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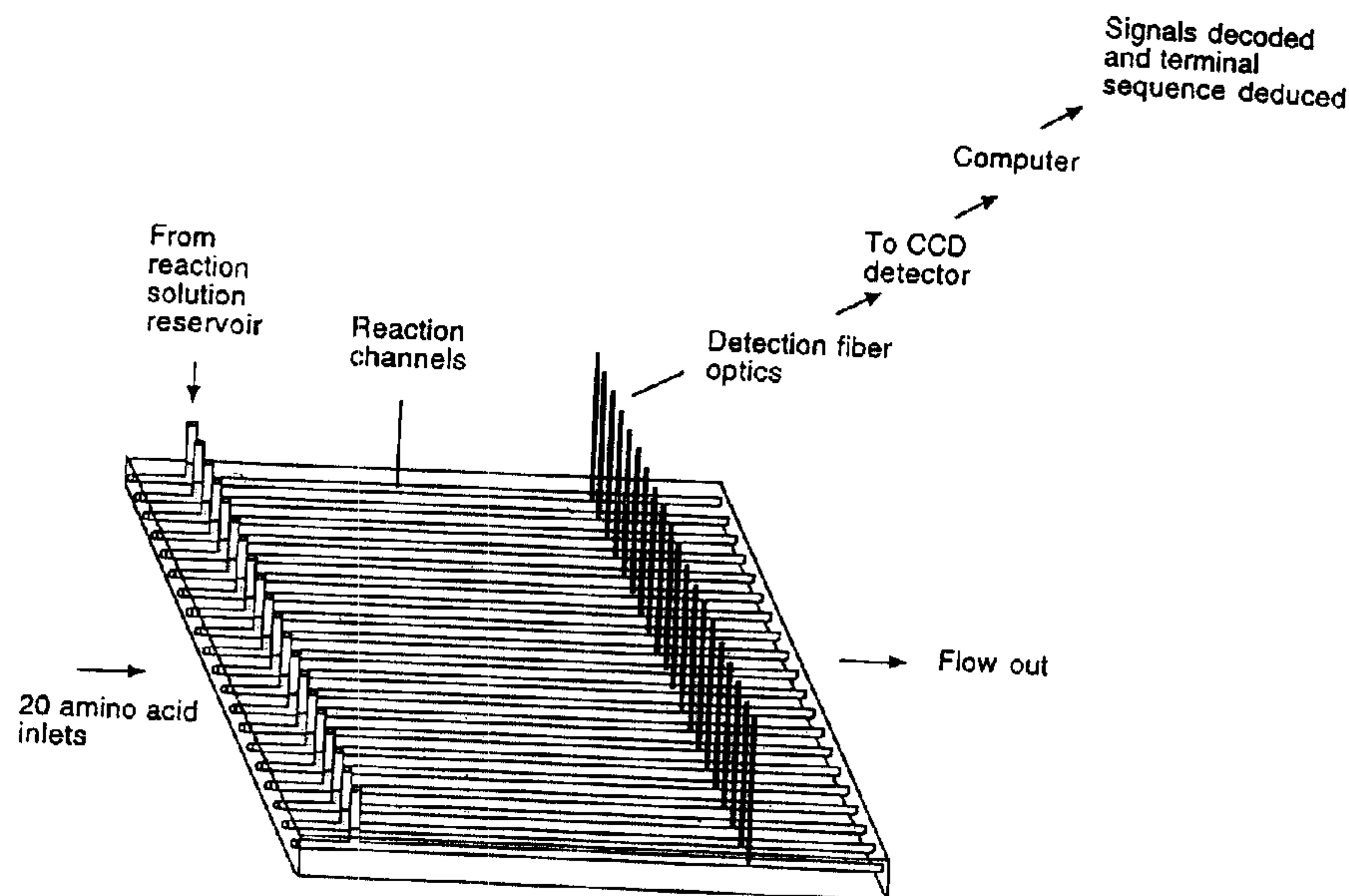
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(54) Titre : JEUX ORDONNES DE MICROECHANTILLONS ET MICROSYSTEMES POUR L'ANALYSE D'ACIDES AMINES ET LE SEQUENCAGE DE PROTEINES

(54) Title: MICROARRAYS AND MICROSYSTEMS FOR AMINO ACID ANALYSIS AND PROTEIN SEQUENCING



(57) **Abrégé/Abstract:**

A method is described where the amino acyl-tRNA synthetase system is used to create very small devices for quantitative analysis of the amino acids in samples or in sequential or complete proteolytic digestions. The methods can be readily applied to the detection and/or quantitation of one amino acid by using only its cognate amino acyl-tRNA synthetase and cognate tRNA. The basis of the method is that each of the 20 synthetases and/or a tRNA specific for a different amino acid is separated spatially or differentially labeled. The reactions catalyzed by all 20 synthetases are monitored simultaneously. Each separately positioned synthetase or tRNA will signal its cognate amino acid. The synthetase reactions can be monitored using continuous spectroscopic assays. Alternatively, since elongation factor Tu;GTP (EF-Tu;GTP) specifically binds all AA-tRNAs, the amino acylation reactions catalyzed by the synthetases can be monitored using ligand assays. Microarrays for amino acid analysis are provided. Additionally, amino acid analysis devices are integrated with protease digestions to produce miniaturized enzymatic sequencers capable of generating either N- or C- terminal sequence data and amino acid analyzers with integrated protein and peptide hydrolysis. The possibility of parallel processing of many samplers in an automated manner is discussed.

## ABSTRACT OF THE DISCLOSURE

A method is described where the amino acyl-tRNA synthetase system is used to create very small devices for quantitative analysis of the amino acids in samples or in sequential or complete proteolytic digestions. The methods can be readily applied to the detection and/or  
5 quantitation of one amino acid by using only its cognate amino acyl-tRNA synthetase and cognate tRNA. The basis of the method is that each of the 20 synthetases and/or a tRNA specific for a different amino acid is separated spatially or differentially labeled. The reactions catalyzed by all 20 synthetases are monitored simultaneously. Each separately positioned synthetase or tRNA will signal its cognate amino acid. The synthetase reactions can be monitored using  
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## Microarrays and Microsystems for Amino Acid Analysis and Protein Sequencing

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## BACKGROUND OF THE INVENTION

10 This invention relates to rapid methods of amino acid analysis on a microscale and to the application of microscale amino acid analysis techniques to studies of protein amino acid composition and amino acid sequence and the amino acid content of samples of interest, including biological samples.

15 In the past few decades the art has developed a multitude of protocols, cartridges, kits, and systems using biomolecular recognition phenomena for the analysis of biological samples for diagnostics, research, therapeutics, and various monitoring processes. Ligand-receptor or binding assays are powerful and well established. Immunoassays with labeled antibodies were introduced around 1960, and shortly afterward sandwich type immunoassays were described. Over the years, the art has produced significant improvements in ligand assay design, reagents, and detection systems. The development of hybridoma technology and monoclonal antibody production resulted in immunoassays with improved specificity and sensitivity. In addition, 20 phage display, combinatorial chemistry, antibody engineering, and directed evolution now make possible the production of proteins, peptides or oligonucleotides which bind a specific molecule

with any desired specificity. A plethora of assays using fluorescent and chemiluminescent labels have been developed. The discovery and use of new solid phases for heterogeneous binding assays was a major advance. In homogeneous binding assays the physiochemical properties of the tracer changes upon binding to the receptor, thus allowing direct monitoring of bound  
5 fraction without prior separation of the bound form from the tracer. Homogeneous assay systems using time-resolved fluorescence, fluorescence polarization, fluorescence resonance energy transfer and scintillation proximity assays are well established. Ligand assays have been miniaturized, automated, and computer-controlled. Innovations in engineering and chemistry have led to delivery systems for nanoliter volumes and sensitive biosensors for real time  
10 monitoring of biomolecular interactions. Ultrasensitive spatially specific detectors have been developed that allow the analysis of thousands of binding interactions on a single microchip. Recombinant DNA technology now enables the production and engineering of binder molecules with novel desired properties (e.g., catalytic antibodies, oligonucleotides and peptide aptamers).

15 Target molecule analysis has taken advantage of biomolecular recognition phenomena as well as new techniques in microfabrication technology and position-sensitive detectors. Such technology has recently revolutionized genome analysis. Microarrays detect gene expression levels in parallel by measuring the hybridization of mRNA to many thousands of genes immobilized at high spatial resolution on a glass surface [Reviewed in Watson et al. (1998) *Curr.*  
20 *Opin. Biotech.* 9:609-614]. Highly resolved detection is generally achieved by laser induced fluorescence of a labeled probe. Results from massively parallel and quantitative gene expression measurements analyzing up to 40,000 genes at a time and whole-genome variant detection methods show the power and accuracy of combining biorecognition phenomenon with miniaturized array based methods [Lipshutz, R. J., et al. (1999) *Nat. Genet.* 21:20-24]. Capillary  
25 array electrophoresis where many capillaries are run and detected in parallel has recently been developed for rapid DNA sequencing [McKenzie et al.(1998) *Eur. J. Hum. Genet.* 6:417-429]. It is now possible to fabricate interconnected and computer controlled and integrated fluid systems with the channel sizes in the micron dimensions. These microflow systems can be mass-produced inexpensively and will soon be in world wide use for chemical and biochemical analysis.

Homogeneous ligand assays based on energy transfer, fluorescence polarization spectroscopy, fluorescence correlation spectroscopy and other optical and electrochemical detection methods provide the sensitivity, speed, and ease needed for high-throughput screening and are well suited for miniaturization. For example, fluorescence techniques now allow binding properties to be  
5 determined in biological assays in real time, in miniaturized and multiplexed formats, and with sensitivities approaching the single molecule level. The development of miniaturized arrays and "Lab on a Chip" technologies represents a combination of several disciplines that include microfabrication, microfluidics, microelectromechanical systems (MEMS), chemistry, biology, and engineering. Many of the limitations of passive binding assays can be overcome by active  
10 microfluidic chip devices which facilitate the rapid transport, mixing and selective addressing of biomolecules to any position on the chip surface. Specially designed microsystems containing a multitude of sub-microliter chambers or microchannels can be used in combination with microfluidics and/or nanopipetting to analyze a multitude of samples simultaneously or nearly simultaneously. On-line microfluidic systems that transport liquid solutions in channels of  
15 micron dimensions have been used for high-throughput DNA genotyping [Zhang et al. (1999) Anal. Chem. 71:38-1145], polymerase chain reactions, and DNA sequencing reactions [Wooley et al. (1996) Anal. Chem. 68:720-723]. Future ligand assays are likely to be performed in miniaturized microflow systems. Miniaturized detectors can be placed in flow channels. Alternatively, scanning or imaging detectors may be used in microfluidic arrays. Sensitive  
20 miniaturized affinity biosensors are also being developed that can monitor binding *in situ* or *in vivo* and in real time [reviewed in Cunningham, A.J. (1998) *Introduction to Biological Sensors*, John Wiley and Sons, New York, NY].

Progress in the computer industry has made possible micromachining of silicon into complex shapes with dimensions of less than 1  $\mu\text{m}$  creating an ever increasing number of  
25 miniaturized devices. Devices can be electrical, such as microelectrodes, and signal transducers; optical such as photodiodes and optical waveguides; and mechanical, such as pumps and microreactors. In the emerging field of microfluidics, the integration of automated microflow devices and sensors allow very precise control of ultrasmall flows on microchip platforms

[Gravesen et al. (1993) *J. Micromech. Microeng.* 3:168-182; Shoji and Esashi (1994) *J. Micromech, Microeng.* 4:157-171]. Micrometer-scale pumps and valves transport liquid solutions from separate reservoirs through channels of micrometer dimensions. Many different flows can be combined in all sorts of ways and mixed on the same chip. Chip technology also allows the integration of intersecting channels, reaction chambers, mixers, filters, heaters, and detection devices to perform on-chip reactions in sub-nanoliter volumes in a highly controlled and automated fashion [Colyer et al. (1997) *Electrophoresis* 18:1733-1741; Effenhauser et al. (1997) *Electrophoresis* 12:2203-2213]. Microflow channels have very low dead volumes, allowing rapid chemistry and detection and real-time monitoring. In addition, if reagents are continuously replenished by micropumps, sequential measurements are possible. By microfabrication of many parallel channels on a single microchip platform, multiple samples can be analyzed simultaneously. Laser-induced fluorescence is generally the detection method of choice for microflow devices. Ultrasensitive spatially specific detection in capillary or microchannel arrays can be achieved with laser-induced fluorescence using imaging detectors or confocal scanners [for a review, see, e.g., Kheterpal and Mathise (1991) *Anal. Chem.* 71:31A-37A]. Integrated, on-line microfluidic systems for transporting the amino acids and other reagents from separate chambers through the amino acid analysis microarrays are thus an aspect of the present invention, thus allowing sequential measurements and rapid (possibly direct) data acquisition. Desirably, the channels are in fluid connection with waste receptacles.

