Development of an Automated SNP Analyzer

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ABSTRACT As we enter the "post-genome sequencing" era, great importance attaches to techniques for analyzing single-nucleotide polymorphisms (SNPs), which are expected to make a crucial contribution to achieving tailor-made medical treatment. This makes it desirable to realize a system that fully automates the process of SNP analysis.

The Production Engineering Development Center has therefore embarked, together with FI-Techno and Precision Systems Science, on a joint project for development of a fully automated DNA analyzer, and has achieved a number of significant technological results relating primarily to DNA detection. By developing a system for transmitting genetic information by means of fluorescence-marked microbeads and a proprietary high-sensitivity detection system that takes advantage of fiber-optic technology, we have developed a unique DNA information analyzer capable of high-speed and high-sensitivity analysis of SNPs. We are now manufacturing an apparatus capable of fully automated analysis of SNPs that can be used in clinical diagnosis applications.

1. INTRODUCTION

With smaller size and greater precision, data transmission equipment using optical fibers and fiber-optic components are being applied in different fields, and specifically are widely used in optical instruments and signal transmission. Biotechnology, meanwhile, is entering a "post-genome" era in which attention is focused on analyzing the single-nucleotide polymorphisms (SNPs), which identify the individual DNA differences that will be a crucial means of realizing tailor-made medical treatment. In these SNP analyzers, such as DNA chips and bead arrays, the importance of high-speed, high-sensitivity measurement means that methods that use fluorescence have become dominant. However DNA chips, which constitute the most common analytical technique for SNPs, are systems that measure surface reactions. They therefore have disadvantages in terms of low reactivity and a measurement sensitivity that is degraded proportionally as densities increase, and of being complicated to operate and requiring longer measurement time. What is more the DNA samples to be measured require pre-processing, presenting problems in terms of automation, so that at present, fully automated systems are not practical. Fully automated analyzers are particularly desirable in the clinical context, with the further need for high reliability, greater convenience, and improved economy.

The Production Engineering Development Center has embarked, together with FI-Techno and Precision Systems Science, on a joint project for development of a fully automated DNA analyzer. In the present work we have succeeded in producing an apparatus for analyzing SNPs and other DNA information, in which the DNA information is loaded onto microbeads, which are advantageous in terms of automated processing, and the DNA information on the beads is then measured in microchannels as fluorescence at high speed and with high sensitivity.

2. DETECTION SYSTEM

2.1 Principle of SNP Analysis

The microbead array method is used under conditions of suspension in an aqueous solution fluid, with the result that the degree of freedom of reaction and reactive rate are high, and the cost of manufacturing the arrays can also be kept extremely low in comparison with DNA chips. Furthermore, with microbead arrays, the adoption of Magtration using magnetic beads, which has been developed by Precision Systems Science, makes it possible to use the same automated processing system for both the pre-processing of the samples and for their measurement, making it the analytical method most suitable for clinical diagnosis applications.

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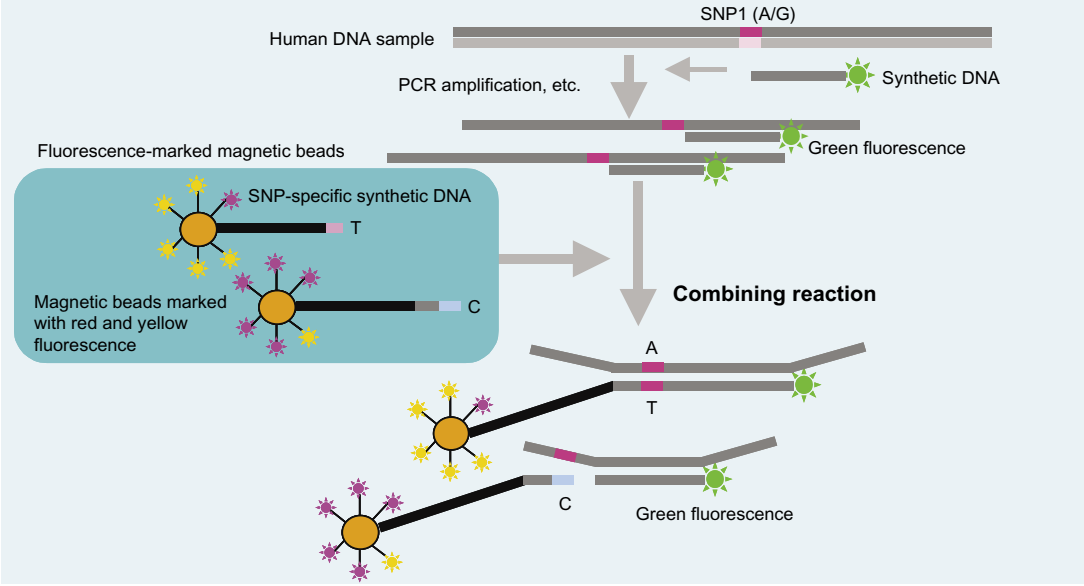

