete, and the slightest amount of MdNTP not incorporated into the

template remains in the solution, that causes a lot of noise when conducting measurements, which is a problem.

An object of the present invention is to provide a method and an apparatus for determining the base sequences of very long DNA [chains] by determining the types of bases of nucleotides incorporated when conducting complementary-chain elongation reactions by polymerase, in order, one at a time, in order to resolve the problems with the DNA base sequence determination methods proposed to date which do not employ electrophoresis.

With the present invention, a single template DNA molecule (sample) is held within the field of view of a fluorescence microscope, complementary-chain elongation is conducted, under control, one base at a time, and each fluorescently labeled base incorporated is subjected to single molecular measurement. In performing DNA base sequencing, it is only necessary to monitor, one by one, the types of bases of nucleotides incorporated in the complementary-chain elongation reaction by polymerase. There are two conditions, namely (1) that the elongation reaction not advance after one nucleotide has been incorporated, and (2), that, after determining the base type of the one nucleotide incorporated, incorporation of the next single nucleotide be made possible, that all nucleotides to be incorporated must be chemically modified so as to simultaneously satisfy. These two conditions can be achieved by combining nucleotides, on the one hand, and caged compounds having fluorophores as labels, on the other, for example. Caged compounds are compounds that mask residues contributing to the activity of physiologically active substances by the nitrobenzyl group or the like, and cause the dissociation of modifying groups by photo-irradiation. For example, a caged compound is a substance wherein the 2-nitrobenzyl group has been introduced, which 2-nitrobenzyl group is released by UV irradiation. This procedure is widely used in the field of biology (Annu. Rev. Biophys. Biophys. Chem. 18, 239 – 270 (1989)). Many different kinds of caged compound are sold by companies such as Molecular Probes, Inc. The chemically modified nucleotides used in the present invention, as diagrammed in Fig. 1, are caged compounds wherein a 2-nitrobenzyl group is bonded to a physiologically active substrate X (a nucleotide), and have the capability to suppress the inherent activity of chemically modified nucleotides, that is, of the activity of being continually incorporated by complementary chain synthesis reactions, the ability to release the caged substance (2-nitrobenzyl group) when subjected to UV irradiation at 360 nm or below, and to convert [the chemically modified nucleotides] to a substrate X or HX exhibiting inherent physiological activity. In Fig. 1, R is H or an alkyl group (such as CH₃) or the like.

The method of manufacturing Texas Red-labeled caged dGTP, as an example of a chemically modified nucleotide having the capabilities noted above, is diagrammed in Figs. 3 to 7. In Fig. 3, water-soluble carbodimide (HOOC (CH₂)₂ COOH) is reacted with a derivative of dGTP (Fig. 2) that is a starting substance when chemically modifying a base (Science 238, 336 – 341, 1987), to obtain a dGTP derivative having a carboxyl group as a linker end. As diagrammed in Fig. 4, on the other hand, nitric acid is reacted with 2-nitroacetophenon (Aldrich, N920-9), a nitro group is inducted at the carbon 4 position, and that nitro group is reduced to convert it to an amino group. As diagrammed in Fig. 5, the compound on the left side in Fig. 4 is reacted with Texas Red (Molecular [sic] Probes, T-

353), causing it to bond. Then, as diagrammed in Fig. 6, $\text{NH}_2\text{-NH}_2$ is reacted with the compound on the right side in Fig. 5, MnO_2 is further reacted therewith, and the acetophenone of the compound on the left side in Fig. 6 is converted to a diazoethane group (J. Am Chem. Soc. 110, 7170 – 7177, (1998)). The compounds on the right side in Figs. 3 and 6 are reacted, whereupon, as diagrammed in Fig. 7, a fluorescently labeled (Texas Red) caged nucleotide (dGTP) is obtained that is dGTP wherein a 2-nitrobenzyl group to which Texas Red has been bound as a label has been introduced. For other base types also, fluorescently labeled caged nucleotides (nucleotides to which are bound caged compounds to which a fluorescent label has been attached) can be synthesized.

As seen in the substance diagrammed in Fig. 7, substances labeled at the position of the base of the nucleotide can be incorporated by polymerase-based complementary-chain elongation reactions, as has been confirmed by various experiments. It has been confirmed, for example, that dideoxy nucleotide ddNTPs, the positions of the bases whereof are labeled by various fluorophores, that is, that the terminators of the complementary chain synthesis, are incorporated by complementary chain synthesis (Nucleic Acids Respectively. 20, 2471 – 2483 (1992)). When the molecular size of the fluorophore is large, steric hindrance occurs, so two or more fluorescently labeled deoxynucleotides are not incorporated continuously (Anal. Biochem. 234, 166 – 174 (1996)). That is, a deoxynucleotide labeled by a comparatively large fluorophore, as diagrammed in Fig. 7, can be incorporated by complementary chain synthesis, but, once it has been incorporated, all further complementary chain elongation will cease. When the compound diagrammed in Fig. 7 is subjected to UV irradiation at 360 nm or below, the chemical structure changes according to the photochemical reaction diagrammed in Fig. 1, as diagrammed in Fig. 8, and caged substances with a fluorophore attached are released from the positions of the bases of the compound diagrammed in Fig. 7. If the like reaction is brought about with a complementary chain incorporated, steric hindrance will be eliminated, and the complementary chain will again be elongated. At that time, the linker portion remains in the base, as diagrammed in Fig. 8, but, because the size of that linker portion is small, complementary-chain elongation is not affected.

That is, if polymerase complementary-chain elongation reactions are conducted with the Texas Red-labeled caged dGTP diagrammed in Fig. 7, and caged dATP, dCTP, and dTTP are labeled with different fluorophores, as substrates, the one-base-at-a-time elongation reaction described above can be controlled, and the type of base incorporated can also be identified by fluorescence measurement. With fluorescence measurement, excitation laser irradiation is conducted, and, in order to excite only the incorporated nucleotides while not exciting the other suspended nucleotides, laser irradiation is conducted after spatially separating the two, or the evanescent irradiation described subsequently is used. By repeating the steps described above, that is, (1) incorporating one fluorescently labeled caged nucleotide using polymerase, (2) exciting the incorporated fluorescent label incorporated by laser irradiation, (3) spectrally separating the emitted fluorescence and determining the type of base from the type of fluorophore, and (4) releasing the fluorescently labeled caged substance by a photochemical reaction through UV irradiation, the fluorescently labeled caged nucleotides can be incorporated one by one, using polymerase, whereupon the base sequence of the template DNA is determined.

Next, the single molecule measurement that detects fluorescence from the fluorophore labeling one incorporated nucleotide is described. Fig. 9 is a simplified diagram of the configuration of the main components of an apparatus for conducting this single molecule measurement. Because single molecule measurement is extremely abhorrent of all impurities and contamination and such like, all procedures are conducted in a clean room, paying particularly close attention to all optical systems. A cell filled with a buffer solution is placed above the objective lens of an inverted fluorescence microscope, and the sample DNA 7 that is to be the template is held, by the technique described below, in the buffer solution, inside the field of view of the fluorescence microscope. The position wherein the sample DNA is held is brought close to the upper surface inside the cell, and the distance from that upper surface is maintained at 100 nm or less. A prism 4 is disposed at the upper surface outside the cell. An excitation laser beam 1 is introduced from the upper right, obliquely through the prism 4, perfectly reflected by the upper surface inside the cell, and conducted toward the upper left, obliquely through the prism 4 once again. When this is done, exciting light called evanescent waves 13 slightly infiltrates the buffer solution in the cell in the vicinity of the upper surface thereof. The intensity of this exciting light diminishes exponentially as the distance from the upper surface inside the cell increases. When a 515 nm Ar laser is used, the intensity will be $1/e$ at a position separated approximately 150 nm from the upper surface. This irradiation method is called the evanescent irradiation method. If this evanescent irradiation method is employed, only substances present within 150 nm of the surface inside the cell will be excited, background light from fluorescence measurement, such as water Raman scattering, can be reduced to almost nothing, and single molecule fluorescence measurement can be performed (Nature 374, 555 – 559 (1995)). The emitted fluorescence is monitored as a fluorescence microscopic image by a supersensitive two-dimensional camera through the objective lens from below the cell. One fluorescently labeled nucleotide (fluorescently labeled caged nucleotide) 9 incorporated by a polymerase reaction 10, with a primer 8 as the origin, is fixed in the template DNA 7, wherefore the fluorescence is observed as a bright spot in a two-dimensional image.

On the other hand, because suspended fluorescently labeled nucleotides that are not incorporated move vigorously about the cell three-dimensionally due to Brownian motion, the fluorophores are not observed one by one in the two-dimensional image as bright spots, but appear as an overall increase in background light. Because the space wherein the fluorophores are excited is limited to the minute range of 150 nm or less from the surface within the cell, the amount of increase in background light is small, and single molecule observation of the fluorophores fixed to the template DNA can be accomplished. The two-dimensional image is divided into four by a prism disposed above a light-receiving optical system, and passed through different filters, whereupon the type of fluorophore is identified at the moment of detection, and, thereby, the type of incorporated nucleotide base is also determined. This fluorescence selection method is described in detail in TOKKAI H2-269936/1990, gazette. The evanescent irradiation method is also used in like manner when releasing the fluorescently labeled caged substance of the incorporated nucleotide 9. The UV pulse laser beam 2 is conducted obliquely from upper right through the prism 4, perfectly reflected by the upper surface of the cell, and then conducted obliquely toward the

upper left, again through the prism 4. The range within 150 nm from the surface inside the cell is UV-irradiated, and the fluorescently labeled caged substance of the incorporated nucleotide is selectively released. At that time, it is possible that the fluorescently labeled caged substance of an extremely small number of suspended nucleotides that happen to be within the range of 150 nm or less from the surface within the cell will also be released, and that non-chemically modified nucleotides that are generated will be incorporated in the subsequent polymerase reaction. In order to reduce that possibility to zero, the buffer solution in the cell is continually made to flow in one direction so that fresh buffer solution is constantly supplied.

Next, the configuration for holding a single molecule of template DNA 7 within the field of view of the fluorescence microscope is described. To the two ends of the sample DNA 7 that is to be the template are bonded beads (solid-phase carriers) 5 and 6 having diameters of approximately 100 nm. The beads are made of a polystyrene material, and one of the beads, namely the bead 6, is magnetized. The sample DNA 7 is introduced into the buffer solution inside the cell, with the bead 5 on the end of the single DNA molecule that is not magnetized being captured within the field of view of the microscope in the cell by a laser trap 3 (Science 271, 795 – 799 (1996)). Optical tweezers having a laser as the typical light source constitute a technique that recently has been rapidly proliferating as a non-contact manipulator (Optics Lett. 11, 288 – 290 (1986)). In particular, laser traps using infrared lasers are being widely used because the effects of irradiation are minimized therewith (TOKKAI No. H2-91545, gazette). When a laser beam is condensed in water by a lens, minute particles in the water can be captured in the vicinity of the focal point thereof with a restraining force of several pN/mW. After the laser trap of the bead (solid-phase carrier) 5 on one end of the single sample DNA 7 has been completed, a magnetic field is applied by an electromagnet 12 in a direction parallel to the upper and lower surfaces of the cell, generating a static magnetic force 11 on the magnetized bead 6 on the opposite end, and stretching out the single DNA molecule 7 (Science 271, 1835 – 1837 (1996)). The stretching-out force is controlled by varying the size of the magnetic field. Maintaining this condition, the stage of the microscope on which the cell is fixed is slowly lowered, and, while capturing the single DNA molecule 7, the upper surface inside the cell is caused to be approached, to within 100 nm or less.

As described in the foregoing, with the present invention, base sequences can be determined from one type of sample DNA, and the base length can be increased close to the limiting length wherewith polymerase can synthesize a complementary chain. There is absolutely no problem even if the polymerase molecule that was engaged in complementary chain elongation separates from the DNA molecule and another polymerase molecule again conducts the continuation of complementary-chain elongation. That is, if active polymerase is sequentially supplied, a base sequence of almost any desired length can be determined. The repeated solution replacement that was a problem with the first prior art is unnecessary with the present invention. Also, with the present invention, since the fluorophore incorporated into the complementary chain is released every cycle, there is no problem of the signal quantity from the fluorophores accumulating, and base sequence determination can be accomplished irrespective of the DNA base length. On the other hand, the continued incorporation of fluorophores into the complementary chain that was a problem with the

second prior art is unnecessary with the present invention. In the second prior art, moreover, it was very difficult to control the release and elongation of bases one by one by controlling enzyme activity. With the present invention, however, elongation is controlled structurally, wherefore the elongation of bases one by one can be controlled irrespective of enzyme activity. Furthermore, changing the solution content, which was a problem with the third prior art, is unnecessary in the present invention. That is, with the present invention, with chemically modified nucleotides not incorporated into the template having been supplied, it is possible to selectively measure only chemically modified nucleotides that are incorporated into the template, and, because the release of chemically modified substance can be selectively applied only to nucleotides incorporated into the template, there is no need to remove chemically modified nucleotides from the solution content which have not been incorporated into the template, during a cycle.

The speed of base sequence determination based on the present invention is dependent on the cycle time for the steps described earlier. The step of incorporating one fluorescently labeled caged nucleotide by polymerase can be accomplished in under 0.1 second. Various types of DNA polymerase are commercially available, but when the elongation rate is the slowest, 30 bases can be incorporated per second. That is, the mean time required to incorporate one base is 0.03 second, so the 0.1 second noted above is a fairly conservative value. The step of exciting one caged nucleotide by laser irradiation, optically separating the emitted fluorescence, and determining the base type from the type of fluorophore, requires 0.5 second.

For single molecule fluorescence measurement, a television rate, that is, an exposure time of 0.03 second or less is sufficient (Nature 374, 555 – 559 (1995)), but, with the present invention, the exposure time is set at 0.5 second in the interest of high-precision measurement. The laser irradiation time is also 0.5 second, synchronized with the fluorescence exposure. Determining the type of base from the type of fluorophore is completed in under 0.1 second, but the determination process can be done simultaneously in the background of the next step, so it does not affect the cycle time. The step of releasing fluorescently labeled caged substance by UV irradiation can be completed in under 0.1 second. The fluorescently labeled caged substance can be released at millisecond order by 10-nanosecond UV pulse laser irradiation (Annu. Rev. Biophys. Biophys. Chem. 18, 239 – 270 (1989)). When the steps noted above are automatically repeated by a computer, even supposing the time required for laser and camera control to be 0.3 second per cycle, one cycle can be repeated in one second. Because one base type can be determined in one cycle, the speed of base sequence determination becomes 1 base/second or less. When the method of the present invention is applied to the base sequencing of the YAC clone (a typical large clone) having a length of 1M bases, this can be finished in 1M seconds, that is, in approximately 12 days, which is shorter than the time required with conventional methods by a margin of an entire decimal place. With the method of the present invention, a plurality of sample DNA can be held within the same microscopic field of view and processed in parallel. Also, base sequences at different positions in the same sample DNA can be simultaneously determined. For example, two strands of the sample DNA of 1M base length noted above can be separated, and each fixed within the field of view of the microscope and measured independently. If the two strands are independently base-

sequenced, the field of view will be decoded from both ends, so that all of the base sequences of a sample can be determined at twice the speed noted above. That is, the base sequences of a single molecule having a length of 1M bases can be determined in a mere six days, thus facilitating very highly efficient genome analysis and genetic diagnostics.