It is now possible to fabricate planar micro-optical elements at a low cost. Applications such as micro-lenses, fiber optical couplers and switches, optical waveguides, and diffractive elements are a few examples. Structures with a periodicity in the submicron range can be fabricated over areas of square centimeter dimensions.

Recent advances in rapid gene analysis have not been duplicated for protein or polypeptide analysis or for amino acid analysis. This lack of development is unfortunate because protein and polypeptide compositional and sequence analyses are pivotal to biological research and the applications for amino acid analysis are vast. – and C- terminal amino acid sequence

information can be used to verify start and stop points of protein coding sequences, to identify proteins and to identify proteolytic degradation products. Terminal sequences as short as four or five amino acid can often be useful in designed oligonucleotides for polymerase chain reaction or hybridization analyses.

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Amino acid analysis and amino acid sequence analysis, fundamental measurements in biological science, have applications in every aspect of biochemical research, biotechnology (agriculture, medicine) clinical medicine, and food technology (agriculture, fishery). The techniques are driven increasingly by the needs for higher sensitivity, faster throughput and lower cost. Historically, the determination of amino acids in protein hydrolysates and other samples has proven to be a very difficult problem for the analytical chemist. The classical method for amino acid analysis uses chromatography to separate the 20 naturally occurring amino acids. Many chromatographic and electrophoretic techniques have been developed to resolve amino acids. However, these methods are complicated because the 20 natural amino acids do not differ from one another in a systematic way. The separation of 20 components is difficult, and the task is further complicated by the similar structures and properties of many of the amino acids, such as leucine and isoleucine, serine and threonine, or tyrosine and phenylalanine. In addition the amino acids have needed to be derivatized to allow detection. Techniques for amino acid analysis need further simplification, and micro-amino acid analysis remains one of the most difficult procedures in analytical chemistry. No real time methods for analyzing the 20 naturally occurring amino acids used in protein synthesis are available. The present invention provides a radical change and meets a longfelt need in the art for a new approach to amino acid analysis. This approach employs biomolecular recognition phenomena to analyze the 20 natural amino acids simultaneously or nearly simultaneously. This new method is particularly suitable for miniaturization and multiplexing and for the creation of rapid, high throughput, and inexpensive systems for amino acid analysis on a microscale. In biochemical and pharmaceutical research, amino acid analysis can be used to quantitate a protein of interest, In clinical medicine amino acid analysis can be used to detect metabolic disorders, for example, in the detection of phenylketonuria (PKU) in newborns and in following the metabolic state and

5 dietary compliance of PKU patients. Amino acid analysis can also be used in quality control analysis of proteins in pharmaceutical, industrial enzyme and food areas and in biotechnology in general. In addition, amino acid analysis has applications in proteome research, where thousands of proteins can be separated in a single experiment.

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10 In addition to serving as the building blocks of proteins, the 20 natural amino acids play important roles in metabolism. Disorders of amino acid metabolism pose formidable challenges for physicians. Biochemical reactions involving amino acids are many and ubiquitous throughout life. Amino acid microbiosensors invented herein may be used to study amino acid metabolism in organisms, tissues, or cell culture. Additionally, these biosensors can be integrated with other enzymes to produce biosensors for a multitude of biologically important metabolites.

15 Furthermore, amino acid compositional analysis and amino acid sequence determination are among the most crucial analytical methods in protein chemistry. The present methods can be used to identify proteins, and the amount of purified protein can be quantified by amino acid analysis. As the only analytical method to determine absolute protein quantities, it is indispensable. Additionally, there remains a high demand for high throughput amino acid analysis for routine quality control and screening of biological samples. For example, amino acid analysis is usually included in the quality control studies of pharmaceutical proteins and peptides  
20 intended for human use. Proteins and polypeptides are increasingly being used as drugs. The number of protein and polypeptide samples requiring amino acid analysis is increasing dramatically in the pharmaceutical industry due in part to the recent advances in combinatorial chemistry and high throughput screening methodologies. With the ever increasing number of peptide and protein drugs, the inadequacies of existing methods of amino acid analysis represent  
25 a significant bottleneck to drug discovery and development. In biochemistry, amino acid analysis is also applied to the determination of the protein-protein interaction ratio in various complexes and to the identification by database search of proteins separated by one- or two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Thousands of proteins can be separated in one experiment using 2D-PAGE; however, current amino acid analyzers can only analyze one sample



at a time. Presently, amino acid analysis is performed using methods and apparatus that are slow, insensitive, time consuming, and expensive. One available automated amino acid analyzer is based on the principles of ion exchange chromatography. Such instruments, still in common use, only achieve nanomole sensitivities, are slow and expensive, and require the use of technical experts. One disadvantage of present methods is the requirement of relatively large amounts of protein for analysis; e.g., as much as 1 microgram of protein. However, many proteins of great biological interest can only be isolated in much smaller amounts. Amino acid analysis of smaller amounts of protein is problematic. Relatively rare (or difficult to purify) proteins are typically isolated in amounts of 1-10 nanograms using common methodologies. This is approximately 100- to 1000-fold less than the amount required for amino acid analysis and protein end group sequencing using methodologies available to date.

In addition to the longfelt need for a simpler, inexpensive method of amino acid analysis with greater sensitivity, a need exists for real-time amino acid analyzers that can monitor trace concentrations of amino acids *in situ* or in process control situations where samples must be run periodically and results are desired quickly. Amino acids have important roles in metabolism and it is desirable to be able to analyze amino acids *in vivo* and *in situ* (e.g. in organisms and cell cultures). This can be accomplished using the amino acid biosensors described herein. Furthermore, the real time amino acid analyzers described herein can be integrated with other enzymatic systems, thus creating integrated microsystems.

Current amino acid analyzers can only analyze one sample at a time. There is a great need for systems and methods to analyze a large number of samples simultaneously. For example, using existing methods, large numbers of protein or peptides of potential therapeutic potential are being produced. These samples must be analyzed using current amino acid analyzers. Because these current methods can only analyze one sample at a time, this is a significant bottleneck to drug discovery. The methods described herein provide for an efficient and sensitive means to analyze a multitude of samples simultaneously.

Proteome projects, which aim to characterize all proteins expressed by a particular genome or in a particular tissue, place enormous demands on the development of ultra high throughput and sensitive amino acid analyzers. The identification of proteins separated by two-dimensional (2D) gel electrophoresis is key to successful proteome analyses. 2D gel electrophoresis enables purification of thousands of proteins to homogeneity in a single experiment [O'Farrell et al. (1975) J. Biol. Chem. 250:4007-4021]. No currently available method exists to identify these proteins rapidly and inexpensively. Often a protein can be identified on the basis of its amino acid composition [Wilkins et al. (1996) Biotechnology 14:61-65] or by N- or C-terminal "sequence tags" of only three or four amino acids [Wilkins et al. (1998) J. Mol. Biol. 278:599-608], but current amino acid analyzers and conventional protein sequencers are too slow, expensive, and insensitive for proteome projects. The amino acid analysis microarrays and microsystems described herein provide methods for inexpensive high throughput amino acid analysis on a microscale. The present rapid methods of amino acid analysis are especially useful in proteome analyses.

C-terminal sequence tags are more specific than N-terminal tags of the same length, but no reliable, sensitive method for C-terminal protein sequencing is currently available. Accordingly, new rapid methods for amino acid analysis and end-group protein sequencing are needed.

The miniaturized, real time amino acid analyzers described herein can be used with exopeptidases, enzymes that remove amino acids sequentially (i.e., one at a time) from a protein's N- or C-terminus, to create integrated protein end group sequencers. These microsystems are suitable for generating either N- or C-terminal sequence tags from intact proteins or peptides on a microscale.

The invention described herein alleviates at least some of the bottlenecks associated with drug discovery and large scale proteome projects created by the inadequacies in the current methods of amino acid analysis and allows rare proteins and peptides that can be isolated only in

minute amounts to be analyzed for amino acid composition.

Thus, the methods of amino acid analysis described herein offer many advantages over the current art. Only a tiny amount of sample is required for each measurement. The new method provides much shorter analysis times, and *in situ* and real time analyses are possible. Mass production of such miniaturized systems is possible, thus enabling disposable, inexpensive amino acid analysis. The microsystems can be integrated in order to construct multifunctional microsystems (e.g. for amino acid analysis and protein and peptide end group sequencing and analysis). The methods provide means for the simultaneous analysis of many samples. The microsystems of the present invention only consume tiny amounts of reagents, greatly reducing reagent costs and disposal expense. The new method allows simplified instrumentation schemes. The new method may be constructed as portable units and systems suitable for use in non-laboratory environments. The new methods are relatively simple to perform. There are a wide range of device applications and designs, including but not limited to, biosensors, microtiter plate kits, and integrated microfluidic systems. The present systems achieve greater sensitivity and lower costs than current methods by orders of magnitude. Whereas existing technology is applicable to nanomole or picomole quantities, the present methods and devices allow the analysis of femtomole and attomole quantities of amino acids. The functional elements are biological molecules, and hence methods known in the art can be used to optimize the system (e.g., such known methods as directed evolution, protein and oligonucleotide engineering, phage display, antibody engineering, and combinatorial chemistry can be used to obtain biomolecules for optimum system operation).

#### SUMMARY OF THE INVENTION

It is an object of the present invention to provide microscale elongation factor-amino acyl-tRNA (AA-tRNA) synthetase systems for analyzing any one of or up to all twenty of the primary amino acids (Primary amino acids are those which, in preparation for protein synthesis, are attached for tRNAs to form amino acyl tRNAs). The primary amino acids are alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine,

leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, tyrosine. By monitoring the reactions catalyzed by these 20 enzymes simultaneously or in a spatially resolved manner, the 20 naturally occurring protein amino acids can be quantitatively identified. Alternatively, a single amino acid can be targeted for measurement by  
5 use of its cognate amino acyl tRNA synthetase and tRNA, coupled with an elongation factor system for detection of the AA-tRNA formed. Binding assays for monitoring the formation of AA-tRNA, and hence methods for determining the concentration of the amino acid cognate to the synthetase and tRNA within the scope of the present invention. The microscale systems of the present invention can be adapted for continuous or discontinuous operation. Elongation  
10 factor 1A from eukaryotic cells or elongation factor Tu (EF-Tu) from prokaryotic cells), when bound to GTP, binds AA-tRNAs with high affinity ( $K_D = 10^{-9}$  M) but not tRNAs. Because of this relationship, known ligand assays can then be used to monitor the concentration of amino acids in a sample. Known methods of miniaturization, detection and automation are used in conjunction with novel aspects of the present invention. Ultrasensitive ligand assays using laser  
15 induced fluorescence have sensitivities approaching the single molecule level. Accordingly, the present methods can be used to measure the concentration of amino acids in small amounts of sample, at high sensitivity and at relatively low cost.