Figure 1 Principle of SNP analysis by measurement of fluorescence-marked beads.

This apparatus analyzes the SNP information on the samples, marking magnetic beads 2.8 μm in diameter by combining them with several fluorescent dyes in various proportions, and applying to them probes corresponding to each of these markers. The labeled samples are made to react with fluorescent dyes not used in marking the beads, determining which of the marked beads the samples are combined with. Figure 1 shows the principle.

2.2 Principle of Measurement Using Fluorescence-Marked Beads

In order to implement the analytical procedure described above, it is necessary to develop an apparatus for detecting the optical information on the beads that is suited to automated operation. And to carry out high-speed analysis for a plurality of SNPs, it is also necessary to measure the multiple types of fluorescent information on the microbeads for each bead individually.

One type of equipment for measuring fluorescence is the spectrophotometer, but it is not capable of measuring beads individually, and flow site meters, which are used in cell research for measuring the fluorescence information of minute particles, are expensive and very hard to reduce in size; they are also unsuited to automated operation.

In the apparatus reported here, a microchannel control system has been developed which assures that the microbeads in the fluid flow through the microchannel in a stable manner one by one, and measurements are made individually for each of the microbeads. In addition, as described below, the fluorescence detection head and microchannel are of unitized construction, and a technique was developed for multi-channel measurement using a fiber array, making possible high-sensitivity measurement of fluorescence-marked beads that is suited to clinical applications. Figure 2 shows the mechanism of measurement and identification using fluorescence-marked beads.

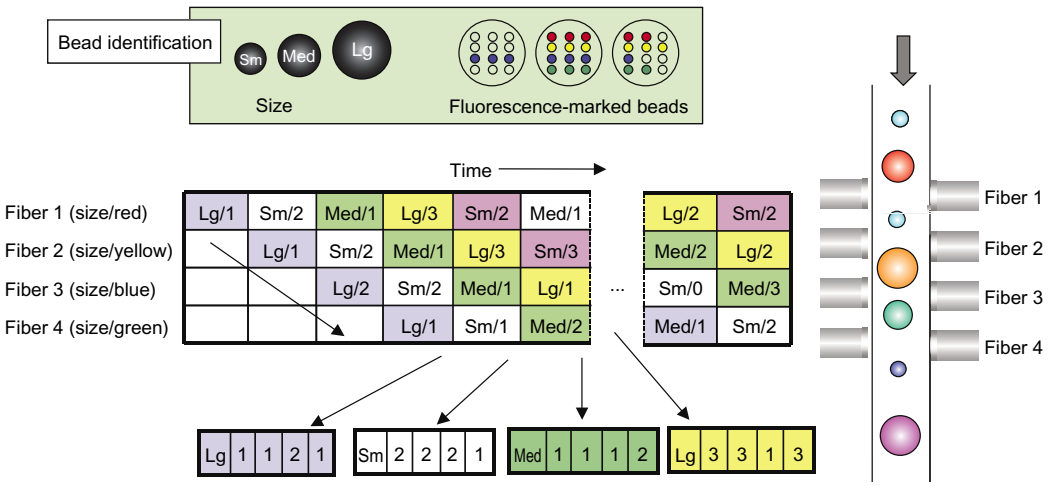

Figure 2 Mechanism of measurement and identification using fluorescence-marked beads.