Brief Description of Drawings

Fig. 1 is a diagram of a photochemical reaction by UV irradiation of a caged compound which is used in the present invention. Fig. 2, Fig. 3, Fig. 4, Fig. 5, Fig. 6, and Fig. 7 are diagrams representing chemical reactions from dGTP derivatives that are departure substances when preparing chemically modified nucleotides in the present invention. Fig. 8 is a diagram representing a photodecomposition reaction of Texas Red-labeled caged dGTP used in an embodiment of the present invention. Fig. 9 is a simplified diagram of the configuration of the main components of an apparatus for performing single molecule measurements in an embodiment of the present invention. Fig. 10 is a simplified diagram of the configuration of a measurement apparatus in an embodiment of the present invention. Fig. 11 is a schematic diagram of the configuration of a cell in an embodiment of the present invention. Fig. 12 is a diagram of the configuration of a prism in an embodiment of the present invention. Fig. 13 is a diagram of the field of view of a microscope in an embodiment of the present invention and an image formation plane that is divided into four. Fig. 14 is a diagram of the configuration of sample DNA having oligonucleotides introduced at both ends, used in an embodiment of the present invention. Fig. 15 is a diagram of the configuration of sample DNA having a bead (solid-phase carrier) and a magnetic bead (solid-phase carrier) bonded at either end, used in an embodiment of the present invention. Fig. 16 is a diagram of the structure of BODIPY-labeled caged dATP used in an embodiment of the present invention. Fig. 17 is a diagram of the structure of BODIPY-labeled caged dCTP used in an embodiment of the present invention. Fig. 18 is a diagram of the structure of BODIPY-labeled caged dGTP used in an embodiment of the present invention. Fig. 19 is a diagram of the structure of BODIPY-labeled caged dTTP used in an embodiment of the present invention. Fig. 20, Fig. 21, Fig. 22, and Fig. 23 are diagrams for describing the fixing of sample DNA in the field of view of a microscopic image monitor in an embodiment of the present invention. Fig. 24 is a diagram that schematically represents four fluorescence microscopic images that have been divided, corresponding to one light emission spot within the field of view of a microscope in an embodiment of the present invention. Fig. 25 is a diagram representing oligonucleotides complementary to 10 types of sample DNA fixed within the field of view of a microscope in an embodiment of the present invention. Fig. 26 is a diagram that schematically represents 10 types of sample DNA captured inside a cell using complementary oligonucleotides, in an embodiment of the present invention. Fig. 27 is a diagram of the structure of fluorescently labeled caged dATP used in an embodiment of the present invention. Fig. 28 is a diagram of the structure of fluorescently labeled caged dCTP used in an embodiment of the present invention. Fig. 29 is a diagram of the structure of fluorescently labeled caged dGTP used in an embodiment of the present invention. Fig. 30 is a diagram of the structure of fluorescently labeled caged dTTP used in an embodiment of the present invention. Fig. 31 is a diagram of the structure of fluorescently labeled caged

dATP used in an embodiment of the present invention. Fig. 32 is a diagram of the structure of fluorescently labeled caged dCTP used in an embodiment of the present invention. Fig. 33 is a diagram of the structure of fluorescently labeled caged dGTP used in an embodiment of the present invention. Fig. 34 is a diagram of the structure of fluorescently labeled caged dTTP used in an embodiment of the present invention. Fig. 35 is a diagram for describing the multiple reflection of a laser beam inside a prism used in an embodiment of the present invention. Fig. 36 is a diagram for describing the multiple reflection of a laser beam inside an optical fiber used in an embodiment of the present invention.

Preferred Embodiments of Invention

In order to describe the present invention in detail, it is described below with reference to the attached drawings.

(Embodiment 1)

All procedures are conducted in a class 1000 clean room so that dust and other impurities are not mixed into the optical systems or sample solutions. The overall configuration of an apparatus relating to this embodiment is described with reference to Fig. 10. The microscope used is an inverted phase-contrast incident-light fluorescence microscope (model IMT2-RFC made by Olympus Co., Ltd.). With this microscope, phase-contrast images and fluorescence image can be switched and [alternately] observed with the field of view fixed. The object lens 27 used is an oil-immersed 100× lens (Plan · Apochromat ×100, 1.40 NA, made by Olympus Co., Ltd.). In this case, the field of view has a diameter of approximately 100 μm. A cell 25 equipped with a temperature regulating function is placed on a stage 26 of the microscope above the object lens 27. The structure of the cell is diagrammed in Fig. 11. The upper surface 37 of the cell is comprised of a quartz slide glass 1 mm in thickness, while the lower surface 38 is comprised of a quartz cover glass 0.1 mm in thickness, with these two glass plates separated by an interval of 0.2 mm. Capillaries [lit. *narrow tubes*] 39 and 40 are connected to the left and right ends of the cell [respectively]. Solution is introduced from one capillary 39 into the interior of the cell, and is discharged from the other capillary 40. The flow rate of solution in the interior of the cell is controlled by a pump. The pump employed is one that uses gravity differential in order to avoid a pulsating flow. A Peltier element is connected to a portion excluding the vicinity of the field of view of the microscope of the upper and lower surfaces of the cell, making it possible to adjust the overall cell temperature within a range of 0°C to 100°C. The object lens 27 and the outer lower surface 38 of the cell are brought into contact through a non-fluorescent oil that does not emit fluorescence when irradiated with light. An electromagnet is disposed on the right side of the cell 25 on the stage 26 diagrammed in Fig. 10, and a magnetic field is generated in the horizontal direction inside the cell, facilitating the reception by a magnetic body inside the cell of an electrostatic force in the direction of the right of the cell.

As diagrammed in Fig. 10, a quartz prism 24 inclined at an angle of 60° is brought into contact with the upper surface 37 outside the cell through non-fluorescent glycerin which does not emit fluorescence by the irradiation of light. Fig. 12 exhibits the shape of the prism 24. Light from a halogen lamp 22, from directly above relative to the stage 26, for observing phase-contrast images, is condensed by a condenser lens 23 and introduced into

the cell through the flat portion of the upper surface of the prism 24. As diagrammed in Fig. 10, from diagonally above the stage 26, at an incidence angle of 68° , a 488nm Ar ion laser beam 15 (20 mW), a 532nm YAG laser beam 16 (20 mW), and a 355nm pulse oscillation YAG laser beam 17 (10 mJ) are made to irradiate the cell 25, through an oblique surface on the right side of the prism 24, and are perfectly reflected by a surface inside the cell. These three types of laser beam are fashioned into a coaxial beam 21, using a perfectly reflecting mirror 18 and dichromic mirrors 19 and 20, which is thereupon condensed to a diameter of approximately $100\text{ }\mu\text{m}$ by a lens having a focal length of 100 mm, and then perfectly reflected at a position in the field of view of the microscope. The perfectly reflected laser beam 21 is output through an oblique surface portion on the right side of the prism 24, as diagrammed in Fig. 10, in an oblique direction relative to the stage 26. The 1064nm YAG laser beam 14 (100 mW), on the other hand, is reflected by the dichromic mirror 28, passed through the object lens 27 and condensed, and then conducted into the cell. A laser beam 21' is condensed by the object lens 27 to a diameter of approximately $1\text{ }\mu\text{m}$, and the focal point thereof set inside the cell within the field of view of the microscope. The dichromic mirror is designed so as to selectively reflect infrared light for incident light at 45° . The output, oscillation, and incidence of the light from the halogen lamp 22 and the four laser beams 14, 15, 16, and 17 is controlled by a computer.

The emitted fluorescence within the field of view of the microscope is formed into substantially parallel flux by the object lens 27, and transmitted through the dichromic mirror 28 and an infrared cut filter 29, whereupon the light path thereof is split into two directions by a beam splitter 30. The beam splitter 30 is capable of freely switching between three stages, namely a half mirror, a perfectly reflecting mirror, and no mirror, and can distribute light with a prescribed ratio of from 0% to 100%. The infrared cut filter 29 is set so as to completely cut 1064nm light which was not cut out by the dichromic mirror 28. The light path in the direction reflected by the beam splitter 30 is subjected to image formation by a 2.5-power photographic lens 35 (NFK $\times 2.5$, made by Olympus Co., Ltd.), and two-dimensionally detected by a video-rate CCD camera 36. This CCD camera 36 is used mainly when capturing sample DNA. Video signals can be observed in real time at video rate by a monitor. The size of the detection plane of the CCD camera is $12.8 \times 12.8\text{ mm}$, and is configured by 512×512 aligned pixels, each pixel measuring $25 \times 25\text{ }\mu\text{m}$. The image magnification at the image formation plane is $100 \times 2.5 = 250\times$, so the size of the field of view is $51.2 \times 51.2\text{ }\mu\text{m}$, with one pixel corresponding to a size of $0.1 \times 0.1\text{ }\mu\text{m}$. The light oriented downward from the object lens 27, on the other hand, after being transmitted through an optical element composed of a quadruple-division prism 31 and four types of interference filters 32, is formed into an image by a 2.5-power photographic lens 33 (NFK $\times 2.5$, made by Olympus Co., Ltd.), and two-dimensionally detected by a cooled two-dimensional CCD camera 34. This cooled two-dimensional CCD camera 34 is mainly used for fluorescence measurement. The size of the detection plane of the cooled two-dimensional CCD camera is $25.6 \times 25.6\text{ mm}$, being configured of 1024×1024 aligned pixels, the size of one pixel being $25 \times 25\text{ }\mu\text{m}$. The image magnification on the image formation plane is $100 \times 2.5 = 250\times$, so one pixel corresponds to $0.1 \times 0.1\text{ }\mu\text{m}$.

As diagrammed in Fig. 13, an image 41 in the field of view that is $51.2 \times 51.2\text{ }\mu\text{m}$ is divided into four by the quadruple-division prism 31 which is inserted into the light path.

The size of [each of] the divided images is [that of] an image 42 [measuring] 12.8×12.8 mm in the detection plane. Image formation is effected for an image 43 that is 25.6×25.6 mm in size so that the four images do not mutually overlap. The interference filters corresponding to the four images have transmission bands exhibiting a transmittance of 70% or better in wavelength regions of, respectively, 500 to 520 nm, 540 to 560 nm, 560 to 580 nm, and 580 to 600 nm, the transmittance whereof outside those transmission bands within a wavelength range of 300 to 1100 nm is 10^{-4} or less. The transmittance at 355 nm, 488 nm, and 532 nm, which are laser wavelengths, is assumed to be 10^{-6} or lower. Fluorescence emitted from one point within the field of view of the microscope is formed into an image on the detection plane of the cooled CCD camera divided into four spots, and coordinates are associated with each of the four points formed as images. As a consequence, of a plurality of bright spots on 1024×1024 pixels, only brightness information on four corresponding points can be selectively transferred to the computer. The four points are transmitted through the four different interference filters noted earlier. The fluorescence intensity ratio between the points will differ according to the type of fluorophore. The fluorescence intensity ratio for the four points can be measured and the type of fluorophore emitted can be determined. The embodiment is now described in detail following the actual measurement procedures. The entire base sequence of λ DNA is determined as a model sample. λ DNA has a length of 48502 bases and the entire base sequence is known, making it ideal as a sample for verifying the principle of the method of the present invention. λ DNA is obtainable in the form of a double-stranded straight chain. At both ends, a complementary double-stranded oligonucleotide is synthesized and bonded to the two ends by a ligase reaction. As diagrammed in Fig. 14, in the synthesized double-stranded oligonucleotide, biotin 49 is used for labeling at the 5' end of an oligonucleotide 47 bonded to the 5' ends of λ DNA 44 and 46, and digoxin is used for labeling at the 3' end of the oligonucleotide 46 bonded to the 3' ends of λ DNA 44 and 45. The base sequence for the portion not complementary with the λ DNA of the synthesized double-stranded oligonucleotide is assumed to have 20 or more bases, and this is made the priming site for the subsequent polymerase reaction.

Meanwhile, as diagrammed in Fig. 15, streptavidin 50 is introduced by acid amide bonding to the outer surface of a polystyrene bead (solid-phase carrier) 52 having a diameter of approximately 0.1 μ m, while an anti-digoxin antibody 51 is introduced by acid amide bonding to the outer surface of the magnetized polystyrene bead (solid-phase carrier) 53 having a diameter of approximately 0.1 μ m. The λ DNA 44 and 45 subjected to ligation are denatured to a single-strand form and *attached* [lit. *added*] to the beads noted above. Because biotin 49 and streptavidin 50, on the one hand, and digoxin 48 and anti-digoxin antibody 51, on the other, bond with a peculiarly high bonding constant, in both the λ DNA 44 and 45 strands, the bead 52 which is not magnetized bonds to the 5' end, while the magnetized bead 53 bonds to the 3' end. Four types of chemically modified nucleotide, having the structures represented in Figs. 16 to 19, are synthesized by the same methods as described with Figs. 3 to 7. Fig. 16 represents caged dATP having BODIPY 493/503 C3 (D-2190, made by Molecular Probes, Inc.) as a label, Fig. 17 represents caged dCTP having BODIPY 530/550 C3 (D-2186, made by Molecular Probes, Inc.) as a label, Fig. 8 represents caged dGTP having BODIPY 564/570 C3 (D-2221, made by Molecular Probes,

Inc.) as a label, and Fig. 19 represents caged dTTP having BODIPY 581/591 C3 (D-2227, made by Molecular Probes, Inc.) as a label. Impurities such as unmodified nucleotides are removed with a column or the like.