Accordingly, one aspect of the invention is a method for amino acid analysis based on biomolecular recognition phenomena. The key recognition event collating the nucleic acid  
20 sequences of genes and amino acid sequences of proteins occurs on amino acyl-tRNA synthetases. Each of the 20 synthetases attaches its cognate amino acid to a specific tRNA, producing AA-tRNAs. Elongation factor 1A.GTP (called EF-Tu;GTP in bacteria) binds the AA-tRNAs and transports them to the ribosomes for protein synthesis. The critical fidelity of protein synthesis depends upon the remarkable ability of the synthetases to recognize their  
25 cognate amino acids and tRNAs and upon the ability of EF-Tu;GTP to discriminate between AA-tRNAs and tRNAs. Rather than separating the amino acids by chromatography, each synthetase or a tRNA specific for a different amino acid is separately positioned, and the amino acids are analyzed from a mixture by monitoring the reactions catalyzed by the synthetases in a

spatially resolved manner. Because EF-Tu;GTP specifically binds all AA-tRNAs but not tRNAs with high affinity, ligand assays can be developed to monitor the amino acylation reactions catalyzed by the synthetases. By reducing amino acid analyses to the simplicity of a ligand assay and by using spatially positioned biomolecular recognition molecules (synthetases, elongation factors, tRNAs) and high-specific-activity labels, paralleled amino acid analysis microarrays and microsystems far more sensitive than current amino acid analyzers are possible.

Aminopeptidases and carboxypeptidases are enzymes that release amino acids sequentially, one-at-a-time, from a protein's amino-terminus and carboxy-terminus, respectively. These enzymes are used in discontinuous kinetic assays for protein end-group sequencing. Due to the nonlinear rate of hydrolysis, those kinetic studies have been generally unsuccessful. Real-time amino acid analysis microarrays that can detect amino acids as they are released by these enzymes are thus a further aspect of this invention. These arrays are integrated with aminopeptidase or carboxypeptidase digestions, creating on-line microfluidic systems capable of generating either N- or C-terminal sequence data. As amino acids are liberated by an exopeptidase, they flow from the digestion chamber through the amino acid analysis microarrays and are detected sequentially. Desirably, the digestion chamber is separated from the analysis arrays by a membrane which allows only small molecules to pass, thus protecting the channels or wells or microarrays from debris or whole proteins which could foul or plug the system. In addition, or alternatively, the protein of interest and/or the exopeptidase can be bound to the interior of the digestion chamber. The terminal sequence is generated by a computer upon analysis of the sequential amino acid detection data. If the sequence information is generated from an unidentified protein, for example, the protein can be identified by automated database searching, provided that it is one for which sequence information is available on accessible databases.

In the present invention, each of 20 synthetases and/or specific tRNAs (one specific for each amino acid) is positioned into or transported through a separate chamber (e.g microwell, microcapillary or microchannel) or immobilized in a separate location on a surface (e.g bead,

microparticle or microchip), or immobilized on a separate bead or particle, or on a separate transducer (e.g., an optical fiber, electrode or piezoelectric crystal). The 20 specific synthetase reactions are monitored in a spatially resolved manner. Multianalyte analysis methods use multiple labels or separately positioned biorecognition molecules and spatially specific  
5 detectors. Spatially resolved microarrays and microflow systems of the present invention can use laser-induced fluorescence and imaging (e.g., CCD detectors), scanning (confocal scanners) detectors, piezoelectric detector systems or surface plasmon resonance detection systems, for example.

The present inventor has coupled the amino acid analysis device to enzyme reactions that  
10 release amino acids, one at a time, from the N-terminus (using aminopeptidases) or more importantly from the C terminus (carboxypeptidases) for protein and peptide end group sequencing, endgroup amino acid analysis or amino acid compositional analysis. Presently, automated methods are not commonly available for identifying C-terminal sequences of proteins. Using carboxypeptidases and aminopeptidases along with amino acid analysis for protein end  
15 group sequencing is not new. Over the years, these enzymes have been used in discontinuous kinetic assays for protein end group sequencing. Proteins are typically digested by these enzymes and samples of the digests are taken at various time points and analyzed later using an amino acid analyzer. However, due to the nonlinear rate of hydrolysis by these enzymes, kinetic assays have been unsuccessful in most cases. When an analyte varies rapidly and unpredictably, as in  
20 the case here, a continuous (real time) assay is needed. However, no continuous amino acid analyzers exist. The systems invented herein make possible continuous microsystems for amino acid analysis. The inventor proposes the creation of integrated continuous amino acid analysis microarrays and microsystems that can detect amino acids continuously in the order that they are released by these enzymes. These miniaturized amino acid detectors can be integrated with  
25 aminopeptidase and carboxypeptidase digestions creating ultrasensitive, on-line microfluidic systems capable of generating either N- or C- terminal sequence data using minute protein samples. As the amino acids are liberated by these enzymes they flow from the site through amino acid analysis microfluidic arrays and are detected continuously in the order that they are

released. The terminal sequence is then regenerated by a computer from the record of the amino acid detection.

Microarrays or microfluidic systems of the present invention are a further aspect of the invention. These systems analyze tiny amounts of amino acid containing samples with high sensitivity. These systems advantageously offer femtomole or attomole concentration detection, which sensitivity is made possible by the use of fluorescence detectors that possess higher sensitivities than do present analyzers.

The present invention also sets forth methods of amino acid analysis based on biomolecular recognition phenomenon using small amounts of reagent and sample in microscale or nanoscale devices. The proposed methods are ultrasensitive, inexpensive and rapid. Furthermore, the methods are suitable for multiplexing to create high throughput systems.

Simultaneous multianalyte testing is now possible, and any known method for multianalyte analysis can be used to construct amino acid analyzers employing the twenty amino acyl-tRNA synthetases and twenty specific tRNAs as recognition elements. Methods of simultaneous multianalyte testing include assays based on more than one label and assays based on spatially separated reaction zones. For example, researchers have used binders in the same assay zone labeled with different fluorescent molecules [Vuori et al. (1991) Clin. Chem. 7:2087-2092; Hemmila, I. (1987) Clin. Chem. 33:2281-2283], different radioactive species [Wians et al. (1986) Clin. Chem. 32:887-890; Gutcho et al. (1977) Clin. Chem. 23:1609-1614; Gow et al. (1986) Clin. Chem. 32:2191-2194], different enzymes [Nanjee et al. (1996) Clin. Chem 42:915-926], metal ions [Hayes et al. (1994) Anal. Chem. 66:1860-1865], colored latex particles [Hadfield et al. (1987) J. Immunol. Methods 97:153-158] and particles of different sizes [Frengen et al. (1995) J. Immunol. Methods 178:141-151]. Various detection schemes are employed in these multianalyte are based on changes in one or more of the following signals: absorbance, steady-state fluorescence, fluorescence lifetime, chemiluminescence, radioactivity, electrochemical response, laser light scattering, and frequency of a piezoelectric quartz crystal,

upon the binding event(s).

Rather than separating 20 amino acids by chromatography, the present methods apply biomolecular recognition phenomena to analyze the naturally occurring amino acids from a mixture simultaneously or nearly simultaneously. These methods of the present invention  
5 advantageously take advantage of the fact that each of the 20 naturally occurring amino acids is recognized with high specificity by a specific amino acyl tRNA synthetase and attached to a specific tRNA forming AA-tRNA. Each synthetase binds only its cognate amino acid. In the methods of the present invention, this binding is monitored in a spatially resolved manner. The amino acid composition of the mixture is determined by monitoring up to all 20 synthetase  
10 reactions simultaneously or using a spatially specific detector (i.e., scanning or imaging detectors). This can be accomplished in a number of formats. For example, simultaneous multianalyte testing is now possible, and any known method for multianalyte analysis can be used to construct amino acid analyzers using the twenty amino acyl tRNA synthetases as the recognition elements. Each of the 20 synthetases or tRNAs are separately positioned, and the  
15 reactions catalyzed by these enzymes monitored in a spatially resolved manner. Each synthetase converts its cognate amino acid into a proportionate amount of product. The AA-tRNAs produced by the synthetases specifically bind elongation factor Tu:GTP, as explained in further detail herein. By using a molecule that specifically binds all the AA-tRNAs, ligand assays are possible in methods for amino acid analysis.

20 Arrays useful in the present invention vary according to their transduction mechanisms and include surface acoustic wave sensors, microelectrodes, solid-state sensors, and fiber-optic sensors. However, optical, electrochemical and piezoelectric crystal arrays are preferred. These systems may be used to analyze amino acids in volumes of less than 1 microliter with a sensitivity many orders of magnitude greater than current analyzers.



An additional aspect of the present invention are methods of end-group analysis of proteins and polypeptides. Proteins that are N-terminally blocked present a challenge because they cannot be directly sequenced by Edman degradation. A blocked N-terminus is common (approximately 80-90% of eukaryotic proteins are N-terminally blocked), while C-terminal blocking is rare. One advantage of the present invention lies in rapid endgroup sequencing and analyses that may be accomplished by the methods described herein.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B generally illustrate enzymatic amino acid analyzers of the present invention. Fig. 1A is a schematic illustration of a multichannel pipettor suitable for simultaneously mixing and dispensing a solution of the primary amino acids into 20 wells in a microtiter plate format. Each of the 20 amino acyl-tRNA synthetases and/or a cognate tRNA specific for a different amino acid is dispensed into a different well. The single letter codes for the amino acids are used here to represent the synthetase or tRNA cognate for a specific amino acid. Each well signals only the amino acid cognate to the synthetase and/or tRNA present in that well. The reactions catalyzed by the synthetases is monitored in all wells simultaneously (using a plate reader and), each synthetase will be dispensed into a separate well and the detector is a spectrophotometer used to quantitate the amino acids. Because each synthetase is positioned in a different well at a known location, the reactions catalyzed by these enzymes can be used to identify and quantitate their cognate amino acids.

Fig. 1B graphically illustrates an ultrasensitive ligand assay for the determination of the ternary complex in a microtiter plate format. In this scheme, EF-Tu;GTP is immobilized on the bottom of the wells. Each of the 20 wells in a row contains a different one of the 20 synthetases in amino acylation buffer and its labeled cognate tRNA. After the amino acid mixture is added to the wells, the ternary complex forms on the bottom of the well in response to the amino acids having a cognate tRNA in the well. After a washing step, the bound fluorescence or other label is quantitated using a fluorometer (plate reader) or other detection system. Depending on the fluorescent labels and instrumentation, detection limits as low as femtomole-attomole

sensitivities can be achieved.

Fig. 2A and 2B generally illustrate the tRNA and synthetase microarrays of the present invention. Fig. 2A is a graphical illustration of a tRNA microarray of the present invention. Twenty tRNAs (one specific for each amino acid) are arrayed as microspots. Each unique amino acid is positioned at a known location on the array. A mixture of amino acids is added along with the 20 synthetases and fluorescently labeled EF-Tu:GTP in reaction buffer. AA-tRNAs are formed on each microspot in proportion to the concentration of amino acid specific for each immobilized tRNA. Fluorescently labeled elongation factor binds the AA-tRNAs, and the labeled complex is detected by laser induced fluorescence using position-specific detectors..

Fig. 2B graphically illustrates synthetase microarrays of the present invention. The 20 synthetases are co-immobilized with EF-Tu;GTP as separate microspots. Each synthetase is co-immobilized with the elongation factor at a known location on the array. Fluorescently labeled tRNAs are added along with the amino acid-containing to be analyzed. Amino acids and tRNAs specific for the microspot bearing their cognate synthetases are converted into AA-tRNAs and 'captured' on the microspot by the EF-Tu;GTP. Amino acids are detected by laser induced fluorescence. The amount of AA-tRNA captured on each microspot, and hence the amount of amino acid in the sample, is determined by laser-induced fluorescence of the captured AA-tRNA.

Fig. 3 generally illustrates a spatially resolved detection system for amino acid analysis microarrays of the present invention. Patterned arrays of biorecognition elements (synthetases, or tRNAs) are immobilized on a surface at known spatially resolved locations. Single letter codes for amino acids represent the synthetases or tRNAs specific for each amino acid. Each patterned microdot signals its cognate amino acid after it is captured into the EF-Tu;GTP-AA-tRNA complex as described above. In cases where the tRNAs are immobilized as microdots, the elongation factors can be fluorescently labeled. And where synthetases and elongation factors are immobilized as microdots, the tRNAs are fluorescently labeled. Upon excitation of the fluorescent labels, a spatially specific detector linked to a computer allows analysis of the pattern

and intensity of fluorescently labeled molecules on the surface. As shown, the fluorescence is imaged through the microscope, and the detector is a high-sensitivity CCD camera. Fluorescence intensity on the arrays is correlated with the amount of amino acids in the sample, and the position of the fluorescent label on the surface identifies the amino acid. Thousands of samples  
5 can be analyzed on a single microarray.