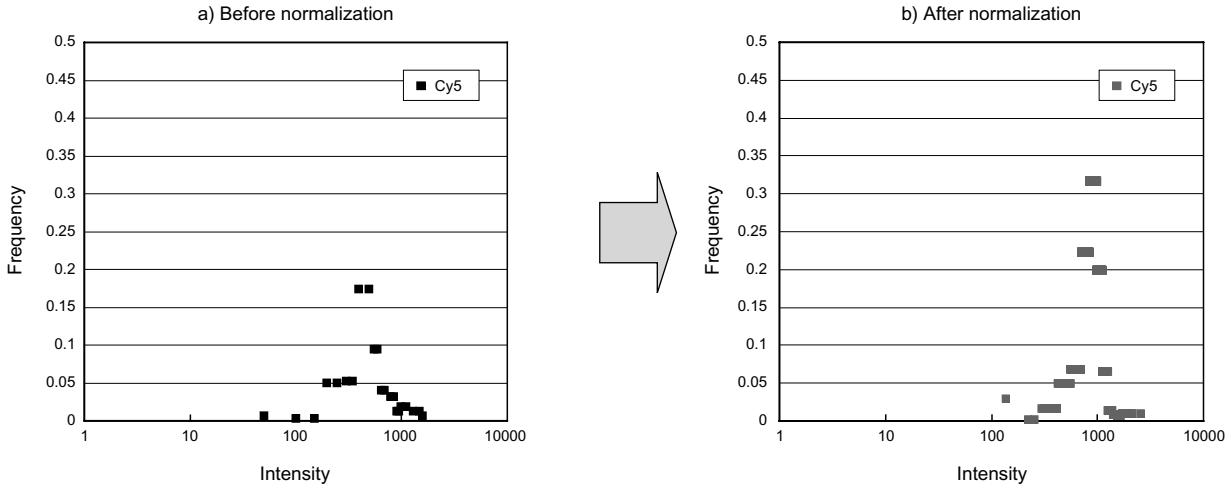

Figure 3 Effect of normalization using data on bead tracks in the microchannel.

2.2.1 Unitized Construction of Fluorescence Detection Head and Microchannel

To detect the low-intensity fluorescence of beads with multi-color markings using a number of fluorescent dyes, further distinguished by multiple gradations of light and dark, it is necessary to expose each of the microbeads in the flow accurately to a specific excitation light beam, and measure the optical information from each bead with high sensitivity. Specifically, since the beads being measured are only 2.8 μm across, the smaller the channel the better. Furthermore the optics are ordinarily configured on the peripheral surface of a glass channel, so that problems like the need to adjust the optical axis to the channel and the occurrence of optical noise due to surface reflection also require attention.

We therefore designed a configuration in which a plurality of optical fibers are disposed along the wall of a precision microchannel measuring some several hundreds of micrometers on a side, with a positional accuracy of 1 μm or better. The channel wall is thus formed by the ends of the fibers, enabling the direct exposure by and detection of the excitation beam on the beads by the optical fiber. This effects a significant reduction in optical noise, limiting the point of measurement to a minute area, and thereby making possible highly sensitive measurement. The precise positioning and alignment of the optical fibers was achieved by applying techniques for manufacturing optical connectors.

This unitized construction of the channel and the light path not only increases sensitivity by more efficient transmission of light energy but also enables more accurate positioning of the optical axes with respect to the bead tracks, thereby achieving improvements in rate of bead detection and eliminating optical axis adjustment.