The λ DNA 44 and 45 having the beads 52 and 53 bonded at both ends are introduced, as is in the single-stranded state, with a buffer solution made to flow into the cell 25 that is secured to the stage 26 of the microscope. First, in the phase-contrast microscope mode, the beam splitter 30 indicated in Fig. 10 is made [to function as] a 100% reflecting mirror, and, with the CCD camera 36 for capturing sample DNA and using a video monitor, the buffer solution flow is stopped, and the field of view 54 indicated in Figs. 20 to 23 is observed. As diagrammed in Fig. 20, on the monitor, the unmagnetized bead 52 bonded to the 5' ends of the sample DNA 44 and 45 is found. The stage is thereupon moved in the horizontal direction (56) and, as diagrammed in Fig. 21, the bead 52 found as noted above is placed at the position 55 at the focal point of a 1064nm infrared laser which has been set in the center on the left side on the monitor. Then, the 1064nm infrared laser is made to oscillate, and the bead 52 found as noted earlier is captured (57) as the prescribed bead by a laser trap. Whether or not this was captured will be immediately known if the stage is moved. The buffer solution is again caused to flow (58), and, as diagrammed in Fig. 22, the other bead and sample DNA not captured are carried outside of the field of view. Next, a magnetic field is generated by an electromagnet in the horizontal direction, the magnetized bead 53 bonded to the 3' end of the sample DNA is pulled to the right on the monitor (59), as diagrammed in Fig. 23, and the sample DNA 44 and 45 sandwiched by the beads at both ends are stretched out. To confirm that these two beads are bound to the two ends of a single DNA, it is only necessary to move the position of the focal point 55 of the infrared laser, and see that the bead 53 on the right side is synchronized with that movement. The magnetic force is regulated so as not to stretch out the DNA molecule excessively. When the λ DNA 44 and 45 are stretched out by this method, the overall length becomes approximately 16.5 μm , which is adequately accommodated within the $51.2 \times 51.2 \mu\text{m}$ field of view of the microscope. The microscope stage is thereupon slowly pulled down, the sample DNA is brought close to the upper surface inside the cell, with the beads at the two ends held in position, and, when the beads or the DNA has just made contact with the upper surface, the stage is fixed. At this time, the sample DNA is held in a space that is 0.1 μm or less from the upper surface. Even when conducting the polymerase reaction described subsequently, the 1064nm laser and the electromagnet will continue to be operated to fix the beads at the two ends, and the λ DNA is captured.

Next, the phase-contrast microscope mode is switched to a fluorescence microscope mode, the beam splitter 30 indicated in Fig. 10 is placed in the no-mirror condition, and all fluorescence is input to a cooled CCD camera 39. The environment in which the microscope is placed is put into a darkroom state so that no light other than laser beams enters the cell. As diagrammed in Fig. 14, a synthetic primer that is an oligonucleotide that is 20 bases long and complementary to the base sequence of the oligonucleotide 47 introduced at the 5' end of the sample DNA is introduced together with buffer solution into the cell. The temperature of the cell is set at 30°C, and the primer is complementary-chain bound to the 5' end of the sample DNA. Following upon that, the cell temperature is set to 37°C, and a mixed buffer solution of the four types of chemically modified nucleotide

diagrammed in Figs. 16 to 19 and Sequenase Version 2.0 T7 DNA polymerase (made by Amersham) is introduced into the cell. This mixed buffer solution is made to slowly and continuously flow in one direction, keeping the environment for the sample DNA always that of a fresh composition. The concentration of all four types of chemically modified nucleotide is 100 nM, and the polymerase concentration is 100 units/L, both of which concentrations are approximately 1/1000 that of a customary polymerase reaction. The Sequenase Version 2.0 marketed by Amersham exhibits extremely high complementary-chain elongation capability, with an elongation speed of 300 base length/second. Even with a low nucleotide concentration of 100 nM or lower, adequate incorporation is observed, and nucleotide analogs such as chemically modified nucleotides are incorporated very well. The polymerase [takes] * the base next after the primer and causes the incorporation of one base only that is complementary to the base sequence of the sample DNA from the four types of chemically modified nucleotides. *[flawed syntax in source]

488nm and 532nm laser beams are output for 1 second, and a 1-second exposure is implemented with the cooled CCD camera 34 synchronized with that laser irradiation. The laser beam 21 is perfectly reflected by the upper surface inside the cell 25, inducing evanescent irradiation within a range of 150 nm or less from the surface inside the cell. Since the sample DNA is captured within a range of 100 nm from the upper surface inside the cell, one chemically modified nucleotide incorporated by the polymerase reaction is subjected to excitation. Chemically modified nucleotides other than that one move around vigorously due to Brownian motion and will not be detected even should they somehow be subjected to evanescent irradiation. As to the image(s) detected by the cooled CCD camera 34, for the entire field of view that is $51.2 \times 51.2 \mu\text{m}$, a first to a fourth channel 61 to 64 are displayed on a monitor 60, as diagrammed in Fig. 24 [source syntax ambiguous]. The images (bright spots) 65, 67, 69, and 71 of the first to fourth channel corresponding to one emitted light point for one incorporated chemically modified nucleotide are automatically recognized and detected.

With the continuous measurements following thereafter, as diagrammed in Fig. 24, only the brightness information for the areas in the vicinity of those four points, that is, for the measurement areas 66, 68, 70, and 72 for the first to fourth channel images, are transferred to the computer, recorded, and subjected to analysis. The four bright spots 65, 67, 69, and 71 change their positions a little at a time with the passage of time, but the four bright spots which change their positions are automatically followed by the computer. The ratio of fluorescence intensity of the four bright spots measured after undergoing image division in correspondence with the emitted fluorescence from one spot will clearly vary according to the spectrum of the fluorophore that has emitted light, that is, according to the fluorophore type, and, if the fluorophores are of the same type, the fluorescence intensity ratio for the four bright spots will be constant. As a consequence, the fluorophore type can easily be identified from the fluorescence intensity ratio measured for the four bright spots. Then, irradiation is performed with a 355nm pulse-oscillation UV laser beam for 10 nanoseconds. This laser beam is perfectly reflected by the upper surface inside the cell, inducing evanescent irradiation within a range of 150 nm or less from the surface in the interior of the cell. Because the sample DNA is captured within a range of 100 nm or less from the upper surface inside the cell, the one chemically modified nucleotide incorporated by the

polymerase reaction will be subjected to UV irradiation, and caged substance to which a fluorophore has been *attached* [lit. *added*] will be released as diagrammed in Fig. 8. Chemically modified nucleotides other than that one will move around vigorously due to Brownian motion, and buffer solution is made to continually flow, so that, even should they somehow be accidentally subjected to evanescent irradiation so that the caged substance to which the fluorophore has been *attached* is released, the released nucleotides will not be incorporated by the polymerase reaction. Following this, by means of polymerase, the next chemically modified nucleotide, one only in number, is incorporated into the complementary chain. Thereafter, the following step is repeated with a certain time interval. Thereupon, 0.4 second after the 355nm pulse oscillation, irradiation with 488nm and 532nm laser beams is implemented, and, synchronously therewith, a 0.5-second exposure is made with the cooled CCD camera, whereupon the type of the fluorophore of the terminal chemically modified nucleotide incorporated by complementary-chain elongation is identified. Then, 0.1 second after the irradiation by the 488nm and 532nm lasers is finished, a 355nm UV laser is caused to pulse oscillate, the caged substance having *attached* thereto the fluorophore of the terminal chemically modified nucleotide incorporated by complementary-chain elongation is released, and the next chemically modified nucleotide is incorporated. This cycle [lasting] a total of 1.0 second, inclusive of the time for transferring data to the computer, is repeated. Fluorescence intensity information for the four spots is transferred to the computer during the 0.5 second when no exposure is being made. The fluorophore type is identified instantaneously from the fluorescence intensity ratios for the four spots and the base type is determined. The results of this determination are output to the monitor in real time. The cycle wherein one base is determined every second is repeated, and the determination of the entire base sequence of the λ DNA that is 48502 bases long can be accomplished in 48502 seconds, that is, in approximately 13.5 hours.

(Embodiment 2)

An example is now described wherein the base sequences of 10 types of DNA sample are determined simultaneously on a large scale. Synthetic oligonucleotides having a known base sequence at either end are introduced, by ligation, independently, to each of 10 types of DNA sample having a length of 100k bases, and biotin is introduced at the 3' end by the same method as that used in embodiment 1. A known base sequence having a length of 50 bases is introduced on the 5' end, but that base sequence is changed by the 10 types of DNA sample. Meanwhile, streptavidin is introduced, by acid amide bonding, to the outer surface of magnetized polystyrene beads each having a diameter of approximately 1 μ m. Each DNA sample subjected to ligation is denatured to a single-stranded state, the magnetic beads are added, and a magnetic bead is bound to the 3' end of each DNA sample. Measurement is done using the same apparatus as in embodiment 1. As is described next, 10 types of single-stranded oligonucleotide are bound locally to the lower surface inside the cell. On the glass surface of the cell, within a range of $51.2 \times 51.2 \mu$ m that is the field of view of the microscope, as diagrammed in Fig. 25, to each of minute sample *capture* areas 73 to 82 at ten locations for capturing a first to a tenth sample DNA, [respectively,] single-stranded synthetic oligonucleotides 83 to 93 [each] having a length of 50 bases and complementary to the known base sequences introduced at the 5' ends of the first to tenth

sample DNA are bonded [source syntax ambiguous]. The sample capture areas measure $1 \times 1 \mu\text{m}$, and are linearly aligned at an interval of $5 \mu\text{m}$. Each type of sample at the 5' end has a different base sequence. The first to tenth sample DNA, to each whereof has been introduced a magnetic bead, at the 3' end, are introduced into the cell in a mixed condition. The cell temperature is set at 25°C , and each sample DNA is complementary-chain bound to an oligonucleotide having a complementary base sequence. Sample DNA not complementary-chain bound is eliminated from the field of view with the flow of buffer solution. One or a number of sample DNAs are bound to each sample capture* area. Next, a magnetic field is applied in the horizontal direction, a magnetic force is made to act on the magnetic bead bound to the 3' end of the sample DNA, and, as diagrammed in Fig. 26, the first to tenth sample DNA 93 to 102 are stretched out. The stretching force is controlled by varying the size of the magnetic field. When a DNA sample having a length of 100K bases is stretched out, the length thereof becomes approximately $34 \mu\text{m}$, and the 3' end of the sample DNA is accommodated in the $51.2 \mu\text{m}$ [range] of the field of view.

The temperature of the cell is set to 65°C , but, because the complementary-chain binding of the oligonucleotide binding to the sample DNA is long, with a length of 50 bases, the complementary-chain binding will be maintained even at 65°C . A mixed buffer solution of the four types of chemically modified nucleotide diagrammed in Figs. 27 to 30 (with the fluorophores indicated by abbreviations) and Ampli Taq DNA polymerase (made by Takara Shuzo Co., Ltd.) are introduced into the cell. With the chemically modified nucleotide diagrammed in Fig. 21, unlike in the chemically modified nucleotides diagrammed in Figs. 16 to 19, the position of labeling to the nucleotide of the caged substance to which the fluorophore has been *attached* [lit. *added*] is changed from a base to the ribose carbon 3' position, but incorporation into a complementary chain by polymerase is nevertheless possible as when using the chemically modified nucleotides diagrammed in Figs. 16 to 19. When a caged substance to which a fluorophore has been *attached* according to the photoreaction diagrammed in Fig. 1 is released, however, by UV irradiation, the nucleotide will become equivalent to the unmodified state. The four types of chemically modified nucleotide diagrammed in Figs. 27 to 30 can be prepared by organic synthesis in like manner as the chemically modified nucleotides diagrammed in Figs. 16 to 19. Figs. 27 to 30 diagram caged dATP, caged dCTP, caged dGTP, and caged dTTP, labeled, respectively, by Cy 3.0, Cy 3.5, Cy 5.0, and Cy 5.5 (made by Biological Detection System, Inc.). This mixed buffer solution containing these [nucleotides] is made to slowly and continuously flow in one direction, keeping the environment for the sample DNA always that of a fresh composition. Polymerase conducts a complementary-chain elongation reaction with [each of] the oligonucleotides bonded to the cell as a primer. The continuous fluorescence measurement that follows is the same as in embodiment 1 except for the following points.

For the fluorescence exciting laser beams, a 532nm YAG laser (20 mW) and a 633nm He-Ne laser (20 mW) are used. The interference filters corresponding to the four images, moreover, have, respectively, transmission bands with 70% or greater transmittance at 540 to 580 nm, 580 to 620 nm, 650 to 680 nm, and 680 to 730 nm, with transmittance outside of those transmission bands assumed to be 10^{-4} or less within a range of 300 to 1100 nm. The transmittance at the laser wavelengths of 355 nm, 532 nm, and 633 nm is assumed to be

10^{-6} or less. A thin metal film is vapor-deposited on the upper surface inside the cell, plasma waves are excited in the metal by perfect reflection of the laser beams, and near-field light is generated by surface plasmons inside the cell. This near-field light can be output with high intensity temperature on even more minute areas by a method using evanescent irradiation, and single-molecule measurement can be realized by higher-sensitivity fluorescence measurement.

In order to irradiate a sample DNA molecule with near-field light by surface plasmon resonance, in a 1-cycle 1.0-second continuous measurement step, a magnetic field is applied in the cell, oriented from below to above, during the 532nm and 633nm laser irradiation time period, the 355nm laser irradiation time period, and the time period between those, for a total of 0.7 second, whereupon the sample DNA is drawn to the upper surface inside the cell. During the remaining 0.3 second, a magnetic field is applied in the opposite direction, and the sample DNA is made to migrate away from the upper surface inside the cell, thus facilitating the incorporation of the next chemically modified nucleotide. Furthermore, the measurement points in the field of view will be 10 points corresponding to the ten samples, which are divided by four in the detected image to become 40 points, but the fluorescence intensity is sequentially recorded and analyzed while following those 40 points with detected images. The ten types of sample DNA are completely separated spatially, as diagrammed in Fig. 26, so they can be independently measured. Depending on the sample DNA, a plurality of molecules will be captured, so fluorescence from a plurality of molecules will be detected. However, because fluorescence is always detected from the same fluorophore, all that will happen from the perspective of measurement is that the fluorescence intensity will increase, which poses no problem at all. Continuing on from the continuous measurement described above, one base determination will be made in one second for one sample, and the entire base sequence for ten samples [each] having a length of 100k bases, totalling 1M bases in length, can be determined in 100k seconds, which is approximately one day.