Fig. 4 is a schematic diagram of an evanescent-wave optical fiber C-terminal nanosequencer. Each synthetase is co-immobilized with EF-Tu;GTP on a different optical fiber. The tRNAs are labeled with fluorescent tags. The formation of the ternary complex (AA-tRNA-  
10 EF-Tu;GTP) on the sensor surface is monitored continuously by fluorescence detection. The biorecognition elements (synthetases, tRNAs, and elongation factors) is optionally enclosed by a semipermeable membrane having a molecular weight cutoff that allows free passage of amino acids but is impermeable to macromolecules. These miniaturized optical fiber amino acid biosensor arrays are coupled with aminopeptidases or carboxypeptidases for protein end group  
15 amino acid sequencing and amino acid analysis. Fluorescently labeled AA-tRNAs are captured on the sensors' surface in response to amino acids cognate to the synthetase immobilized at each of the 20 fiber ends. Excitation and emission light travels through each fiber. Emission light is imaged through a microscope objective onto a CCD detector for continuous detection. The increase in fluorescence on each fibers' end is plotted as a function of time to generate the  
20 terminal sequence of the protein being sequenced.

Figs. 5A, 5B, and 5C generally illustrate a process of continuous-flow protein sequencing on a chip, as described herein. Fig. 5A is a schematic drawing of a continuous flow C-terminal nanosequencer. The carboxypeptidase digestion takes place in a tiny chamber, and free amino  
25 acids are collected by a microdialysis or ultrafiltration probe. As the amino acids are released by the carboxypeptidase, they cross the membrane and enter the flow stream. This flow stream enters a continuous flow mixer and then an array of 20 reaction chambers or channels. For simplicity, only one reaction chamber is shown. In each of the 20 reaction channels or chambers, the flow stream is joined by a second flow stream carrying a fluorescently labeled tRNA specific

for a different amino acid and the cognate synthetase. EF-Tu;GTP is immobilized downstream in each reaction channel. Amino acids cognate for the labeled tRNA in each channel or chamber are converted into a proportionate amount of AA-tRNA, which is captured by the immobilized EF-Tu;GTP. A laser beam focused on the immobilized EF-Tu;GTP allows detection of the fluorescently labeled AA-tRNAs as they bind the elongation factor. The emitted light is imaged to a CCD detector, and analysis of the record of the binding of AA-tRNAs to EF-Tu;GTP in the 20 chambers yields the C-terminal sequence of the protein.

Fig. 5B further illustrates the continuous flow C-terminal nanosequencer of the present invention. In this format, each of the 20 synthetases is immobilized in a separate reaction channel. Amino acids sequentially liberated from the protein's end traverse the microdialysis probe and enter the flow stream which is joined by a second flow stream carrying fluorescently labeled tRNAs (one cognate for each of the 20 primary amino acids) in reaction buffer. The two flows are mixed on the chip and enter the reaction channel array. In each reaction channel, amino acids having a cognate synthetase immobilized are converted into a proportionate amount of AA-tRNA. The newly formed AA-tRNAs bind to EF-Tu;GTP immobilized downstream and are continuously detected as described above.

Fig. 5C is a schematic drawing of a flow optical fiber nanosequencer of the present invention. The two flow streams are combined and mixed on the chip. One stream contains exopeptidase digestion buffer and is pumped through a microdialysis probe that traverses a tiny digestion chamber. Liberated amino acids cross the microdialysis membrane and enter the flow stream. The second flow stream contains a mixture of fluorescently labeled tRNAs (one specific for each amino acid) in reaction buffer. The two flows are combined and mixed on the chip. This stream containing the mixture of amino acids and fluorescently labeled tRNAs flows through the biosensor array. Amino acids are attached to their cognate tRNAs by the immobilized synthetases and bind co-immobilized elongation factors. The bound AA-tRNAs are then quantitated by fluorescence detection.

Figs. 6A-6C illustrate three ways for the immobilization of amino acid analysis biorecognition elements onto surfaces as arrays, e.g., microwells, microchannels or microspots. In Fig. 6A, the synthetase and elongation factor are co-immobilized to the surface using a single polypeptide tether which contains a defined epitope in the approximately central region. The epitope is bound by an antibody affixed to the surface of the assay locus at a known location. The antibodies may be arrayed onto surfaces using known methods [see, for example, Patel et al. (2000) *J. Biomater.Sci. Polym. Ed.* 11, 319-331; Morozov et al. (1999) *Anal.Chem.* 71, 3110-3117; Lueking et al. (1999) *Anal. Biochem.* 270, 103-111]. The antibodies may be arrayed onto surfaces using known methods [for example, see Patel et al. (2000) *J. Biomater. Sci. Polym. Ed.* 11, 319-331; Morozov et al. (1999) *Anal. Chem.* 71, 3110-3117; Lueking et al. (1999) *Anal. Biochem.* 270, 103-111].

Each of the 20 synthetases may be co-immobilized with elongation factors in this way having a different epitope tag recognized by a unique antibody for each of 20 synthetases. The 20 antibodies may then be arrayed at known positions at high spatial resolution. Hence, each of the 20 synthetase-elongation factor pairs will be immobilized at a known position by binding to its specific antibody. Functional groups for the covalent attachment of the peptide to the synthetase and elongation factor may occupy the N- and C-terminal positions of the peptide tethers. Amino and thiol groups are especially useful for the covalent attachment of proteins to surfaces including peptides. A suitable tether may therefore have amino groups (e.g. lysines) or thiol groups (e.g. cysteines) at the N- and C-termini. Suitable peptide tethers should be composed of polar amino acids and be flexible. For example, tethers composed of glycine and serine would be uncharged, polar and flexible. Commercially available crosslinking reagents will be useful in the conjugation of the synthetases and elongation factors to tethers. Alternatively, the peptide tethers may be added to the synthetase/elongation factor pairs by the construction of elongation factor-synthetase fusion proteins. [for review see Nilsson, J et al (1997) *Protein Expr Purif* 11, 1-16].

In fig. 6B the synthetase and elongation factor are joined to one end of tethers; at the other end of each tether is a biotin molecule. The biotin-avidin or biotin-streptavidin system is

used to co-immobilize the synthetases and elongation factor. Since each streptavidin has four binding sites for biotin, the streptavidin may be used to co-immobilize the elongation factor and synthetases in close proximity. This will facilitate the rapid capture of the AA-tRNAs formed by the synthetases by the co-immobilized elongation factor. Flexible tethers or spacer arms will prevent steric problems and allow the immobilized proteins to interact with their macromolecular substrate. The biotin-streptavidin co-immobilized synthetase-elongation factor pair may be immobilized onto a surface including biosensor transducers. This may be achieved, for example, by binding to an immobilized biotin molecule. Alternatively, as shown schematically, a peptide bearing unique epitope may be conjugated to the streptavidin and used to immobilize the each of the 20 synthetase-elongation factor pairs on an array of 20 antibodies as described above. In an alternative approach, the avidin or streptavidin may be attached to the elongation factor or synthetases by making elongation factor-avidin or synthetase-avidin fusion proteins. The avidin fusion proteins may be constructed as taught in Airene et al. (1999) *Biomol. Eng.* 16, 87-92.

In Fig. 6C each of the 20 synthetases are co-immobilized with the elongation factors as described in Fig 6B above. In this embodiment, a different oligonucleotide having a unique sequence is conjugated to the synthetase-elongation factor pairs. The conjugates are then arrayed at known positions by the hybridization of the conjugated oligonucleotides to their complimentary oligonucleotides which are immobilized onto surfaces in known positions. Methods for arraying oligonucleotides are well known in the arts.

In Fig. 6B, the synthetase and elongation factor are each joined to one end of a tether; at the other end of each tether molecule is a ligand, such as biotin. These moieties are indirectly linked to the assay surface by an antibody which binds to an epitope on a tether molecule, which carries the ligand binding partner at the other (e.g., avidin or streptavidin). In Fig. 6C, each synthetase and the elongation factor are again indirectly linked to the surface, but in this embodiment, there is an oligonucleotide immobilized directly on the assay surface, and a complementary oligonucleotide linked to the ligand binding partner mediates attachment.

Figs. 7A-7B show two modes for ultrasensitive amino acid analysis in continuous microflow displacement systems. An array of 20 channels, each perfused with a separate reaction mixture containing a different synthetase, its cognate tRNA and EF-Tu;GTP, permits the continuous, simultaneous analysis of the 20 amino acids on a microscale. For simplicity, only one channel is shown. A ternary complex probe, a molecule or substance that specifically and reversibly binds the ternary complex, is immobilized in each reaction channel. A known density of labeled ternary complex is bound to the ternary complex probe. The ternary complex may be further stabilized by using a nonhydrolyzable GTP analog. Ternary complex probes can include, without limitation, antibodies, antibody fragments, oligonucleotides, peptide nucleic acids, proteins, peptides, carbohydrates ribosomes, ribosome fragments or any other substance binding the ternary complex and permitting a displacement assay. The reaction channels have an inlet for sample containing amino acids, an outlet, and the channels are in fluid connection with at least one reservoir. A reaction solution containing a synthetase, its cognate tRNA and EF-Tu;GTP is pumped continuously through each reaction channel by microfluidic pumping. A different synthetase and cognate tRNA along with EF-Tu;GTP is therefore pumped through each of the 20 reaction channels. The amino acid sample is pumped through the 20 reaction channel from a main channel or capillary in fluid connection with all 20 inlets. As the mixture of amino acids flows into the reaction channel array, amino acids having a cognate synthetase and tRNA in each reaction channel are converted into AA-tRNAs which then bind the EF-Tu;GTP to form a stable ternary complex (EF-Tu;GTP-AA-tRNA). The newly formed ternary complex then displaces a proportionate amount of labeled ternary complex that is reversibly immobilized in each microchannel. The displaced labeled ternary complex then flows past an integrated and computer controlled spatially specific detector and is continuously detected.

In Fig. 7A a continuous microflow ELISA for amino acid analysis is described. The ternary complex is labeled with an enzyme that can catalyze an amplification reaction (one enzyme molecule yields many detectable product molecules). Enzymes may be used that convert a nonfluorescent substrate into a fluorescent product. Alternatively, enzymes that produce luminescent or electrogenic products may be employed. Enzymes that may be used to label the

ternary complex (or ternary complex probes) include, but are not limited to, alkaline phosphatase and horseradish peroxidase. Examples of fluorogenic substrates for alkaline phosphatase include Attophos and 5-fluorosalicyl phosphate. A fluorogenic substrate for horseradish peroxidase is 3-p-hydroxyphenyl-propionic acid. Alkaline phosphatase and horseradish peroxidase may also be used as enzyme labels for electrogenic and chemiluminescent substrates. For alkaline phosphatase and horseradish peroxidase 1,2-dioxetane arylphosphate, and 4-iodophenol-enhanced luminol are excellent chemiluminescent substrates and achieve attomole-zeptomole detection limits. As shown, the enzyme labeled ternary complex is reversibly immobilized in the reaction channel by binding to the immobilized ternary complex probe. A reaction mixture containing a specific synthetase, its cognate tRNA, and EF-Tu;GTP is continuously pumped through each reaction channel by microfluidic pumps. As the amino acid sample enters the reaction channel array, as described above, the amino acid having a cognate synthetase and tRNA in each channel will be converted into a proportionate amount of ternary complex that will displace a proportionate amount of enzyme labeled ternary complex. The displaced labeled ternary complex flows down stream where the flow is joined by a second flow stream. This second flow contains the substrate for the enzyme label in reaction buffer and is transported from a separate reservoir in fluid connection with the reaction by microfluidic pumping as shown. The reaction products of the enzyme label are continuously detected down stream by an integrated spatially specific detector(s). The type of detector will depend on the enzyme substrate used. For example, if a fluorogenic substrate is employed laser induced fluorescence detection may be achieved using a confocal scanner or CCD detector. With spatially specific detectors, the amino acids will be identified by the position of the channel sending the signal to the detector. The 20 amino acids will thereby be identified and quantitated.