2.2.2 Multi-channel Design Using Optical Fiber Arrays

Disposing a plurality of optical fibers along the direction of flow of the fluid makes it possible for each of the fibers to perform measurements individually for a different marker. For each fiber, measurements are performed by introducing the excitation beam into the fiber and exposing a bead to irradiation from the fiber. The fluorescent light thus excited is detected by the same fiber, subjected to wavelength selection by an optical filter, and the fluorescence information carried by the bead is measured. The measured values are digitized by multi-level comparison and by correlating the measured information from each measurement point with information on flow rate and time the multiplicity of optical data carried on the bead is measured. Since each measuring point is in a different location, it is possible to measure the spectra of various types of fluorescence with high sensitivity. Since the transmitted light is measured at each measurement point simultaneously, the passage of each bead can by unambiguously confirmed, and using the values obtained for transmitted light, bead diameter can be measured and the tracks of the beads in the channel can be determined. Measured values for fluorescence show a large dispersion without pre-processing, but by normalizing using measured values for transmitted light obtained at the same time, it is possible to effect a significant improvement in the signal-to-noise ratio for fluorescence values (see Figure 3).

Figure 4 shows data from measurements of fluorescence-marked beads using a typical flow site meter for purposes of calibration. These results confirm that beads of five levels of fluorescence intensity can be identified.

Mounting the optical fibers at high density has made it possible to miniaturize the detection head, and since the excitation beam and the fluorescence received from the beads are transmitted on fine, flexible fibers, it has been possible to dispose the lasers, photodetectors and other optical components in a different location and accommodate them in the unit compactly. Specifically in the case of multiple colors a problem arises in that the constituent optical systems cannot physically be accommodated compactly in the detection head, but since the detection head developed here is a fiber array only, the number of fluorescent dyes measured can be easily

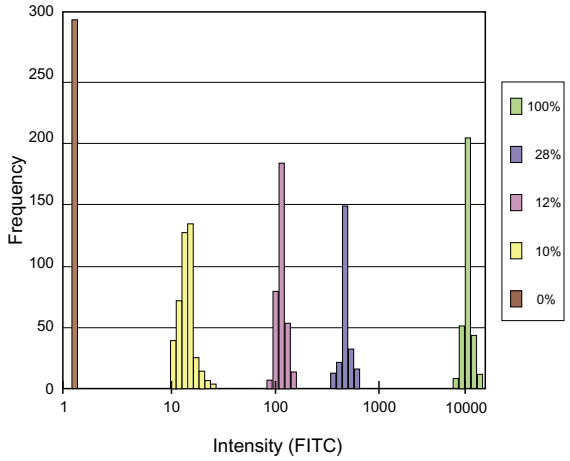

Figure 4 Result of measurement of fluorescence-marked beads by flow site meter for purposes of calibration.

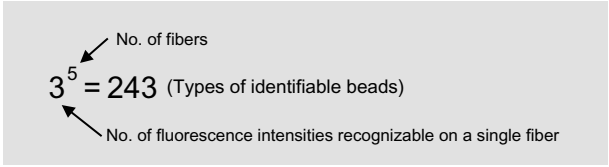

Figure 5 Bead identifying capability.

increased, and as shown in Figure 5, the bead identifying capability has been significantly expanded.

2.2.3 Controlling Tracks in the Bead Channel

If the flow channel is too large with respect to the size of the beads being measured, significant measurement dispersion can occur, and if it is too small, the beads can cause blockages that impede stable flow. Accordingly a fluid control technique using sheath flow was introduced, thereby controlling bead track dispersion in the microchannel, improving the bead detection ratio, and suppressing blockage.

In this sheath flow technique, the sample flow including the beads and a sheath flow enveloping the sample flow are introduced, each at a different pressure, into the channel, so that the flow resistance of the sample flow is drastically reduced, minimizing the gradient of its rate of flow so that the sample flow is squeezed more narrowly with respect to the channel diameter. The difference between the pressures set for the sheath flow and the sample flow regulates the diameter of the sample flow, concentrating the bead tracks at the desired position and reducing the measurement dispersion to the beads. This reduction in measurement dispersion is confirmed by the experimental results shown in Figure 6.