(Embodiment 3)

In this embodiment, the intracellular distribution of mRNA is measured. Approximately 10,000 types of mRNA that are expressed in certain types of cells are extracted, and then introduced into a fluorescence cell set in place under an fluorescence microscope. In the measurement apparatus used in this embodiment, the measurement apparatus used in embodiment 2 is altered in the following respects. The excitation laser is changed from two lasers, namely the 532nm YAG laser and 633nm He-Ne lasers, to a single 488nm Ar ion laser. Four types of interference filters combined with a quadruple-division prism have transmission bands with a transmittance of 70% or greater at 500 to 540 nm, 540 to 570 nm, 570 to 590 nm, and 590 to 730 nm respectively, with the transmittance outside of those transmission bands assumed to be 10^{-4} within a range of 300 to 1100 nm. The transmittance at the laser wavelengths of 355 nm and 488 nm, moreover, is assumed to be 10^{-6} or less. Chemically modified nucleotides having the structures diagrammed in Figs. 31 to 34 are used, but with these, unlike with the chemically modified nucleotides diagrammed in Figs. 27 to 30, each nucleotide is labeled with two fluorophores. In Figs. 31 to 34, a caged dATP, caged dCTP, caged dGTP, and caged dTTP, respectively, are diagrammed, with FAM, JOE, TAMRA, and ROX (made by Applied Biosystems, Inc.) labels, respectively, at the carbon

3 position in the nitrophenyl group bound to the ribose carbon 3' position, and all having FAM labels at the carbon 5 position in the nitrophenyl group. Energy transfer is induced inside the same nucleotide between FAM and JOE, FAM and TAMRA, and FAM and ROX. As a consequence, not only FAM, but JOE, TAMRA, and ROX also can be excited with high efficiency by the 488nm Ar ion laser. Every fluorophore is also bound to a nucleotide through a nitrophenyl group, and is released from the nucleotides by irradiation with a 355nm UV pulse laser. Impurities such as unmodified nucleotides are removed with a column or the like.

The objective lens is an oil-immersed lens having a power of 100 \times to 60 \times (Plan-Apochromat \times 60, 1.40 NA, made by Olympus Co., Ltd.). The photographic lens immediately in front of the camera is a lens having a power of 2.5 \times to 1.67 \times (NFK \times 1.67, made by Olympus Co., Ltd.). The cooled two-dimensional CCD camera used has a detection plane that is 35.8 \times 35.8 mm, configured by an array of 1024 \times 1024 pixels each of which measures 35 \times 35 μ m. The image magnification at the image formation plane is 60 \times 1.67, that is, 100 \times , so one pixel corresponds to 35 \times 0.35 μ m. An image in the 179 \times 179 μ m field of view is divided into four by a quadruple-division prism inserted in the light path, and the four [resulting] images, divided to a size of 17.9 \times 17.9 mm, are formed into images having a size of 35.8 \times 35.8 mm so as not to mutually overlap in the detection plane, as diagrammed in Fig. 13. In order to enhance the intensity and uniformity of the irradiation with the 488nm and 355nm laser beams within the field of view of the microscope, a multiple reflection is used which perfectly reflects the laser beams, repeatedly, in the interior of the prism, as diagrammed in Fig. 35. As diagrammed in Fig. 36, the laser beams may be introduced into an optical fiber 103, and perfectly reflect, repeatedly, in the interior of the fiber, and measurements made using the near-field light in the vicinity of the outer surface of the fiber. If multiple fibers are bundled and measurements carried out simultaneously, the measurement area can be enlarged. The 5' end of a single-stranded synthetic oligonucleotide comprised of poly-T labeled with FAM can be bound substantially uniformly to the lower surface inside the cell within the field of view of the microscope at an average ratio of one per 10 \times 10 μ m area. The binding positions in the field of view can be verified as fluorescence emission spots by irradiation with a 488nm laser. The number of emission spots within the field of view, that is, the number of poly-T bonds, is approximately 330. The emission spots can be mutually and spatially separated and detected, and the fluorescence thereof can be independently measured. A total of 1320 coordinates are recorded on the detection plane for 330 emissions spots divided quadruply, which are made measurement points in fluorescence measurements thereafter. Irradiation is performed continuously with high-power 488nm laser beams, decomposing the label FAM and causing it to fade.

Because the mRNA introduced into the cell has a poly-A portion, that poly-A portion binds complementarily to fixed poly-T. Because this complementary chain binding occurs completely randomly, the composition of the mRNA complementary-chain bound to the 330 poly-Ts, within the field of view that can be measured simultaneously, reflect the intracellular frequency of occurrence and distribution. As a consequence, if the 330 bound mRNAs can be identified, it is then possible to find out what kinds of, and how many, mRNAs are manifesting inside the cells. This identification, in this embodiment, is

accomplished by directly determining the base sequences of the mRNAs, and then making reference to an existing database. When conducting a cDNA elongation reaction using reverse transcriptase and the poly-T as a primer, direct base sequence determination is performed, with mRNA as the template, using the chemically modified nucleotides diagrammed in Figs. 31 to 34 in the substrate, by the same method as in embodiment 2. Needless to say, base sequence determination can be performed by synthesizing cDNA beforehand and making that the template. mRNA has a length ranging from 1k to several k bases, and the length thereof when stretched out is 1 μm or less. As a consequence, during a complementary-chain elongation reaction, 330 emission spots will be mutually separated by an average distance of 10 μm , so there will be no mutual overlap or interference. The relative positional relationship between poly-A and poly-T complementary chain binding, that is, the priming positions of the complementary-chain elongation reaction, will differ randomly according to the 330 measurement positions. However, the signals obtained from the measurement positions constitute information obtained from single mRNA molecules, so the indeterminacy of this priming site will not be a problem. This is a major advantage of base sequence determination methods that use single molecules. The base sequences of 330 kinds of mRNA can be decoded at a speed of one base per second, whereupon these base sequences can be determined over their entire lengths within 30 minutes or less. The base sequence information so obtained is compared to an existing database, whereupon what kind of protein is coded by the gene is determined, and, simultaneously, the frequency distribution thereof is displayed. The number 330 is thought to be sufficient as a sampling number, but, if necessary, the sampling number may be increased and the measurement described above repeated.

In this embodiment, poly-T fixed in the cell was used as the primer, but any oligonucleotide which contains a base such as inosine, 3-nitropyrrole, or 5-nitroindole that can be complementary-chain bound with all of the four bases (adenine, cytosine, guanine, and thymine) may be used as a primer as well. Such an oligonucleotide can complementarily bind at any position on a template nucleic acid and complementary-chain elongation can be performed with that position as the starting point. As when using poly-T as a primer, the indeterminacy of this starting point is not a problem in this embodiment wherewith base sequence information can be obtained from a single molecule of the template nucleic acid. Furthermore, although mRNA base sequence determination has been described for this embodiment, when nothing more than mRNA distribution measurements are conducted, it will be sufficient to determine base sequences having a few tens of bases or fewer. When a base sequence is obtained that is not registered in a database, that constitutes the discovery of a new gene. Besides the embodiments described in the foregoing, with the method of the present invention, the behavior of polymerase and various other enzymes can be studied from elementary reactions, and enormous contributions can be made to chemistry, molecular biology, biophysics, medicine, and other fields. For example, the speeds of elementary reactions such as the polymerase complementary chain synthetic elongation reactions, that is, the speed whereby nucleotides or chemically modified nucleotides are incorporated can be measured, and high time-resolution capabilities can be obtained when conducting detailed investigations of how, for example, incorporation reaction rates are changed by the type of nucleotide, or how the

incorporation reaction rate is changed by the type of chemical modifier of a chemically modified nucleotide, or how these reaction rates are changed by the type of polymerase used in the complementary chain synthesis elongation reaction.

Based on the present invention, the base sequences of single DNA molecules having several hundred thousands of bases can be determined, the determination of large DNA base sequences, which conventionally has been very inefficient, requiring much time and effort, can be done quickly and easily, and preprocessing prior to base sequence determination can be very sharply curtailed.

Claims

1. A nucleic acid base sequence determination method for complementary-chain binding an oligonucleotide (8, 83 to 92) to a portion of a template nucleic acid (7, 44, 45, 93 to 102) at least a portion whereof is a single strand, performing a complementary chain synthesis elongation reaction (10) with said oligonucleotide as the starting point, and determining the nucleic acid base sequence of said template nucleic acid, having the steps of:

(a) binding said oligonucleotide to the single-stranded portion of said template nucleic acid;

(b) complementary-chain binding one chemically modified nucleotide (9), that has been chemically modified so that said complementary chain synthesis elongation reaction does not continue to advance, to the single strand of said template nucleic acid;

(c) performing a chemical reaction to alter the chemical structure of the nucleotide bound to said template nucleic acid, in a condition wherein said chemically modified nucleotide that is not bound to said template nucleic acid coexists, in order to produce a condition wherein the advance of said complementary chain synthesis elongation reaction is made possible; and

(d) repeating said step (b) and said step (c); wherein:

the alteration of the chemical structure of said chemically modified nucleotide bound to the single strand of said template nucleic acid is controlled; and the base sequence of said nucleic acid template is sequentially determined one base at a time.

2. The nucleic acid base sequence determination method according to claim 1, wherein the alteration of said chemical structure in said step (c) is performed by a photoreaction.

3. The nucleic acid base sequence determination method according to claim 1, wherein said chemically modified nucleotide is a caged compound.

4. The nucleic acid base sequence determination method according to claim 1, wherein said chemically modified nucleotide is a 2-nitrobenzyl derivative.

5. The nucleic acid base sequence determination method according to claim 1, wherein said chemically modified nucleotide is a chemically modified nucleotide that is at least chemically modified at the ribose carbon 3 position.

6. The nucleic acid base sequence determination method according to claim 1, wherein said chemically modified nucleotide is a nucleotide that is at least chemically modified at a base position.

7. The nucleic acid base sequence determination method according to claim 1, wherein at least one fluorophore is bound to said chemically modified nucleotide.

8. The nucleic acid base sequence determination method according to claim 1, wherein: at least one fluorophore is bound to said chemically modified nucleotide; and said fluorophore is different for each type of base in said chemically modified nucleotide.

9. The nucleic acid base sequence determination method according to claim 1, wherein: at least one fluorophore is bound to said chemically modified nucleotide; and at least one of said fluorophore is released from said chemically modified nucleotide by said chemical reaction.

10. A measurement method for measuring the reaction rate of a polymerase complementary chain synthesis elongation reaction that is a method for complementary-chain binding an

oligonucleotide (8, 83 to 92) to a portion of a template nucleic acid (7, 44, 45, 93 to 102) at least a portion whereof is a single strand, performing a complementary chain synthesis elongation reaction (10) with said oligonucleotide as the starting point, and measuring the reaction rate of the polymerase complementary chain synthesis elongation reaction, having the steps of:

(a) binding said oligonucleotide to the single-stranded portion of said template nucleic acid;

(b) complementary-chain binding one chemically modified nucleotide (9), that has been chemically modified so that said complementary chain synthesis elongation reaction does not continue to advance, to the single strand of said template nucleic acid;

(c) performing a chemical reaction to alter the chemical structure of the nucleotide bound to said template nucleic acid, in a condition wherein said chemically modified nucleotide that is not bound to said template nucleic acid coexists, in order to produce a condition wherein the advance of said complementary chain synthesis elongation reaction is made possible; and

(d) repeating said step (b) and said step (c); wherein:

the alteration of the chemical structure of said chemically modified nucleotide bound to the single strand of said template nucleic acid is controlled; and the reaction rate of the polymerase complementary chain synthesis elongation reaction is measured.

11. A nucleic acid base sequence determination method for complementary-chain binding an oligonucleotide (8, 83 to 92) to a portion of a template nucleic acid (7, 44, 45, 93 to 102) at least a portion whereof is a single strand, performing a complementary chain synthesis elongation reaction (10) with said oligonucleotide as the starting point, and determining the nucleic acid base sequence of said template nucleic acid, having the steps of:

(a) binding said oligonucleotide to the single-stranded portion of said template nucleic acid;

(b) complementary-chain binding one chemically modified nucleotide (9) wherein four types of nucleotide have been chemically modified so that said complementary chain synthesis elongation reaction does not continue to advance, to the single strand of said template nucleic acid;

(c) performing a photochemical reaction to alter the chemical structure of the nucleotide bound to said template nucleic acid, in a condition wherein said chemically modified nucleotide that is not bound to said template nucleic acid coexists, in order to produce a condition wherein the advance of said complementary chain synthesis elongation reaction is made possible; and

(d) repeating said step (b) and said step (c); wherein:

the alteration of the chemical structure of said chemically modified nucleotide bound to the single strand of said template nucleic acid is controlled; and the base sequence of said nucleic acid template is sequentially determined one base at a time.

12. A nucleic acid base sequence determination method [comprising] the steps of:

(a) binding one caged nucleotide (9) to which a fluorescent label has been added, to one strand (7, 44, 45, 93 to 102) of a template nucleic acid;

(b) exciting said fluorescent label by laser irradiation (1, 21), measuring the fluorescence emitted, and determining the base type;

(c) releasing said fluorescent label from said caged nucleotide bound to said template nucleic acid, by UV irradiation (2), in a condition wherein said caged nucleotide that is not bound to said template nucleic acid coexists; and

(d) repeating said steps (a) to (c); [and also the steps of:]

releasing said fluorescent label, after having sequentially bound caged nucleotides, to which said fluorescent label has been added, one at a time, to said template nucleic acid; and

sequentially determining the base sequence of said nucleic acid template one base at a time.

13. A nucleic acid base sequence determination method [comprising] the steps of:

(a) binding one chemically modified nucleotide (9) that has been chemically modified to one strand (7, 44, 45, 93 to 102) of a template nucleic acid;

(b) detecting said bound chemically modified nucleotide;

(c) releasing said chemical modification from said chemically modified nucleotide bound to said template nucleic acid, in a condition wherein said chemically modified nucleotide that is not bound to said template nucleic acid coexists; and

(d) repeating said steps (a) to (c); [and also the steps of:]

releasing said chemical modification, after having sequentially bound said chemically modified nucleotides, one at a time, to said template nucleic acid; and

sequentially determining the base sequence of said nucleic acid template one base at a time.

14. The nucleic acid base sequence determination method according to claim 13, wherein said chemically modified nucleotide is a caged nucleotide to which a fluorescent label has been added.

15. A nucleic acid base sequence determination method wherein:

one chemically modified nucleotide (9) that has been chemically modified is bound to one strand of a template nucleic acid (7, 44, 45, 93 to 102); and

said chemical modification is released from said chemically modified nucleotide bound to said template nucleic acid, in a condition wherein said chemically modified nucleotide that is not bound to said template nucleic acid coexists, and the base sequence of said nucleic acid template is sequentially determined one base at a time.

16. The nucleic acid base sequence determination method according to claim 15, wherein said chemically modified nucleotide is a caged nucleotide to which a fluorescent label has been added.