Fig. 7B shows an ultrasensitive amino acid analyzer using a continuous microflow displacement format and laser induced fluorescence detection. Multiple labels may be used to increase the sensitivity of the assay. Labeled ternary complex is reversibly immobilized in each reaction channel and each reaction channel has an amino acid inlet, an outlet, and is in fluid connection to a unique reservoir containing a specific synthetase, cognate tRNA, and EF-Tu;GTP



as described above. As the amino acid sample flows through the reaction channel(s) each amino acid reacts with its synthetase and cognate tRNA forming AA-tRNAs that bind the elongation factor forming a ternary complex. The newly formed ternary complex will displace a proportionate amount of fluorescently labeled ternary complex, which will flow down stream and pass through a laser beam. The emission light will pass through optical filters and be continuously detected using a spatially specific detector (e.g. high-sensitivity CCD camera) linked to a computer. The amount of amino acid cognate to the synthetase and tRNA in each channel will be proportional to the amount of fluorescent label flowing past the detector. Since each microchannel is perfused with a different one of the 20 synthetase and its cognate tRNA, each reaction channel will signal a different amino acid, the one cognate for the synthetase and tRNA present. The location of each channel in the array will allow the identification of the 20 amino acids. A spatially specific detector such as a high-sensitivity CCD camera or a confocal scanner will permit spatially resolved detection of the 20 amino acids simultaneously and continuously with a sensitivity orders of magnitude greater than current amino acid analyzers. Indeed, fluorescently labeled molecules have been detected quantitatively in continuous flow at the single molecule level.

Figs. 8A-8C illustrate the application of microarray technology to amino acid analytical methods of the present invention. Fig. 8A shows the general microarray assay, in which an epifluorescent microscope and a CCD camera detect the light emitted in the assay by individual spots of defined location on a high density array amino acid analysis microchip. A linked computer decodes the signals to provide the amino acid quantitative data/or sequence analysis data when the arrays are coupled to carboxypeptidases or aminopeptidases. Arrays may be covered with a membrane (e.g. a microdialysis membrane) having a molecular weight cutoff that allows free passage to amino acids but is impermeable to macromolecules. Peptides or small proteins to be sequenced are conjugated to a surface (e.g. a polymer such as dextran) to prevent their passage through the membrane. As shown, fluorescently labeled tRNAs are captured in synthetase arrays and fluorescently labeled elongation factors are captured in tRNA microarrays. A tiny drop of exopeptidase digestion buffer containing the protein to be sequenced and the

exopeptidase (e.g. carboxypeptidase(s) for C-terminal sequencing) are placed on the membrane over each array. The arrays can be patterned on the surfaces of optical waveguides, and evanescent wave excitation can be employed for excitation of captured fluorescent probes. The formation of the EF-Tu:GTP-AA-tRNA ternary complexes may be followed continuously by following the position-dependent increase in fluorescence on the waveguides' surface using a CCD detector. Alternatively, the excitation and emitted light can be transported to/from each microspot through optical fibers. The emission light passes through optical filters and can be imaged through a microscope objective to a CCD detector for continuous detection.

Fig. 8B shows an embodiment where each synthetase and the elongation factor are indirectly immobilized as microspots via a ligand-ligand binding system.

Fig. 8C shows an embodiment in which 20 tRNAs, one specific for each of the protein amino acids, are arrayed as microspots. Amino acids in the sample are attached to the tRNAs by their cognate synthetases, and fluorescently labeled EF-Tu;GTP binds the arrayed AA-tRNAs.

Fig. 8D illustrates an embodiment of a high density C-terminal end group analysis microarray. The 20 aminoacyl-tRNA synthetases are coimmobilized with the elongation factor to a different microspot. The carboxypeptidases are immobilized to a different zone on the same surface, and the protein substrate is added to each well by a nanopipetting robot. Liberated amino acids are converted to AA-tRNAs and captured on the surface of each microspot and the amount of AA-tRNA on each spot is quantified by fluorescence detection. Alternatively, the carboxypeptidases and protein substrate are immobilized to a bead that is placed in the synthetase array as shown schematically in Fig. 8D. Evanescent wave excitation can be employed so that the formation of the ternary complex on the microspots is monitored continuously without a wash step.

Fig. 9 schematically illustrates an integrated microsystem for end group protein sequencing using a continuous flow displacement scheme for amino acid analysis. An array of

20 microfluidic reaction channels is employed to detect and quantitate the 20 protein amino acids. Only one reaction channel is shown in the figure. In each reaction channel a known density of ternary complex probe (e.g. an antibody that specifically recognizes the ternary complex) is immobilized and saturated with fluorescently labeled ternary complex.

5 Alternatively, the ternary complex is immobilized and saturated with a fluorescently labeled ternary complex antibody. Each reaction microchannel is perfused with a reaction solution containing a different one of the 20 synthetases, its cognate tRNA and the elongation factor complexed with GTP (or a nonhydrolyzable GTP analog). Each of the 20 reaction solutions is transported from a separate reservoir that is in fluid connection with a reaction channel by  
10 microfluidic pumping. Each of the 20 reaction channels has an inlet in fluid connection to a main channel or chamber. As shown, carboxypeptidase digest of the protein substrate is carried out in an enclosed ultrafiltration membrane placed in a flow stream. The membrane is impermeable to macromolecules but allows free passage of amino acids. As amino acids enter the reaction channels, those amino acids having a cognate synthetase and tRNA in each channel  
15 are converted into a proportionate amount of AA-tRNA which then binds the elongation factors, forming the ternary complex (AA-tRNA-EF-Tu;GTP). This newly formed ternary complex displaces a proportionate amount of the reversibly bound fluorescently labeled ternary complex, the displaced complex then flows through a laser beam to excite the fluors, and the fluorescence is continuously detected. A spatially specific detector (e.g. a high-sensitivity CCD detector)  
20 integrated with a computer continuously records the emission light intensities in all channels. The record of the displaced fluorescently labeled ternary complexes going through the focused laser beam in the 20 channels is recorded, and the computer in communication with the detector transforms this data to compute the amino acid sequence of the protein.

Fig. 10 is a diagram of an apparatus for amino acid analysis and end group protein  
25 sequencing in an integrated microfluidic system where a CCD detector serves as an active support to quantitatively detect and image the distribution of labeled target molecules near the spatially addressable pixels. Binding of fluorescently or radiolabeled AA-tRNAs to elongation factors is continuously detected with integrated data acquisition and computation to generate a

sequence.

Fig. 11 is a diagram of an apparatus for amino acid analysis in which a confocal scanner is used to detect signal information in the microchannels.

5 Figs. 12A and 12B are schematic drawings of a microflow system for amino acid analysis using an integrated computer controlled wash step and a laser scanner and fluorescent detector. Computer controlled microfluidic pumps and valves are employed to periodically switch from reaction buffer to wash buffer before the immobilized capture reagent is excited by the laser to generate and read the fluorescent signals from the captured fluorescently labeled molecule over time.

10 Fig. 12 C provides a schematic summary of amino acid analysis using microchannels and detection of fluorescent light using optical fibers to deliver the evanescent wave excitation and carry the emission light to the detector. The elongation factor;GTP is immobilized to the tips of 20 optical fibers, and each fiber is inserted into a different reaction channel, each of which is specific for a particular one of the 20 primary amino acids. Each channel is in fluid connection  
15 with a different reservoir from which a reaction mixture containing a different synthetase, and fluorescently labeled tRNA as described above. As the sample containing amino acids flows into the reaction channel array, the amino acid having a cognate synthetase and tRNA in the channel is converted into fluorescently labeled AA-tRNA which then binds the elongation factor immobilized on the optical fibers. Upon excitation with a light of the appropriate wavelength,  
20 for example, laser light, the signal generated are quantitatively analyzed continuously by fluorescent detection. The signals from the optical fibers may be imaged through a microscope objective onto a CCD detector integrated with a computer where the signal information is decoded and amino acid quantities and/or sequence is generated.

25 Figs. 13A-13B illustrate the embodiment of the present amino acid analysis in which the flow channel has a scintillator surface and the label in the system is a radioactive compound. As

specifically exemplified, a radiolabeled tRNA of a particular specificity is used, together with the cognate synthetase, in each microchannel. Elongation factor is immobilized on the interior surface of the microchannel. When a labeled AA-tRNA is captured on the surface by the immobilized elongation factor, there is continuous signal generation, and a CCD detects the signal and the data are fed to a computer. In Fig. 13B, there are miniature light detectors in the bottom of each flow channel, and the signals due to light emitted as a result of the radiolabeled molecule bound within the channel are continuously monitored and decoded to provide quantitative and/or amino acid sequence information where the amino acid detection is integrated with exopeptidases as described above.

Fig. 14 provides a schematic illustration of the embodiment wherein the assays are carried out on the surfaces of nanoparticles (beads) which are of twenty distinguishable types, each having a distinctive signal generated when the ternary complex of an AA-tRNA and elongation factor are formed. tRNAs can be labeled with a donor fluor and the elongation factors can be labeled with an acceptor fluor, and a fluorescence energy transfer type of assay can be used to monitor formation of the ternary complex. The signals are decoded by an associated computer to give quantitative data and/or amino acid sequence information.

Fig. 15 is a schematic illustration of an amino acid optical fiber biosensor. The amino acid biosensor uses immobilized elongation factor (e.g., EF- Tu;GTP) to bind and detect fluorescently labeled AA-tRNAs. The elongation factor is immobilized on an optical fiber placed within a microchannel. When complexed with GTP, the elongation factor selectively binds all AA-tRNAs, which are produced in amounts proportional to the amount of each particular amino acid in the sample. This biosensor is suitable for the analysis of any one of the 20 protein amino acids up to all 20 when engineered as an array. Each reaction channel is in fluid communication with a microchannel delivering a continuous flow of reaction solution containing a synthetase and its cognate fluorescently labeled tRNA specific for the particular amino acid being analyzed in each reaction channel. Optical fibers are used to excite and detect fluorescence in the evanescent field so that only the surface bound fluorescent molecules are

detected. This allows continuous and real time amino acid detection without a wash step. The elongation factor binds the AA-tRNAs only when complexed to GTP but not GDP. Hence, the bound AA-tRNAs can be eluted with a buffer containing GDP. Other elution buffers are those containing high salt (e.g. 1 M NaCl). After the AA-tRNA are eluted, the immobilized elongation factors are reactivated by washing by a regeneration buffer containing GTP and the biosensors can be reused. The binding of the fluorescently labeled AA-tRNAs to the immobilized elongation factor can be monitored in arrays of microchannels simultaneously, continuously, and in tiny volumes, i.e., picoliters).

Fig. 16 illustrates an ultrasensitive solid phase immunoassay for amino acid analysis. The steps are coupling of a specific ternary complex probe (e.g., an antibody or aptamer that specifically binds the ternary complex, EF-Tu;GTP-AA-tRNA) to a solid support (e.g., the bottom of a microtiter plate well or a microflow channel), addition of an amino acid-containing sample along with a synthetase and cognate tRNA specific for each amino acid being analyzed in each well or chamber, and elongation factor complexed with GTP, and, after a suitable incubation time, washing to remove unbound compounds, and addition of a labeled second probe that specifically binds the captured complex along with the enzyme's substrate, in cases where enzyme labels are employed. The detection probe is labeled with a high specific activity label such as a fluor or radioisotope, or it can be labeled with an enzyme that catalyzes an amplification reaction to create an ultrasensitive enzyme-linked ligand assay for amino acid analysis. Examples of labeled probes for detection include antibodies that recognize the captured ternary complex or oligonucleotides or peptide nucleic acid probes that hybridize to the single stranded regions of the captured AA-tRNAs. Enzyme labels where one enzyme produces many labels typically achieve attomole-zeptomole detection limits. Competitive, noncompetitive, displacement, and homogeneous ternary complex immunoassays are useful for the amino acid analytical methods and devices of the present invention.