Further, the physical diameter of the channel is increased, thereby effectively addressing the problems of bead blockage.

2.2.4 Identification of SNPs Using a Prototype System

Decoding genetic information involves distinguishing the four types of nucleotides --adenine (A), cytosine (C), guanine (G) and thymine (T), and determining the sequence in which the nucleotides are arranged. By determining the sequence of these four nucleotides we can obtain the genetic information that will show the predisposition of individuals to disease. This is the purpose of analyzing single-nucleotide polymorphisms (SNPs).

Figure 7 shows the protocol used in an experimental SNP analysis to evaluate the performance of this apparatus. In this experiment the analysis was performed on one SNP having two fluorescent dyes; it differs from the principle described in section 2.1 in terms of the marking procedure, but is identical in terms of the principle for measuring SNPs by fluorescence marking. Fluorescence measurement was done by spectrophotometry, and the SNP site of the specimen was identified as A/A when the fluorescent dye was FITC alone, as A/G when the dyes were FITC and Cy5, and as G/G when the dye was Cy5 alone.

The result of analysis of one SNP using this prototype apparatus is shown in Figure 8. Bead identification was

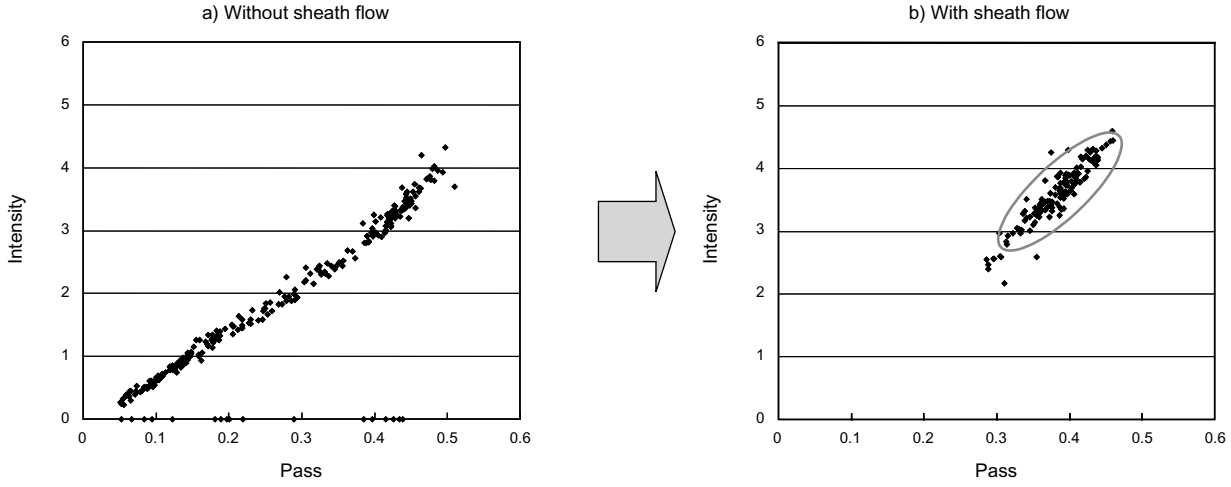

Figure 6 Decrease in measurement dispersion due to sheath flow.