17. A nucleic acid base sequence determination apparatus for complementary-chain binding oligonucleotides (8, 83 to 92) to a portion, or portions, of a template nucleic acid (7, 44, 45, 93 to 102) at least a portion whereof is a single strand, performing a complementary chain synthesis elongation reaction (10) with said oligonucleotides as the starting points, and determining the nucleic acid base sequence of said template nucleic acid, having, in order to detect, after binding said oligonucleotides to the single-stranded portion of said template nucleic acid, one chemically modified nucleotide (9) that has been complementary-chain bound to said template nucleic acid, chemically modified so that said complementary chain synthesis elongation reaction does not continue to advance:

- a laser (1, 15, 16) for exciting fluorophores by which said chemically modified nucleotides are labeled;
- a lens (27) for condensing fluorescence emitted from said fluorophore;
- a filter (32) for selecting a wavelength of said fluorescence; and
- a photodetector (34) for detecting said fluorescence; [and further] having:
- a fluorescence microscope for detecting said fluorescence;
- analysis means for identifying the base type(s) of said chemically modified nucleotide from the output of said photodetector; and
- a light source (17) for generating, by a photochemical reaction, an alteration in the chemical structure of a chemically modified nucleotide bound to said template nucleic acid, in a condition wherein said chemically modified nucleotide that is not bound to said template nucleic acid coexists, in order to bring about a condition wherein the advance of said complementary chain synthesis elongation reaction is made possible; wherein:
- one said chemically modified nucleotide is complementary-chain bound to one strand of said template nucleic acid;
- the alteration of chemically modified nucleotides bound to said template nucleic acid is repeated;
- the alteration of the chemical structure of said chemically modified nucleotide bound to the single strand of said template nucleic acid is controlled;
- and the base sequence of said nucleic acid template is sequentially determined one base at a time.
18. The nucleic acid base sequence determination apparatus according to claim 17, wherein said laser performs evanescent irradiation.
19. The nucleic acid base sequence determination apparatus according to claim 17, wherein said laser performs surface plasmon resonant irradiation.
20. The nucleic acid base sequence determination apparatus according to claim 17, wherein the light source for said chemical reaction emits ultraviolet light or an ultraviolet laser beam.
21. The nucleic acid base sequence determination apparatus according to claim 17, wherein the light for said photochemical reaction is evanescent irradiation.
22. The nucleic acid base sequence determination apparatus according to claim 17, wherein the light for said photochemical reaction is surface plasmon resonant radiation.
23. The nucleic acid base sequence determination apparatus according to claim 17, [further] having: means capable of holding each of a plurality of types of said template nucleic acid at different positions within the field of view of said fluorescence microscope; and the base sequences of the plurality of types of said template nucleic acid, respectively, are simultaneously determined.
24. The nucleic acid base sequence determination apparatus according to claim 17, wherein base sequences at different sites on said template nucleic acid are simultaneously determined.
25. The nucleic acid base sequence determination apparatus according to claim 17, wherein said template nucleic acid is held in a solid-phase carrier.
26. The nucleic acid base sequence determination apparatus according to claim 17, wherein a plurality of locations on said template nucleic acid are bound to a solid-phase carrier.

27. The nucleic acid base sequence determination apparatus according to claim 17, wherein said template nucleic acid is held in a bead or beads (5, 6, 52, 53).

28. The nucleic acid base sequence determination apparatus according to claim 17, wherein: said template nucleic acid is held in a bead or beads (5, 6, 52, 53); and said bead or beads are held by a laser-based light trap.

29. The nucleic acid base sequence determination apparatus according to claim 17, wherein: said template nucleic acid is held in a magnetized bead (6, 53); and said bead is held by a magnetic force.

30. The nucleic acid base sequence determination apparatus according to claim 17, wherein said template nucleic acid is bound by complementary-chain binding to an oligonucleotide bound beforehand to a solid-phase carrier.

31. The nucleic acid base sequence determination apparatus according to claim 17, wherein said template nucleic acid is joined by an electrostatic force to a solid-phase carrier.

32. A measurement apparatus for measuring the reaction rate of a polymerase complementary chain synthesis elongation reaction that is a method for complementary-chain binding an oligonucleotide (8, 83 to 92) to a portion of a template nucleic acid (7, 93 to 102) at least a portion whereof is a single strand, performing a complementary chain synthesis elongation reaction (10) with said oligonucleotide as the starting point, and measuring the reaction rate of the polymerase complementary chain synthesis elongation reaction, having, in order to detect, after binding oligonucleotides to the single-stranded portion of said template nucleic acid, one chemically modified nucleotide (9) that has been complementary-chain bound to said template nucleic acid, chemically modified so that said complementary chain synthesis elongation reaction does not continue to advance:

- a laser (15, 16) for exciting fluorophores by which said chemically modified nucleotides are labeled;

- a lens (27) for condensing fluorescence emitted from said fluorophore;

- a filter (32) for selecting a wavelength of said fluorescence; and

- a photodetector (34) for detecting said fluorescence; [and further] having:

- a fluorescence microscope for detecting said fluorescence;

- analysis means for identifying the base type(s) of said chemically modified nucleotide from the output of said photodetector; and

- a light source (17) for generating, by a photochemical reaction, an alteration in the chemical structure of a chemically modified nucleotide bound to said template nucleic acid, in a condition wherein said chemically modified nucleotide that is not bound to said template nucleic acid coexists, in order to bring about a condition wherein the advance of said complementary chain synthesis elongation reaction is made possible; wherein:

- one said chemically modified nucleotide is complementary-chain bound to one strand of said template nucleic acid;

- the alteration of chemically modified nucleotides bound to said template nucleic acid is repeated;

- the alteration of the chemical structure of said chemically modified nucleotide bound to the single strand of said template nucleic acid is controlled;

- and the reaction rate of the polymerase complementary chain synthesis elongation reaction is measured.