Fig. 17 illustrates a microsystem for amino acid analysis with direct spatially resolved CCD detection of the formation of the ternary complex on a microchip detector. Biomolecular

probes (e.g. elongation factors, synthetases, antibodies, or tRNAs) are immobilized directly onto the pixels of a CCD camera, or they can be attached to a cover slip placed on the surface of a CCD. As shown, the 20 different tRNAs, one specific for each of the 20 amino acids, are immobilized, and the labeled (e.g. fluorescently or radiolabeled) elongation factors are captured  
5 by binding to the newly formed AA-tRNAs after synthetases attach their cognate amino acids to the immobilized tRNAs forming AA-tRNAs. Alternatively, the tRNAs can be labeled and the elongation factors immobilized. Each amino acid is attached to its cognate tRNA by its specific synthetase, and the newly formed AA-tRNAs bind the immobilized elongation factors and are detected. Upon binding of the labeled molecule to the CCD array, photons or radioisotope decay  
10 products are emitted at the pixel locations where the labeled molecule has bound. Electron-hole pairs are generated in the silicon when the charged particles or radiation from the labeled molecule are incident on the CCD gates. Electrons collected beneath adjacent CCD gates are sequentially read out on a display module. The number of photoelectrons generated at each pixel is directly proportional to the number of labeled molecules bound and hence the amino acid  
15 cognate to the synthetase and tRNAs in each channel or microwell. Other proximity assays such as scintillation proximity assays for amino acid analysis are within the scope of the present invention.

Fig. 18 illustrates the situation wherein the polypeptide or peptide to be synthesized and the carboxypeptidases are co-immobilized on the same surface via flexible tethers. With this  
20 approach, the digestion chamber can be made very tiny (e.g., 1 microliter or less). The long flexible tethers allow the substrate protein to be confined to the surface but have freedom to move about the surface by diffusion and collide with the immobilized carboxypeptidase(s). Even though the absolute amount of the target protein to be sequenced is low, the concentration at the surface is high. Hence, the immobilized protein substrate does not need to diffuse very far  
25 before it collides with a carboxypeptidase, and the reaction rates are increased in comparison to what it occurs with enzyme and substrate free in solution. Suitable tethers include, without limitation, dextrans and nucleic acid strands. The particles can include beads (e.g., magnetic nanoparticles) or other surfaces. Phospholipid vesicles or liposomes can be used as surfaces for

the co-immobilization of exopeptidase and substrate protein or peptides. As digestion of the target protein or peptide occurs, an "effluent stream" of liberated amino acids flows into the detector arrays, which are in fluid communication with the digestion chamber. In addition, the co-immobilized enzymes are generally not substrates in the digestion mixtures.

5           The proteins and peptides are conjugated to the surface of vesicles using known methods. For example, they may be tethered covalently to the phosphatidylethanolamine in liposomes using amino reactive coupling. Phospholipid vesicles permit diffusion within the plane of the bilayer and will therefore permit frequent collisions of the co-immobilized elements. This ensures suitable reaction rates. These particles can be dispensed into wells containing the  
10 arrayed biorecognition elements for amino acid analysis described above or they can flow into microchannels for amino acylation reactions, ternary complex formation and signal detection and decoding. This type of assay permits end group amino acid sequence analysis using the high-density amino acid analysis microarrays described above. Alternatively, they can be  
15 immobilized in flow streams with the effluent stream of liberated amino acids flowing through amino acid detection microfluidic arrays. Carboxypeptidases can be immobilized via the C-terminus, or the C-terminus can be blocked (for example, by conjugation of the C-terminus with biotin) to prevent autolysis.

Figs. 19A and 19B are schematic drawings of a microsystem amino acid analyzer using electrochemical detection. A continuous flow displacement format is shown. In Fig. 19A the  
20 ternary complex is labeled with an enzyme label and reversibly bound inside a microchannel using an antibody that specifically binds the ternary complex. The reaction channel has an inlet for amino acids, an outlet, and is in fluid connection with two reservoirs. A reaction mixture containing a specific synthetase, cognate tRNA and EF-Tu;GTP is continuously pumped through the reaction channel from one reservoir. As amino acids flow into the reaction channel, those  
25 having a cognate synthetase and tRNA in the channel are converted into AA-tRNAs which bind the EF-Tu;GTP to form a ternary complex. The newly formed ternary complex displaces a proportionate amount of enzyme-labeled immobilized ternary complex that then flows



downstream. The substrate for the enzyme label in a suitable buffer is pumped through the reaction channel downstream of the immobilized labeled ternary complexes. As the displaced enzyme labeled ternary complexes flow through the zones having its substrate, the enzyme generates its electrochemically detectable substrate, which is detected at the microelectrode placed in the flow channel. Microelectrode arrays are constructed for the continuous detection of all 20 primary amino acids simultaneously by using 20 reaction channels, each perfused with a different synthetase and cognate tRNA.

Fig.19 B shows an amino acid analyzer microbiosensor that uses continuous flow displacement with electrochemical detection. An antibody that specifically and reversibly binds the ternary complex is immobilized on the working electrode. The antibody is saturated with ternary complex labeled with an enzyme that catalyzes a reaction that produces a product that can be detected electrochemically. A reaction mixture containing substrate for the enzyme along with a specific synthetase, cognate tRNA and EF-Tu;GTP is pumped through the reaction channel from a reservoir using microfluidic pumping. Amino acids enter the channel, and those having a cognate synthetase and tRNA are converted into ternary complexes that displace a proportionate amount of the labeled ternary complex from the microelectrode. This results in a proportionate decrease in signal at the microelectrode. The decrease in signal at the microelectrode is proportionate to the concentration of the amino acid having a cognate synthetase and tRNA in the channel. As above, using the microelectrode arrays and methods of the present invention facilitates the analysis of all 20 primary protein amino acids simultaneously.

Nonenzyme electrochemical labels can also be conjugated to the tRNAs or elongation factors and used for electrochemical detection. For example, ferrocene is a electrochemical label that may be conjugated to the elongation factor or tRNAs. Takenaka et al. (1994) Anal. Biochem. 218:436-443 describe a method for the conjugation of ferrocene to DNA and an electrochemical detection system that allows femtomole detection of labeled DNA probes binding to complementary sequences.

Fig. 20 is a schematic drawing of a continuous flow enzymatic amino acid analyzer and end group sequenator using optical detection. The 20 synthetase reactions are arrayed into 20 reaction channels or capillaries in fluid connection with a central circular chamber. Each of the 20 reaction channels is in fluid connection to a separate reservoir containing a unique synthetase and optionally, a cognate tRNA and EF-Tu;GTP in reaction buffer. The exopeptidase digestion chamber is placed in a flow stream that connects with the central circular chamber having the 20 inlets to the reaction channels. The digestion chamber can be a sealed hollow fiber having a molecular weight cutoff that allows free amino acids to pass freely but is impermeable to macromolecules. Alternatively, the protein substrates can be co-immobilized with the exopeptidases on a surface (as in Fig. 18) and held in the flow stream by a porous support. As the amino acids are liberated from the protein's terminus by the exopeptidase(s), they flow through the digestion chamber and past a continuous flow mixer. The amino acids flow through the 20 channels. The amino acid having a cognate synthetase in a particular channel reacts to form a proportionate amount of product. In this embodiment the flows are driven by centrifugal force as the system spins. A light source and computer linked detector are integrated into the system. The product of the synthetase reactions are continuously monitored by the detector. The detector is a scanning spectrophotometer or fluorometer, depending upon the assay used to follow the synthetase reactions. The amino acids are identified from the location of the chamber in the array giving the signal.

Figs. 21A-21B illustrate a computer-controlled integrated microsystem for amino acid analysis and protein end group sequencing using affinity capture and detection of labeled AA-tRNAs on immobilized EF-Tu:GPT with automated calibration, washing, detection, elution, and regeneration of the capture site. The elongation factor is immobilized in the microflow channel as described above. Briefly, the elongation factor is expressed as a fusion protein having a C-terminal flexible tether (optimally composed of glycine residues to ensure flexibility and neutral polar amino acid residues such as serine) having an attachment site (e.g., a C-terminal cystine for thiol specific coupling to surfaces or a hexa-His tag or other ligand specific tag). The elongation

factors are optionally immobilized onto transducers such as optical fibers or microelectrodes that are positioned in the microflow channel. The automated system allows automated calibration with known concentrations of amino acids pumped through the microfluidic array from calibration reservoirs by microfluidic pumping, prewashing to remove unbound or weakly bound material, detection, elution of the labeled tRNAs with an elution buffer, and regeneration of the capture sites with a regeneration buffer all under computer control. All fluidic handling (volumes and flow rate of respective solutions) and data acquisition or image acquisition (series of fluorescent images) are synchronized by means of a computer. The wash buffer reservoir, calibration reservoirs, elution buffer reservoirs, and regeneration buffer reservoirs are in fluid connection with the microfluidic array such that solutions from these reservoirs are pumped through the microfluidic reaction channel arrays as shown in cases where a labeled probe (for example fluorescently labeled oligonucleotides or peptide oligonucleotides that bind to the captures AA-tRNAs) are used for detection, additional reservoirs are added to automatically deliver the probes. An amino acid sample inlet port is also depicted. The 20 reaction solution reservoirs joining each reaction channel in the array as described above (Fig. 5A) are not shown. The digestion chamber (not shown) is as described above, and amino acids flow continuously from the digestion chamber through the main channel. A computer controlled valve diverts the flow of the amino acid mixture pumped from the digestion chamber into a waste reservoir during the automated steps of washing, detection, elution, and capture site regeneration. An additional reaction channel can be added to serve as a control. In this case the reaction solution reservoir is the source of reaction solution buffer through the control channel without a synthetase, therefore, no AA-tRNAs are formed in the control channel.

Fig. 22 is a schematic diagram of microsystem for amino acid analysis with integrated on chip enzymatic hydrolysis. Enzymatic hydrolysis of the protein or peptide sample occurs on-chip in a separate peptidase digestion chamber. The digestion chamber is in fluid connection with the amino acid analysis microfluidic arrays. A mixture of endopeptidases and exopeptidases are employed to achieve total hydrolysis. The proteases are immobilized on beads and placed into the digestion chamber or optionally are immobilized on the bottom of the

chamber. In cases where polypeptides and peptides are analyzed, it is sufficient to use a mixture of carboxypeptidases and aminopeptidases to achieve total hydrolysis. In cases where larger proteins are analyzed, other proteases are immobilized in the digestion chambers along with the carboxypeptidases and aminopeptidases. Commercially available proteases that may be used in this system to achieve total enzymatic hydrolysis include but are not limited to, carboxypeptidase Y, carboxypeptidase P, carboxypeptidase A, aminopeptidase M, aminopeptidase L, trypsin, subtilisin, proteinase k, pepsin, papain, endoproteinase Glu-C, (proteinase V8), endoproteinase Asp-N, endoproteinase, Arg-C, elastase, collagenase, chymotrypsin, cathepsin C, acylamino acid peptidase. The peptidase digestion occurs in the microfabricated digestion chamber that is in fluid connection with the amino acid detection microfluidic array as described above. The digestion is sealed from the flow stream connecting it to the microfluidic amino acid detection array using valves that are opened and closed by a computer. A circular digestion chamber is shown which may have circular pumping of the digestion buffer thereby providing convection within the channel to facilitate rapid and complete digestion. After the digestion has been completed, which can be determined by having an optical detector integrated into the digestion chamber, the amino acid mixture is pumped from the digestion chamber through the amino acid analysis microfluidic array using microfluidic pumping. The amino acids are analyzed on the chip as described above (e.g., in Fig. 5A).