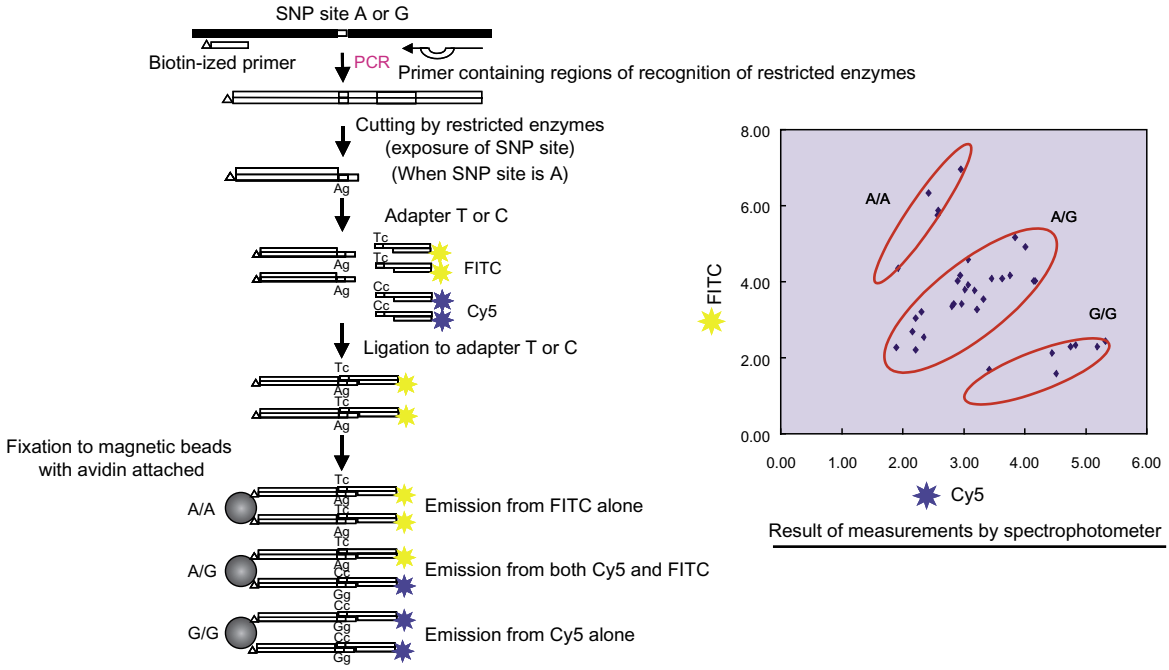

Figure 7 Protocol for SNP analysis by measurement of fluorescence, and results obtained.

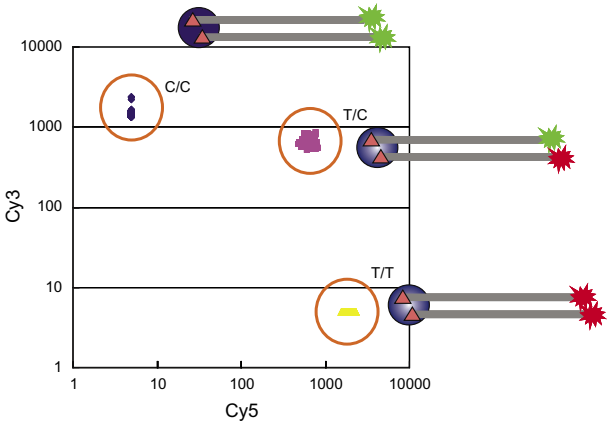

Figure 8 Result of analysis of one SNP using prototype system.

performed using Cy3 and Cy5 as the fluorescent dyes. From these results it was possible to confirm that SNP identification was possible using various combinations of fluorescence intensity.

2.2.5 Multiplexing of Measurement

In section 2.2.4 we looked at analysis of one SNP based on the presence or absence of two types of fluorescent dye, but high-speed, high-throughput analysis can be achieved by causing multiple SNPs to react in the same solution and multiplexing the measurement. For this purpose, according to the principles set forth in section 2.1, it is necessary to perform high-speed identification of a number of types of fluorescence-marked beads. And under this principle, since the marked beads can be measured individually, multiplexing can be realized by measuring a suspension containing a number of types of marked beads in a single process step. Since at present it has been confirmed that with a single type of fluorescent dye five levels of intensity can be distinguished, it is possible, marking with two types of fluorescent dye and with samples labeled with a single fluorescent dye, to analyze twelve SNPs simultaneously. Further, the ability to analyze multiple SNPs from a minute sample makes it possible to perform analyses at an unprecedentedly low cost.