Fig. 1

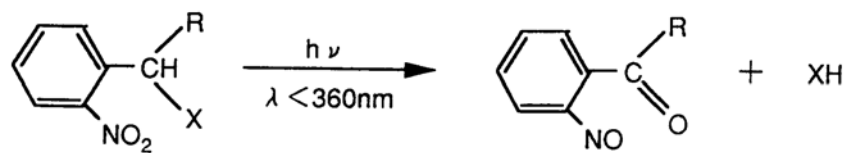


Fig. 2

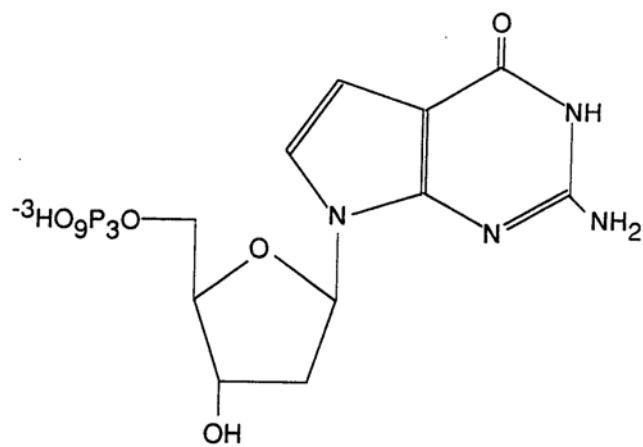


Fig. 3

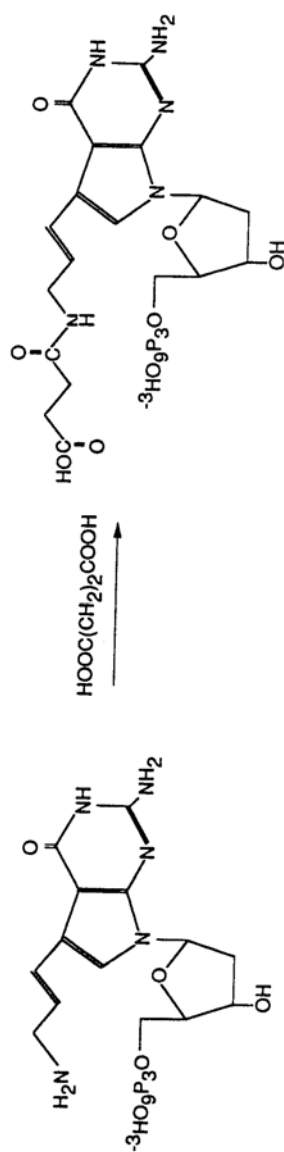


Fig. 4

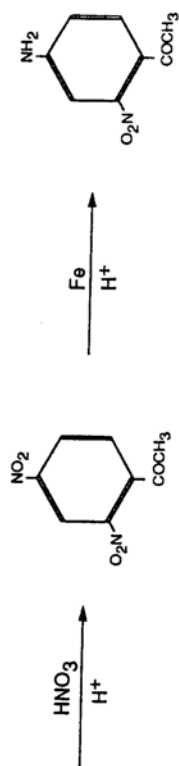


Fig. 5

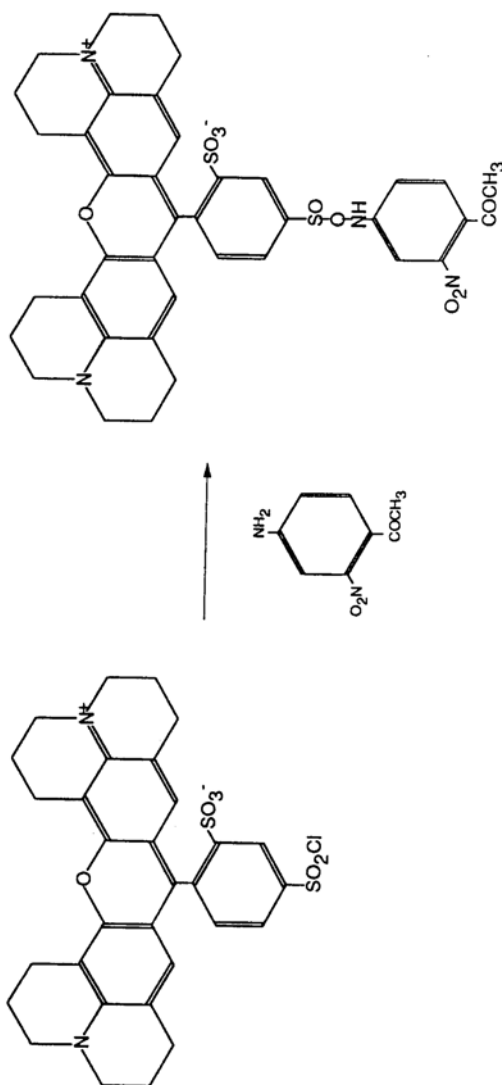


Fig. 6

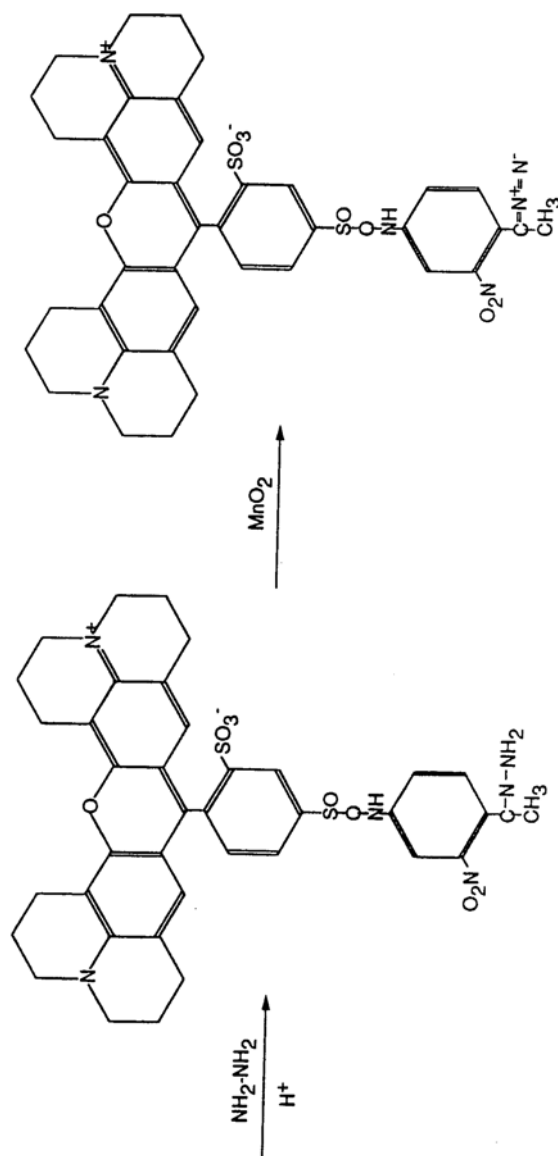


Fig. 5

Fig. 7

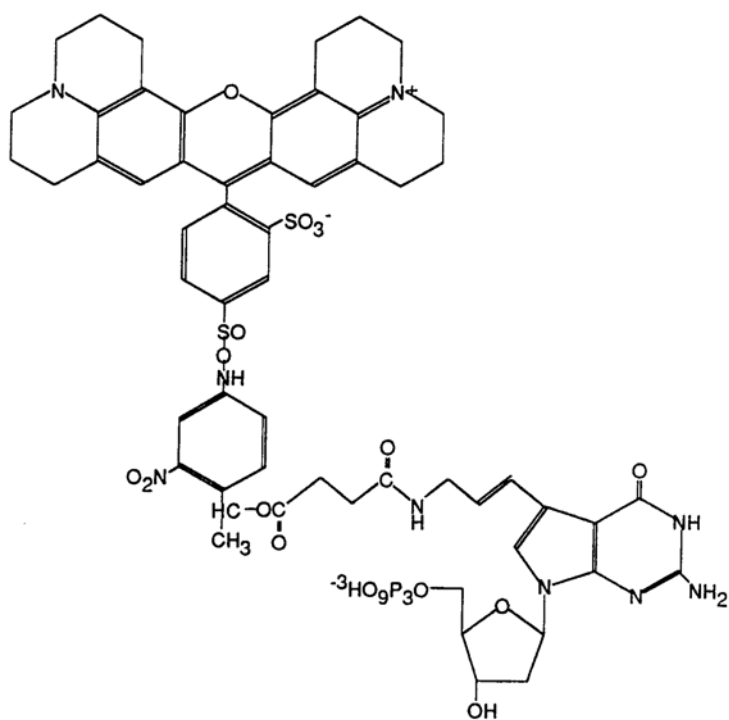


Fig. 8

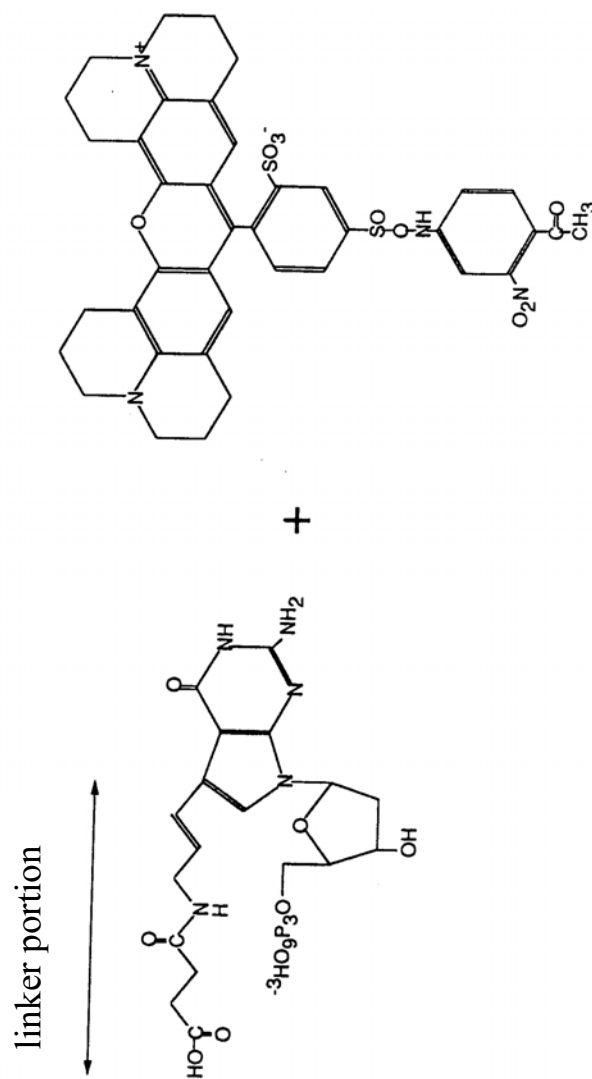


Fig. 10

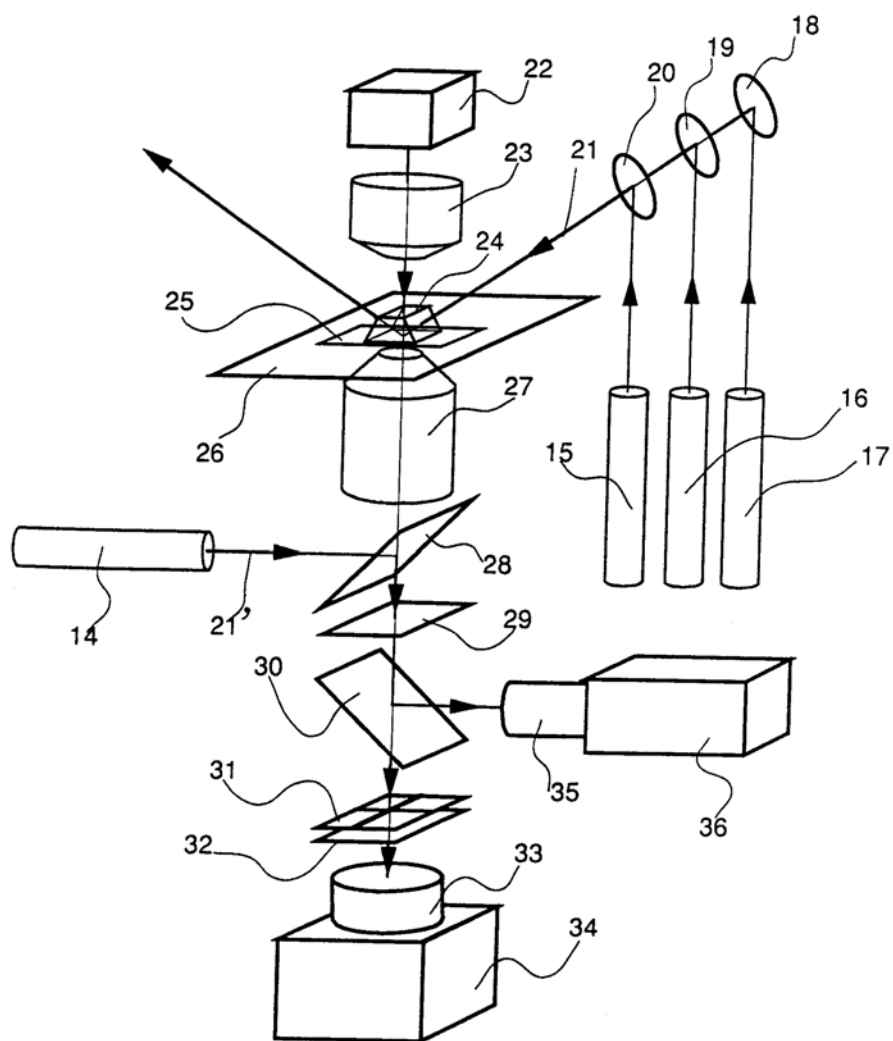


Fig. 11

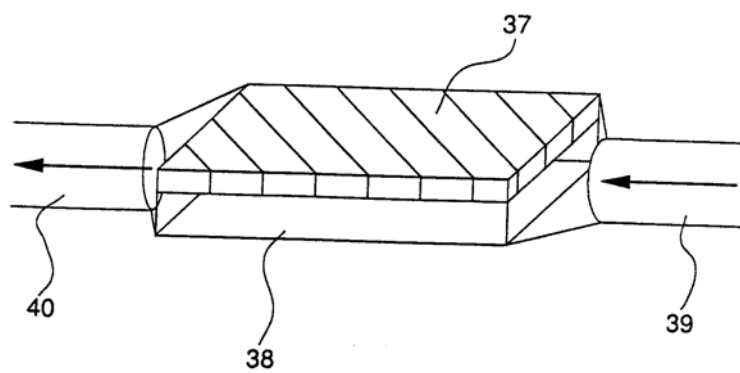


Fig. 12

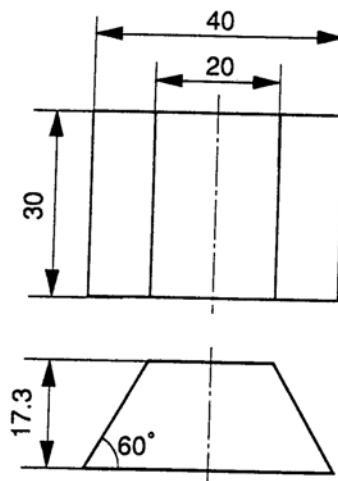


Fig. 13

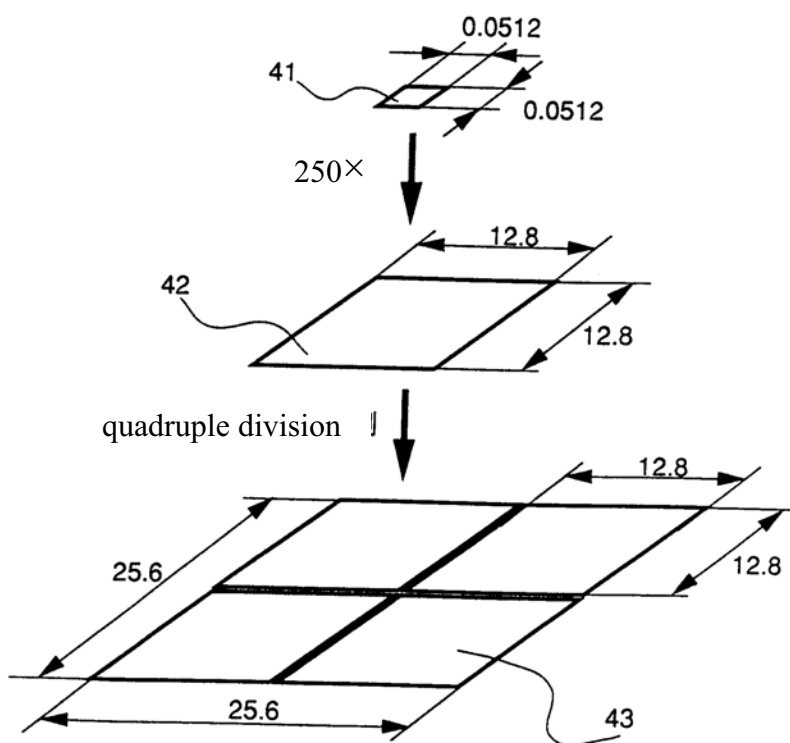


Fig. 14

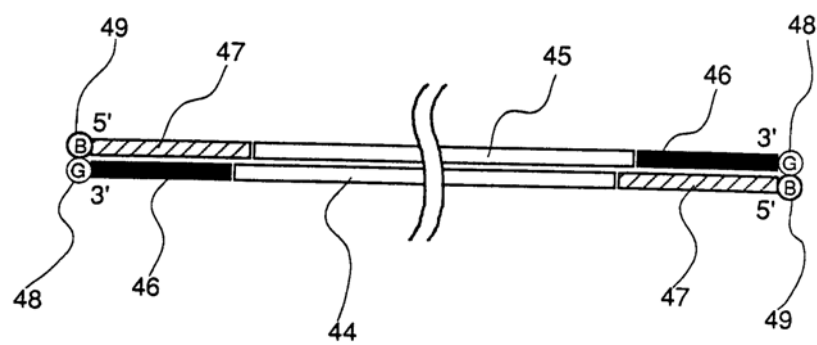


Fig. 15

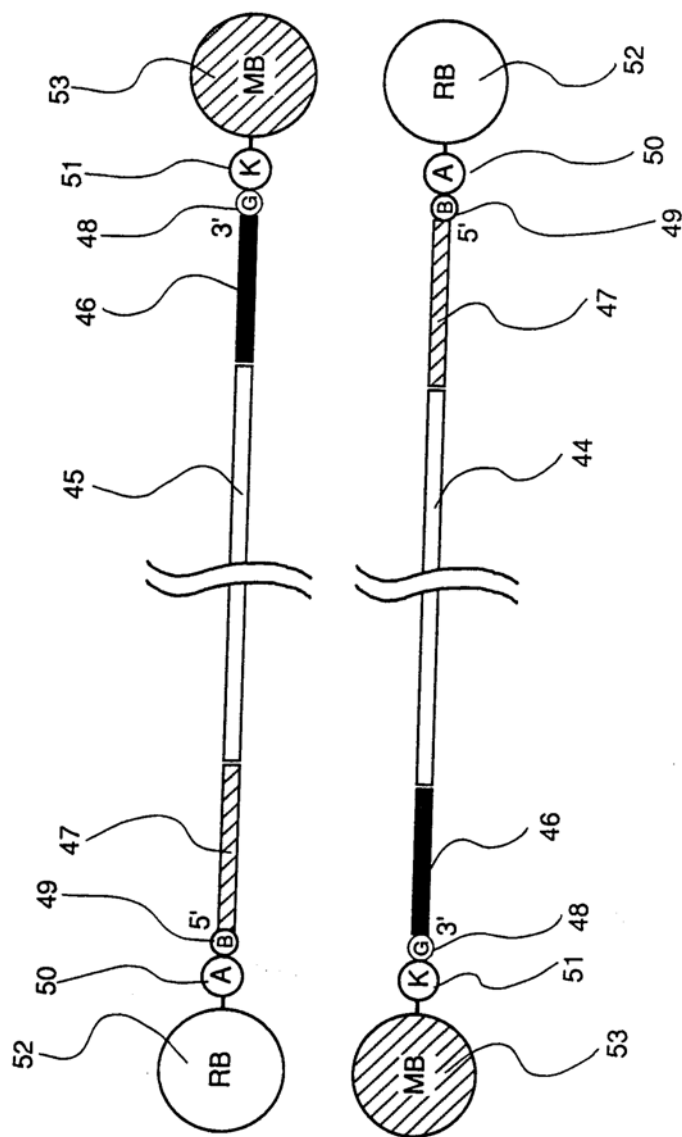


Fig. 16

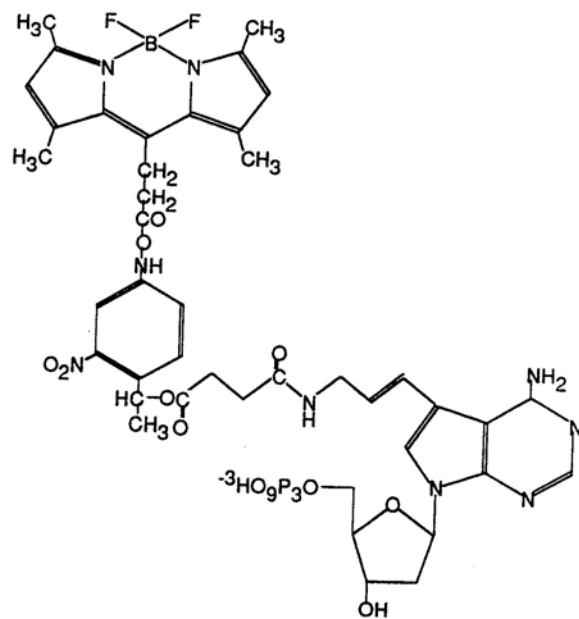


Fig. 17

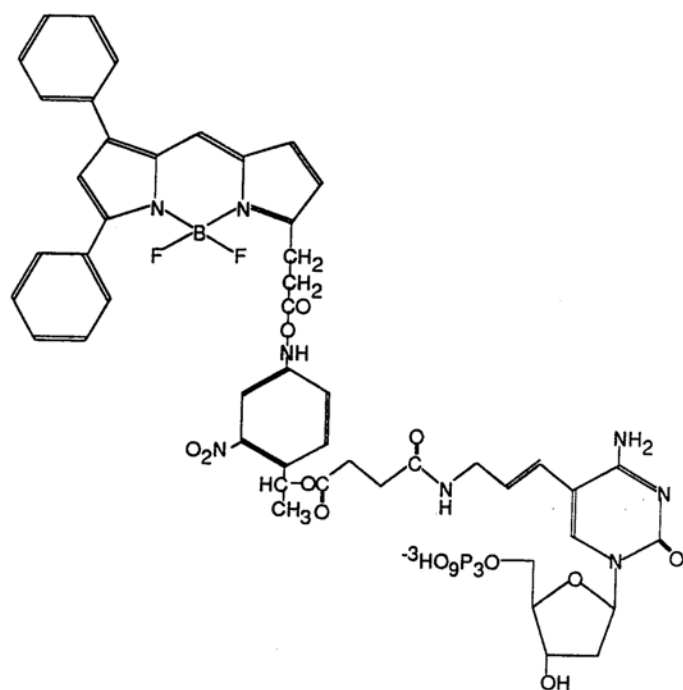


Fig. 18

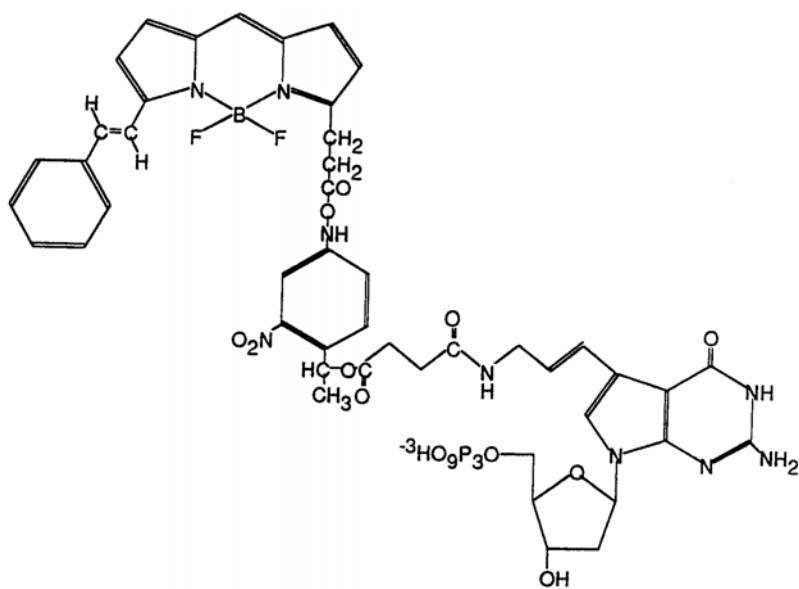


Fig. 19

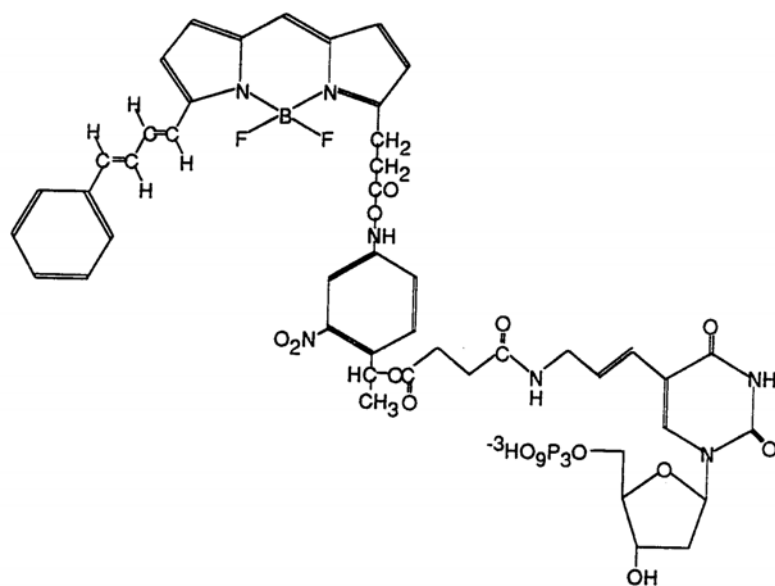


Fig. 20

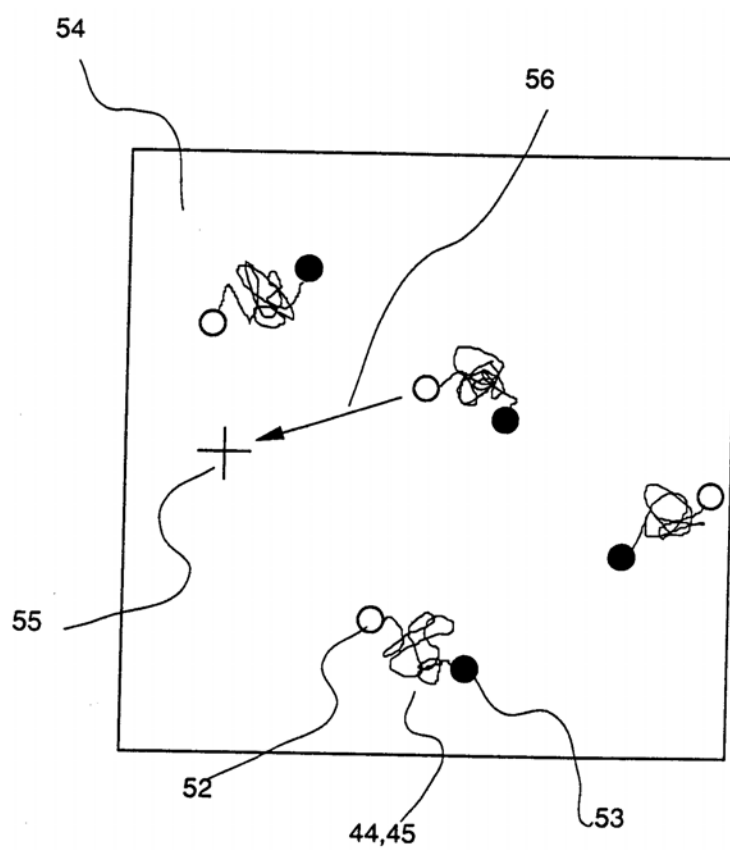