Fig. 23 is a schematic illustration of a single amino acid affinity biosensor. The specific example is detection of phenylalanine in a sample. The elongation factor is immobilized on an optical fiber probe, and a synthetase and fluorescently labeled tRNA cognate for the amino acid being analyzed by the sensor is held next to the sensor by semipermeable membrane. Amino acids cross the semipermeable membrane into the biosensor containing phenylalanine aminoacyl-tRNA synthetase and fluorescently labeled phenylalanine tRNA. The synthetase attaches the phenylalanine to its cognate tRNA. The newly formed AA-tRNA specifically binds the immobilized elongation factor and is captured on the surface and detected. The membrane has a molecular weight cutoff such that amino acids can freely cross the membrane but macromolecules (tRNAs and synthetases) cannot. This simple affinity biosensor format can be

used for any of the 20 naturally occurring amino acids by substituting the appropriate cognate synthetase and tRNA in place of the phenylalanine specific synthetases and tRNAs.

## DETAILED DESCRIPTION OF THE INVENTION

5           The present invention will now be described more fully hereinafter with reference to the accompanying drawings, in which preferred embodiments of the invention are shown. This invention may, however, be embodied in different forms and should not be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art.

10           Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Each publication, patent application, patent, and other reference cited herein is incorporated by reference in its entirety to the extent that it is not inconsistent with the present disclosure.

15           The twenty "primary" amino acids which commonly occur in proteins have their usual three- and one-letter abbreviations routinely used in the art: A, Ala, Alanine; C, Cys, Cysteine; D, Asp, Aspartic Acid; E, Glu, Glutamic Acid; F, Phe, Phenylalanine; G, Gly, Glycine; H, His, Histidine; In, Ile, Isoleucine; K, Lys, Lysine; L, Leu, Leucine; M, Met, Methionine; N, Asn, Asparagine; P, Pro, Proline; Q, Gln, Glutamine; R, Arg, Arginine; S, Ser, Serine; T, Thr, Threonine; V, Val, Valine; W, Try, Tryptophan; Y, Tyr, Tyrosine.

20           The term binder or biorecognition element or molecule is used herein to refer to a molecule that specifically binds another molecule or substance. The term ligand is used interchangeably with binder. Preferred binders may be any biomolecule or fragment thereof and

drugs. Examples of binders include but are not limited to proteins (especially antibodies and receptors and elongation factors) and fragments thereof which retain the binding activity and specificity of the native molecule, carbohydrates, drugs, metals, cofactors, lipids, metals, metal chelators, peptides, nucleic acids, peptides, peptide nucleic acids, hormones, inhibitors, dyes, amino acids, and nucleotides. The analyte binder is the binder being analyzed. The binding elements that reversibly bind the analyte binders and labeled analyte binder analogs are referred to as "capture binders" herein.

In the present context, a reaction fluid comprises an appropriate buffer, at least one amino acyl tRNA synthetase and at least one cognate tRNA.

As used herein cognate refers to the specific relationship between an amino acyl tRNA synthetase and a tRNA, i.e., they are specific for the same primary amino acid.

A sample, as used herein, can be any material in which the determination of the presence or quantity of at least one primary amino acid is carried out. The sample can be a biological sample including, but not limited to, blood, serum, cerebrospinal fluid, fermentation broth, proteolytic digest or cell culture medium. Pharmaceutical and nutritional supplement compositions are also within the scope of "sample".

As used herein, a molecular sieve separates molecules according to size. A molecular sieve can include a selectively permeable membrane, a microdialysis membrane or a microdialysis probe. In the present context, free amino acids pass while proteins and other molecules of greater than about 6 kDa are retained.

In the present application, two components of a device are in fluid communication with one another if liquid can pass from one to the other. The flow of liquid can be due to diffusion, capillary action, gravity or due to the action of a pump, such as a microfluidic pump.

As used herein, two components are in electronic communication if an electronic signal can pass from one to the other (in at least one directions). For example, a signal from a detectable tag in a ternary complex is passed, in electronic form, from the detection system to a computer microprocessor where the signal can be processed and/ or recorded.

5           When an electronic signal is decoded, information about the presence and/or quantity of at least one primary amino acid is generated by the computer or microprocessor.

10           A biorecognition molecule is one which specifically recognizes another molecule. The biorecognition element can be a partner in an immobilization system, for example, of a component is linked to biotin, streptavidin or avidin can serve as a biorecognition element. If the streptavidin or avidin is bound to a surface, it can then mediate binding of a molecule linked to biotin. An antibody can serve as a biorecognition element for a particular molecule for which it is specific, or an oligonucleotide can serve as a biorecognition element for a nucleic acid molecule (or region thereof) which is complementary to the oligonucleotide, and surface localization can be thus mediated. Elongation factor IA or Tu:GTP can serve as a biorecognition  
15           element for an amino acyl-tRNA. Other examples are obvious to one of ordinary skill in the art.

20           A ternary complex probe, as used herein, specifically binds to the complex of amino acyl-tRNA bound to an elongation factor. It can be a complex-specific antibody, or it can be a molecule which specifically binds to a ligand carried either by the synthetase or the tRNA molecule (such as a biotin residue, with avidin or streptavidin binding thereto. For flow displacement systems the antibody or other ternary complex probe used to reversibly bind the labeled molecule is chosen to have a relatively fast dissociation rate constant. Dissociation rate constants can be determined rapidly using biosensor technology [Pellequer et al. (1993) J. Immunol. Meth. 166:133-143].

25           A variety of different arrays and detectors can be employed in the practice of the present invention. Arrays used in the subject invention can be biosensor, microparticle, microbead,

microsphere, microspot, microwell, microfluidic arrays, and the like. The substrates for the various arrays can be fabricated from a variety of materials, including plastics, polymers, ceramics, metals, membranes, gels, glasses, silicon and silicon nitride, and the like. The arrays can be produced according to any convenient methodology known to the art. A variety of array and detector configurations and methods for their production are known to those skilled in the art and disclosed in United States Patent Nos: 6,043,481; 6,043,080; 6,039,925; 6,025,129; 6,025,601; 6,023,540; 6,020,110; 6,017,496; 6,004,755; 5,976,813; 5,872,623; 5,846,708; 5,837,196; 5,807,522; 5,736,330; 5,770,151; 5,711,915; 5,708,957; 5,700,637; 5,690,894; 5,667,667; 5,633,972; 5,653,939; 5,658,734; 5,624,711, 5,599,695; 5,593,839; 5,906,723; 5,585,639; 5,584,982; 5,571,639; 5,561,071; 5,554,501; 5,534,703; 5,529,756; 5,527,681; 4,472,672; 5,436,327; 5,429,807; 5,424,186; 5,412,087; 5,405,783; 5,384,261; 5,474,796; 5,274,240; and 5,242,974. The disclosures of these patents are incorporated by reference herein.

A mixture of amino acids is added along with the 20 synthetases and fluorescently labeled EF-Tu:GTP in reaction buffer. AA-tRNAs are formed on each microspot in proportion to the concentration of amino acids specific for each immobilized tRNA. Fluorescently labeled elongation factor binds the AA-tRNAs and is detected by laser-induced fluorescence using position specific detectors. The arrays may be positioned into the bottom of microwells, microchannels or on the surfaces such as planar waveguides. If the arrays are positioned on planar waveguides, excitation in the evanescent field may permit the binding of AA-tRNAs to the elongation factor continuously without a wash step. When using planar waveguides the detector will be a CCD camera.

The area of Micro-Total Analysis Systems ( $\mu$  TAS), otherwise known as "microsystems" or "Lab-on-a-chip", is a new research field that aims to fabricate miniaturized sensing devices and systems. Andreas Manz predicted that it would be possible to integrate microscopic versions of the devices necessary to process chemical or biochemical samples, thereby achieving completely automated and computer controlled analysis on a microscale. Micro/miniaturized total analysis systems developed so far may be classified into two groups.



One is a MEMS (Micro Electro Mechanical System), which uses pressurized flow controlled by mechanical flow control devices (e.g microvalves, micropumps or centrifugal pumps). The other types use uses electrically driven liquid handling without mechanical elements. Currently, microsystems are being produced in both academic and commercial settings. The term

5 "microsystem" is used herein to describe both types of miniaturized systems. A variety of integrated microsystems, MEMS, and microsystem devices are well known to the art. See, for example, United States Patent Nos. 6,043,080; 6,042,710; 6,042,709; 6,036,927; 6,037,955; 6,033,544; 6,033,546; 6,016,686; 6,012,902; 6,011,252; 6,010,608; 6,010,607; 6,008,893; 6,007,775; 6,007,690; 6,004,515; 6,001,231; 6,001,229; 5,992,820; 5,989,835; 5,989,402;

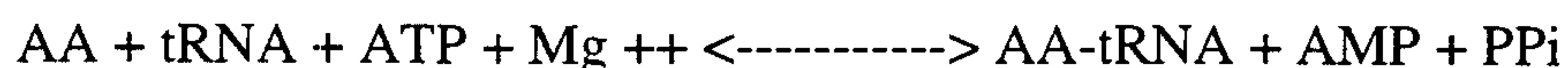
10 5,976,336; 5,972,710; 5,972,187; 5,971,355; 5,968,745; 5,965,237; 5,965,001; 5,964,997; 5,964,995; 5,962,081; 5,958,344; 5,958,202; 5,948, 684; 5,942,443; 5,939,291; 5,933,233; 5,921,687; 5,900,130; 5,887,009; 5,876,187; 5,876,675; 5,863,502; 5,858,804; 5,846,708; 5,846,396; 5,843,767; 5,750,015; 5,770,370; 5,744,366; 5,716,852; 5,705,018, 5,653,939; 5,644,395; 5,605,662; 5,603,351; 5,585,069; 5,571,680; 5,410,030; 5,376,252; 5,338,427;

15 5,325,170; 5,296,114; 5,274,240; 5,250,263; 5,180,480; 5,141,621; 5,132,012; 5,126,022; 5,122,248; 5,112,460; 5,110,431; 5,096,554; 5,092,973; 5,073,239; 4,909,919; 4,908,112; 4,680,201; 4,675,300; and 4,390,403, all of which are incorporated by reference herein.

All proteins from bacteria to man are composed of the same set of twenty amino acids

20 (exclusive of post-translational modifications). Each of the 20 naturally occurring amino acids has one specific synthetase and one or more iso-accepting tRNAs. The enzymes are highly selective for the amino acids they bind and even more selective in their attachment of the amino acids to their specific tRNAs. The method for amino acid analysis of the present invention is effective due to the fact that each synthetase and/or one isoaccepting tRNA specific for each

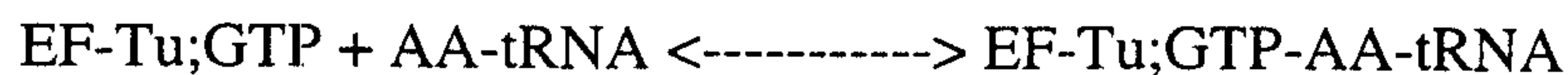
25 amino acid is separated into a different chamber or immobilized onto a separate transducer or a spatially separated zone, and the reactions catalyzed by the 20 synthetases are monitored using a position-sensitive detector, as illustrated in Fig. 1A. The extent of these reactions, desirably under optimal conditions, is generally proportional to the concentration of the amino acids in solution. The amino acids (AAs) are converted into AA-tRNAs by the following overall reaction:



Methods of monitoring this reaction in a reaction sample are known to those skilled in the art. In one method, inorganic pyrophosphatase is included in the chambers of the present invention and the amount of inorganic phosphate produced in a continuous spectrophotometric assay  
 5 monitored. The reaction catalyzed by inorganic pyrophosphatase is



These assays can detect micromolar levels of phosphate and may be used to construct enzymatic amino acid analyzers. By using a macromolecule that specifically binds the AA-tRNAs with high affinity, ligand binding assays that are far more sensitive than spectroscopic assays to  
 10 follow the amino acylation reactions are produced, as described herein. The art has produced powerful screening methodologies that allow selection of biological molecules (antibodies, antibody fragments, peptides, proteins, and nucleic acids) that bind a specific molecule with the desired specificity and affinity. Protocols that allow one to select (directly or after in the laboratory evolution) a biomolecular ligand that specifically binds the desired molecule are well  
 15 established in the art. Hence, molecules that specifically bind AA-tRNAs are used to follow the reactions catalyzed by the synthetases. Biomolecules that specifically bind AA-tRNAs with the desired affinities can be selected using known methods. Examples of molecules that specifically bind the AA-tRNAs include, without limitation, antibodies or parts of antibodies, oligonucleotides (e.g. DNA or RNA aptamers), peptides, or proteins or parts of proteins. For  
 20 example, the protein elongation factor Tu, when complexed with GTP (EF-Tu;GTP), binds each of the AA-tRNAs with high specificity, thus forming a stable ternary complex (EF-Tu;GTP-AA-tRNA). EF-Tu; GTP (or a fragment thereof which retains the sensitive and specific binding of AA-tRNA) is a useful binder of (i.e., ligand for) AA-tRNAs in this invention. Accordingly, if EF-Tu;GTP is present with the synthetases and tRNAs the following reaction  
 25 will occur :