2.2.6 Reducing Measurement Times

In an effort to reduce measurement times and cope with large numbers of samples, it was decided to reduce the length of the flow channel by adopting the commercially available microplate specification for the sample fluid and using a unitized construction for the intake nozzle and detection head. This configuration means that measurement is completed immediately after nozzle intake, significantly increasing operating speeds, approaching to approximately 10 seconds for a sample fluid of one well. Figure 9 is a simplified schematic of detection head and Figure 10 shows a photograph.

3. CONCLUSION

Applying the precision micro-optics technologies for optical fibers and optical connectors, we have developed a detection system that offers higher sensitivity, higher speed and lower cost, and is optimally suited to a microbead array analysis technique for clinical diagnostic applications. We are now manufacturing an SNP analyzer shown in Figure 11, which incorporates this detection system and is capable of providing fully automated

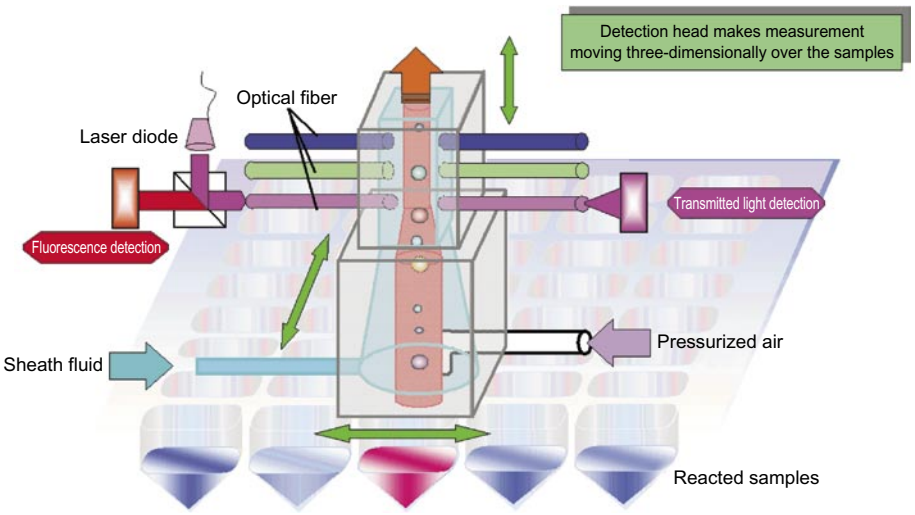

Figure 9 Simplified schematic of detection head.

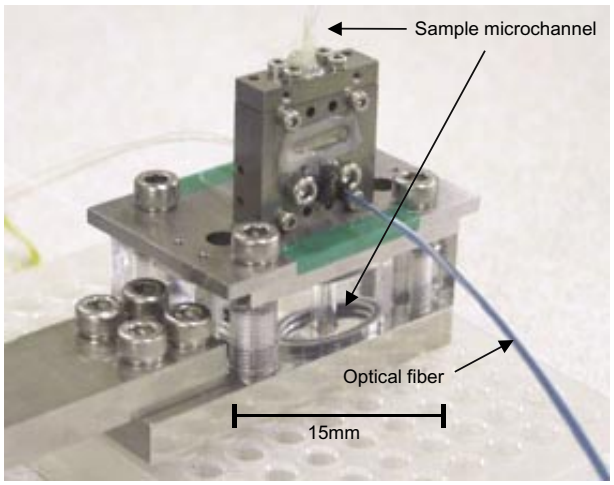

Figure 10 Photo of detection head.

operation from DNA extraction to measurement.

The fully automated analyzer shown in Figure 11 is for research applications in SNP analysis, but it is our belief that by means of this technology we come closer to the genetic diagnostic capability that will make tailor-made medical treatment a reality. It is our plan that, based on this system and in response to market needs, we will produce analyzers of practical use is clinical diagnostic applications.

Figure 11 Photo of SNP analyzer.

REFERENCES

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