Fig. 21

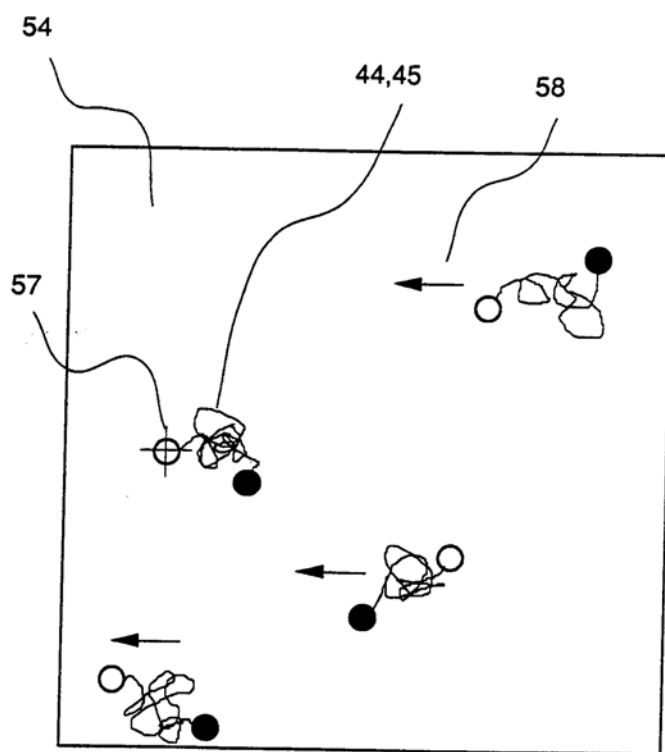


Fig. 22

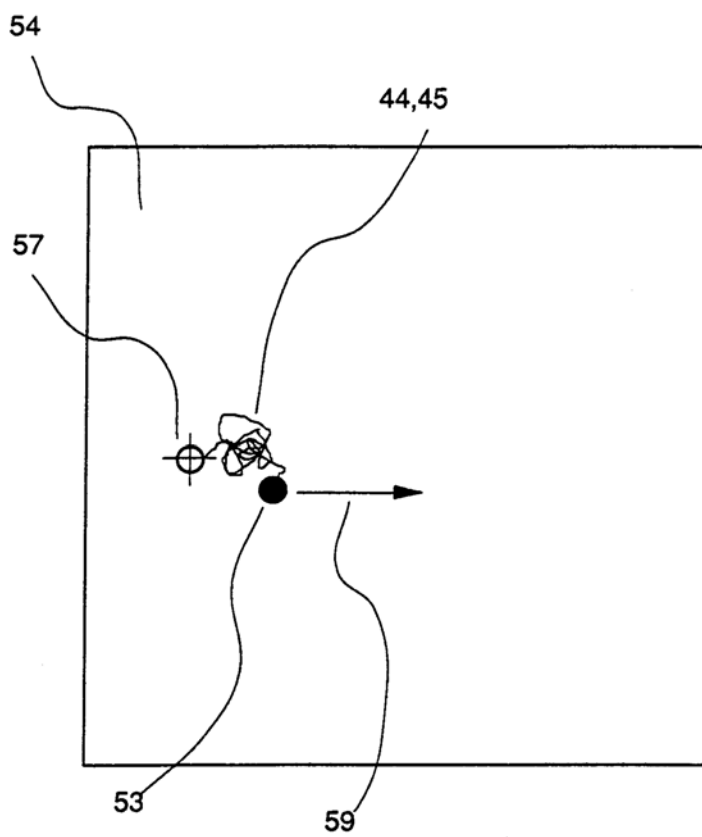


Fig. 23

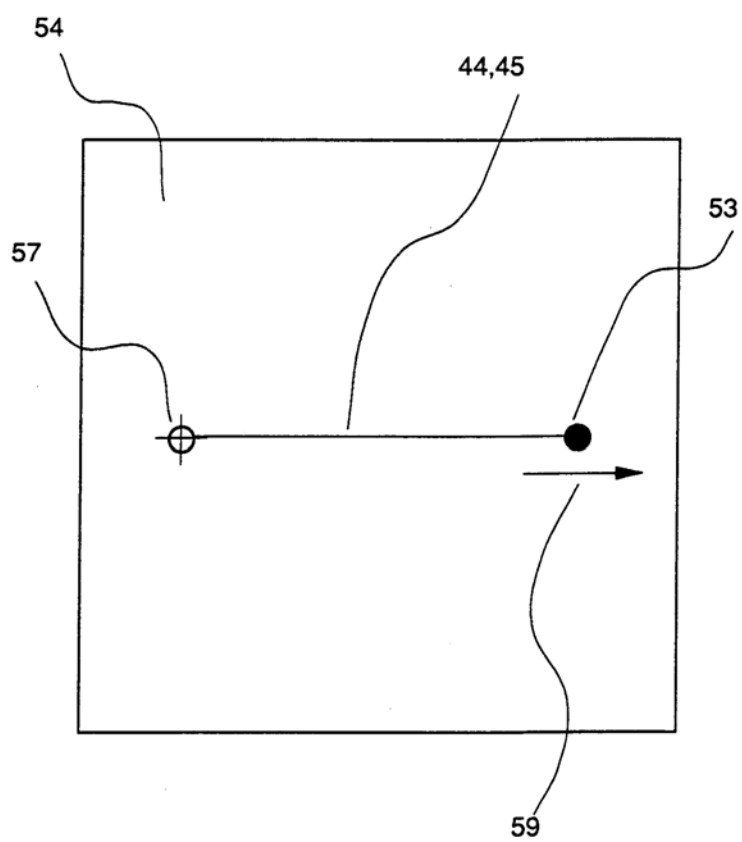


Fig. 24

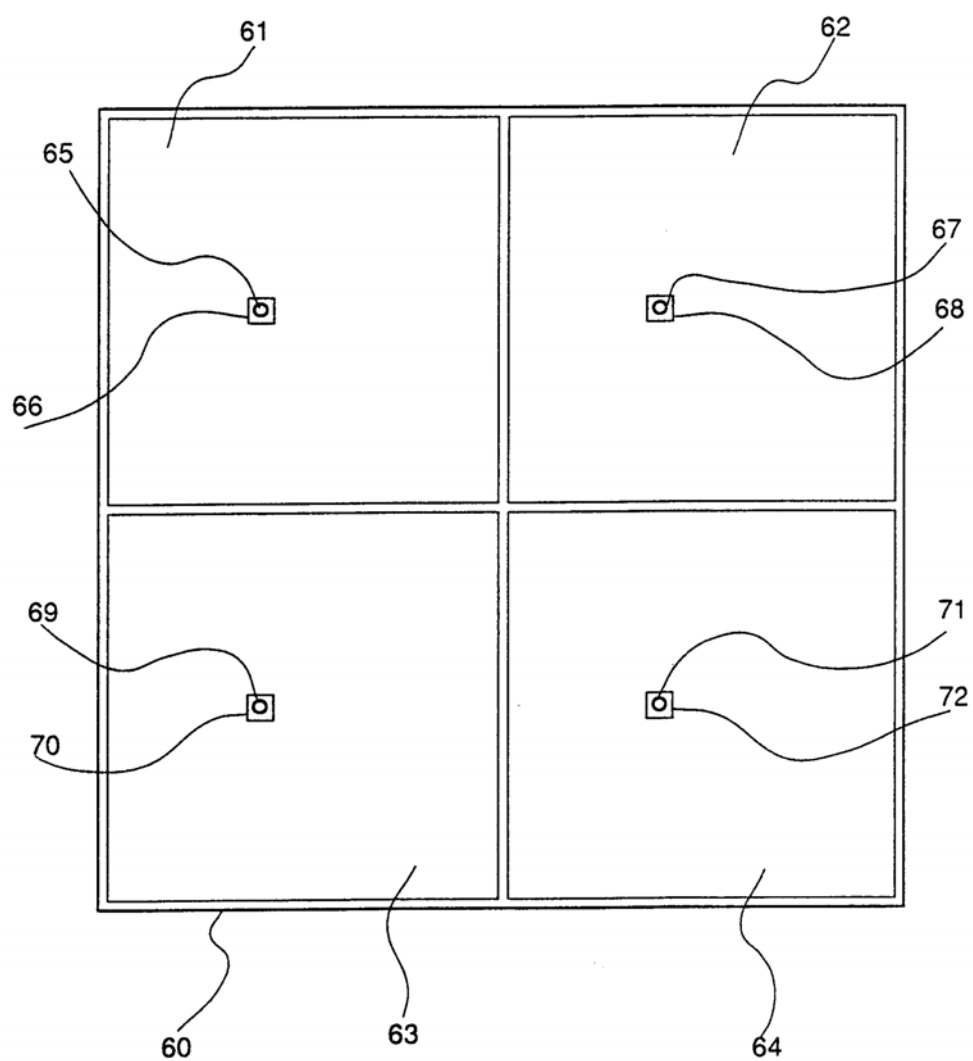


Fig. 25

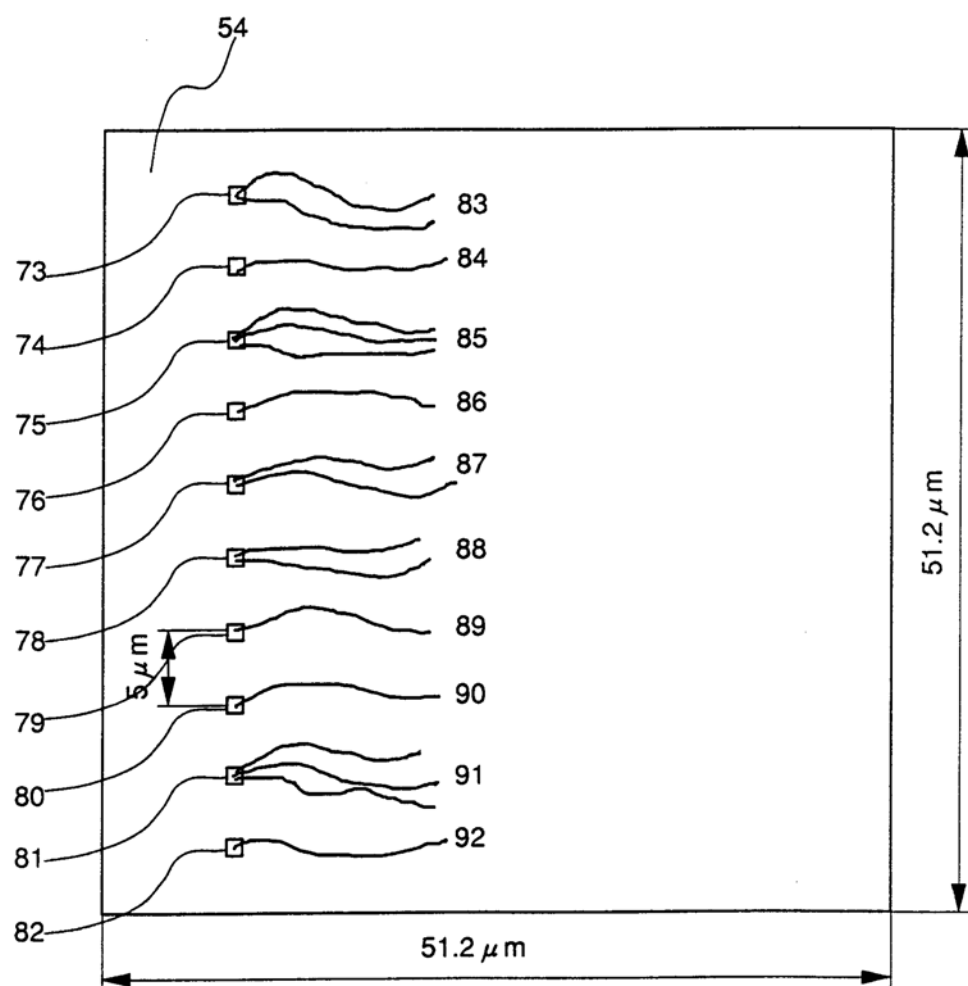


Fig. 26

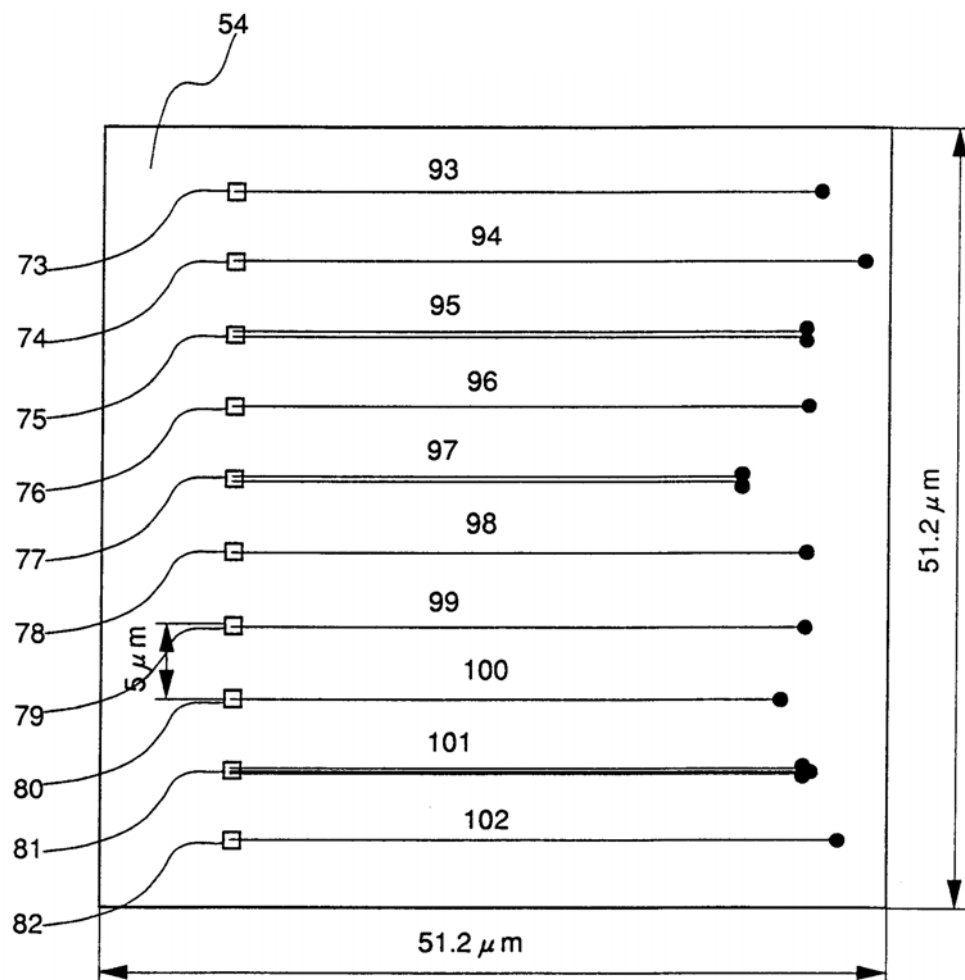


Fig. 27

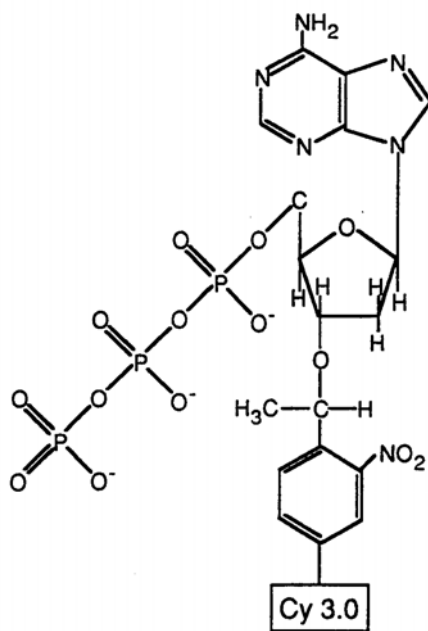


Fig. 28

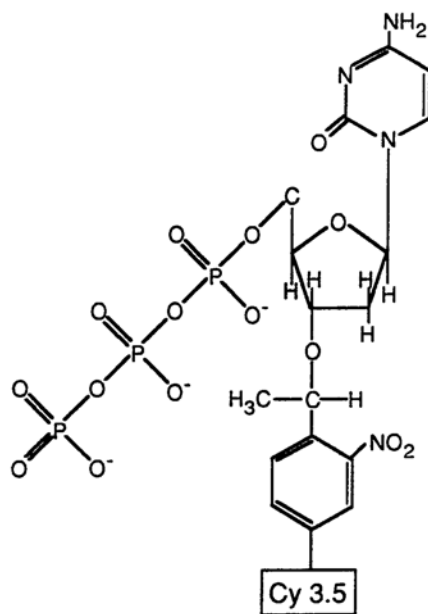


Fig. 29

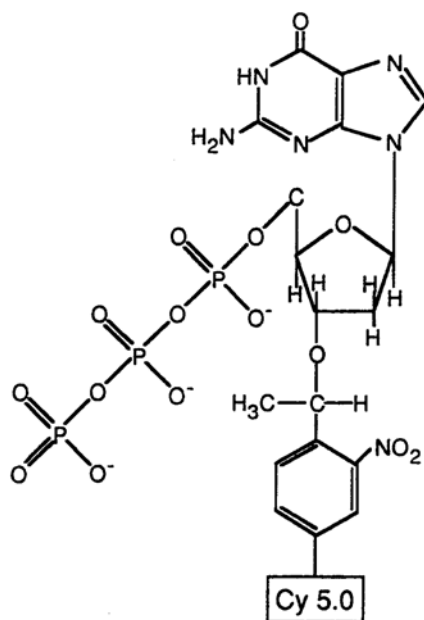


Fig. 30

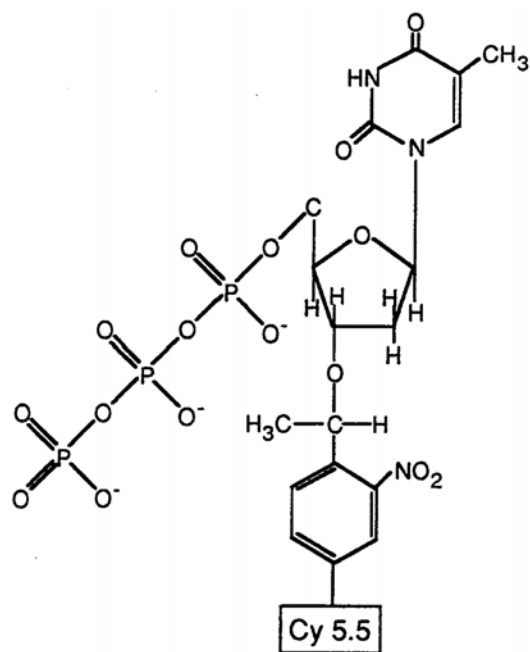


Fig. 31

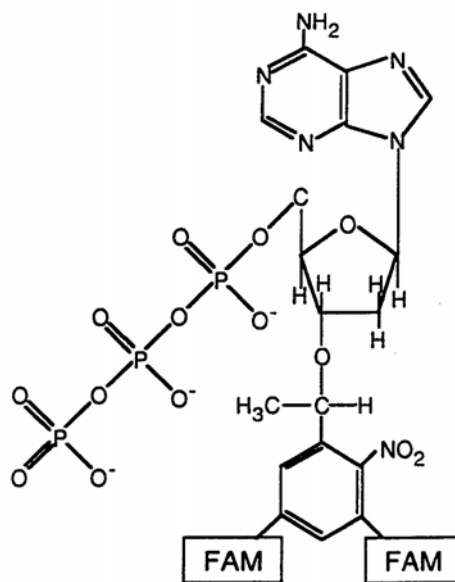


Fig. 32

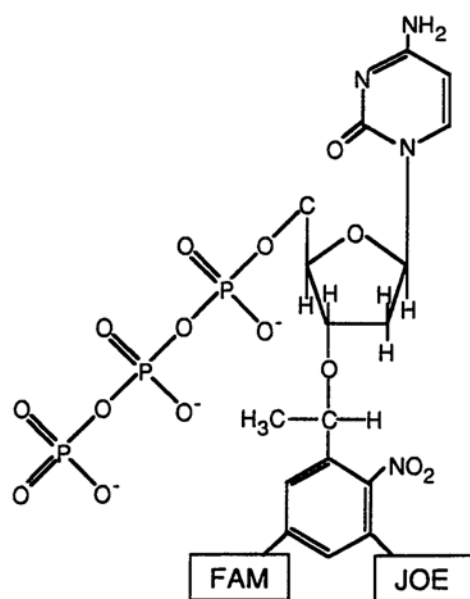


Fig. 33

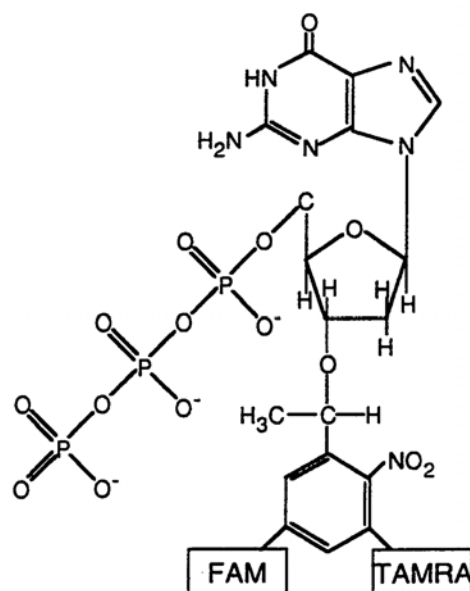


Fig. 34

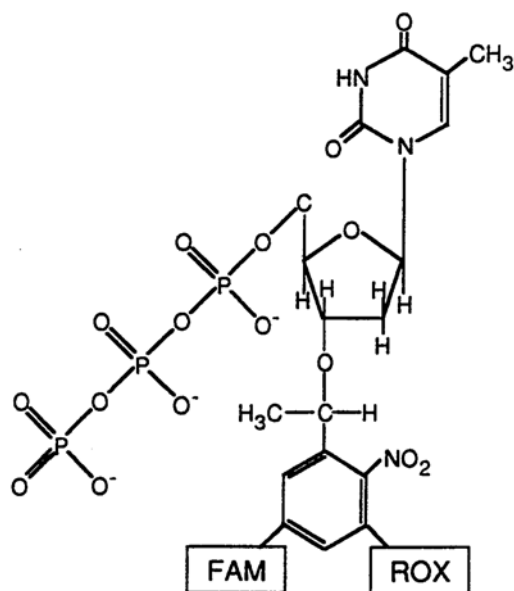


Fig. 35

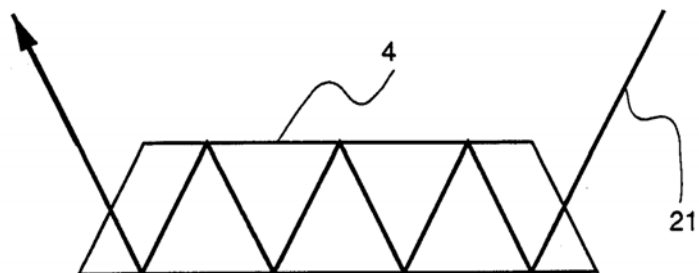
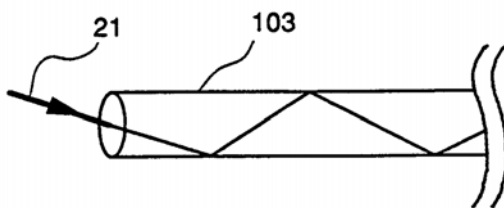


Fig. 36



INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP97/00239

A. CLASSIFICATION OF SUBJECT MATTER Int. Cl ⁶ C12Q1/68, C12M1/00, G01N33/50, C12N15/10 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) Int. Cl ⁶ C12Q1/68, C12M1/00, G01N33/50, C12N15/10 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WPI, BIOSYS		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP, 02-057978, A (Takara Shuzo Co., Ltd.), February 27, 1990 (27. 02. 90) (Family: none)	1 - 32
A	JP, 04-505251, A (Amersham Int. PLC), September 17, 1992 (17. 09. 92) & WO, 90/13666, A & EP, 471732, A	1 - 32
A	JP, 08-332100, A (Hitachi, Ltd.), December 17, 1996 (17. 12. 96) (Family: none)	1 - 32
A	JP, 07-213298, A (Perseptive Biosystems Inc.), August 15, 1995 (15. 08. 95) & EP, 639607, A & US, 5527675, A	1 - 32
A	JP, 07-313168, A (Hideya Terunuma), December 5, 1995 (05. 12. 95) (Family: none)	1 - 32
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search April 16, 1997 (16. 04. 97)		Date of mailing of the international search report April 30, 1997 (30. 04. 97)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.		Authorized officer Telephone No.