The ternary complex may be further stabilized by substituting a nonhydrolysable GTP analog such as GDPNP, as known in the art [Wagner et al. (1995) *Biochemistry* 34:12535-12342; Nissen et al. (1995) *Science* 270:1464-1472]. Either EF-Tu;GTP or EF-Tu;GDPNP has a high affinity for all AA-tRNAs, but it effectively discriminates against tRNAs which are not amino acylated. The amount of ternary complex formed is generally proportional to the concentration of the amino acid cognate to the tRNA and synthetase present. Hence, the amino acids are identified and quantitated by following the formation of the ternary complex. This can be accomplished by labeling either the tRNAs, elongation factor, ternary complex, or a ternary complex probe ( a molecule that specifically binds the ternary complex (e.g antibody, aptamer, peptide, ribosome or ribosomal subunit) and quantifying the label. tRNAs have been fluorescently labeled [see, e.g., Janiak et al. (1990) *Biochemistry* 29:4268-4277]. The fluorescently labeled tRNAs are functional in amino acylation and in binding to elongation factors. Alternatively, the ligand assays may be based on label-free affinity biosensors (e.g. surface plasmon resonance-based biosensors or piezoelectric biosensors). Ligand assays using fluorescent labels typically achieve femtomole or attomole sensitivities [Zubritsky, E. (1999) *Anal. Chem.* 71-39A-43A].

As set forth above, additional aspects of the present invention include ultrasensitive ligand assays for amino acid analyses. In this method, separately positioned synthetases or tRNAs are used in devices for quantitatively or semi-quantitatively discriminating among the 20 amino acids. The synthetase reactions may be followed in one of several methods known in the art. The synthetase reaction may be monitored by following the binding of AA-tRNAs to EF-Tu;GTP. So far as the inventor is aware, this reaction has not been used to follow the synthetase reaction. Although the elongation factor is one preferred binder of the AA-tRNAs, another molecule that specifically binds AA-tRNAs can be used with the same effects. Where the binder is referred to a EF-Tu;GTP it is to be understood that this protein is a preferred binder and that other molecules that specifically bind AA-tRNAs can be used with the same results.

Systems for following the formation of the AA-tRNA-EF-Tu;GTP complexes are an additional aspect of the present invention. A variety of ligand assay formats may be used to monitor the binding of the AA-tRNAs to EF-Tu;GTP. By immobilizing one component of the ternary complex (either the tRNA or EF-Tu;GTP) and labeling the other component, the formation of the ternary complex can be followed as the labeled molecule is 'captured' onto the surface. The EF-Tu;GTP can be immobilized to a surface and the formation of the ternary complex on the surface can be monitored by capturing labeled tRNA molecules, as illustrated in Fig. 1B. Alternatively, the formation of the AA-t-RNA-ligand complex can be measured in a homogeneous ligand assay. In this case, each of the 20 specific synthetases/tRNAs are positioned in separate wells, immobilized on separate beads or transported (e.g. by microfluidic pumps) through separate microchannels, depending on the physical configuration chosen for the assay. The binding of the AA-tRNA to its specific ligand (e.g.EF-Tu;GTP) can be monitored using an ultrasensitive homogeneous ligand assay (for example, fluorescent techniques). As mentioned above the binding of AA-tRNAs to their ligand is monitored in microfluidic arrays or microwells using position-sensitive detectors.

Recently, immobilized EF-Tu;GTP from *Thermus thermophilus* has been used to purify AA-tRNAs by affinity chromatography [Robeiro et al. (1995) Anal. Biochem. 228:330-335; Chinali et al. (1997) Ann. Rev. Biophys. Biomol. Struct. 26:567-596] . Like the free factor, the immobilized EF-Tu;GTP binds AA-tRNAs but not unacylated tRNAs. Hence, immobilized elongation factor retains its specificity for AA-tRNAs. Accordingly, tRNAs (one specific for each amino acid) are immobilized at set locations, and the formation of the ternary complex on all 20 tRNAs is monitored simultaneously or nearly simultaneously. In this case, the ternary complexes are detected with labeled elongation factors or labeled probes that bind the ternary complex. Other ligand assay formats of the present invention employ ternary complex probes (systems that specifically recognize charged AA-tRNA-EF-Tu;GTP complexes) to capture and/or detect the ternary complex. Macromolecules that specifically recognize the ternary complex include, but are not limited to, ribosomes and ribosomal subunits (e.g. cognate mRNA-charged small ribosomal subunits). Optionally, antibodies or oligonucleotide aptamers that specifically

bind the ternary complex are used.

5           Ultrasensitive ligand assays have relied on labeling one component of the reaction with a high-specificity-activity label (e.g. radioactive, fluorescent, or electrogenic labels) or a label that can participate in an amplification reaction (e.g., enzyme labeling). Label detection is the key  
10           determinant of sensitivity. Fluorescence is a very sensitive detection method, and laser-induced fluorescence is desirably used for sensitive amino acid detection in the present invention. Since tRNAs, elongation factors, ternary complexes or ternary complex probes can be labeled with multiple copies of fluorescent tags or with amplifying labels, one can develop ligand assays for amino acid analysis with a sensitivity many orders of magnitude greater than in commonly  
15           available methods for amino acid analysis.

Affinity biosensors are another aspect of the present invention.

20           Microarrays for amino acid analysis are an additional aspect of the invention. Generally, array technology is the method of choice for high throughput analysis. Miniaturized arrays (microarrays) for the quantitative analysis of many amino acid samples in parallel are an aspect  
25           of the present invention as described herein. Microarrays, wherein thousands of different bioaffinity molecules are immobilized on a surface in a defined and spatially resolvable fashion (usually as spots 10-100  $\mu\text{m}$  in diameter) and used to capture ligands, have been developed for both nucleic acids and proteins [Fodor et al. (1991) *Science* 251:767-773; Lueking et al. (1999) *Anal. Biochem.* 270:103-111]. Microarray technology is applied in the present invention to provide amino acid analysis microchips. The 20-fold spatial distribution of the recognition elements in the microarrays of the present invention is achieved by arraying either 20 specific tRNAs or the 20 specific amino acyl-tRNA synthetases.

30           Techniques for detection of amino acids in the integrated microsystems and microarrays include, but are not limited to fluorescence emissions, optical absorbance, chemiluminescence, Raman spectroscopy, refractive index changes, acoustic wave propagation measurements,

electrochemical measurement, and scintillation proximity assays. There are many demonstrations in the literature of single molecules being detected in solution using fluorescence detection. A laser is commonly used as an excitation source for ultrasensitive measurements and the fluorescence emission can be detected by a photomultiplier tube, photodiode or other light sensor. Array detectors such as charge coupled device (CCD) detectors can be used to image the analytes spatial distribution on an array.

Several microchip fluorescent detection systems are commercially available. These include the Hewlett Packard's BioChip Imager with an epi-fluorescence confocal scanning laser system having a 50 micrometer, 20 micrometer, or 10 micrometer resolution. This instrument detects less than 11 molecules of the dye Cy5/square micrometer and has a dynamic range of four orders of magnitude. General scanning's ScanArray 3000 is a scanning confocal laser with a 10 micrometer resolution that can detect 0.5 molecule of fluorescein/micrometer square (or less than 0.15 attomole of end labeled nucleotide) taking 4 minutes to scan a 10 micrometer by 10 micrometer chip. Molecular Dynamics' Avalanche confocal scanners have a resolution of 10 micrometers and can detect less than 10 molecules of Cy3 molecules/square micrometer on chips taking 5 minutes to scan the entire chip.

Methods for the spatially resolved and ultrasensitive detection of fluorescently labeled molecules in microfluidic channels are disclosed in U.S. Patent Nos. 5,933,233 and 6,002,471.

Instrumentation for the detection of single fluorescent molecules are described in U.S. Patent No. 4,979,824 and reviewed in Sinney et al, (2000) *J Mol Recognit*, **13**, 93-100; Nie, S. and Zare, R.N. (1997) *Annu Rev Biophys Biomol Struct* **26**, 567-96; Rigler, R. (1995) *J Biotechnol* **41**, 177-186; Chan, W.C. and Nie, S (1998) *Science* **281**, 2016-8; and Nie, S. and Emory, S.R. (1997) *Science* **275**, 1102-6. CCD imagers for confocal scanning microscopes are disclosed in U.S. Patent Nos. 5,900,949; 6,084,991; and 5,900,949. Capillary array confocal scanners are described in U.S. Patent Nos 5,274,240. CCD array detectors suitable for microchips are described in U.S. Patent No. 5,846,706, and 5,653,939. Detector systems for optical waveguide microarrays are disclosed in U.S. Patent Nos. 6,023,540, 5,919,712, 5,552,272, 5,991,048,

5,976,466, 5,815,278, 5,512,492.

Mass sensing biosensors such as piezoelectric sensors are known, for example, as disclosed in U.S. Patent Nos. 4,236,, 4,735, and 6,087,187 and are suitable for use in the present invention to construct amino acid biosensor arrays.

5 The method depicted in Fig. 18 illustrated methods for the co-immobilization of carboxypeptidases with their substrate proteins to facilitate rapid digestion of tiny amounts of protein in tiny chambers. The method will also reduce the losses of protein samples by adsorption onto surfaces. Proteins need to collide in order to react and therefore it is not typical or obvious to immobilize two proteins that are to interact. However, by using long polar and  
10 flexible spacer arms or immobilizing the proteins onto a surface that permits collisions of immobilized proteins, not only will the two proteins interact, but the rate of reaction will be greatly increased due to increasing the local concentrations on the surface and thereby increasing the rate of collisions. If the coimmobilized proteins are tethered to liposomes via flexible tethers, they will be confined to a surface but will undergo frequent collisions due to the diffusion of the  
15 lipids to which they are attached within the two-dimensional plain of the lipid bilayer. This will decrease diffusional limitations by converting a three-dimensional random walk into a two-dimensional random walk and greatly decrease the number of collisions that reacting proteins make with the solvent before colliding with substrates. I have suggested conjugating the proteins to be coimmobilized to lipids in liposomes via crosslinking reagents or by other methods as  
20 reviewed in Keinanen K et a. (1994) FEBS Lett **346**, 123-6. Methods for the conjugation of proteins to liposomes are well known in the arts. The carboxypeptides (e.g. carboxypeptidase Y) may be expressed with C-terminal extensions that are both flexible and polar that will be used as tethers. By using an amino acid composition that is rich in glycine the tethers will be flexible since glycine has no side chain and only a hydrogen atom instead, it adds flexibility. Serine is a  
25 polar and uncharted amino acid, hence, it can be encoded into the tether. The C-terminal residue of the carboxypeptidase-tether may be a cysteine. This will permit thiol specific conjugation to surfaces or protein substrates of carboxypeptidases.

The particles bearing the coimmobilized protein substrate and exopeptidase may be placed in the microarrays for amino acid analysis for high throughput end group analysis. For example these particles may be placed into the microwell arrays shown in Gif. 8D.

5 Fig. 20A is a schematic drawing of a continuous flow enzymatic amino acid analyzer and end group sequenator using optical detection. The 20 synthetase reactions are arrayed into 20 reaction channels or capillaries in fluid connection to a central circular chamber. Each of the 20 reaction channels is in fluid connection to a separate reservoir containing a unique synthetase and optionally a cognate tRNA and EF-TU;GTP in reaction buffer. The exopeptidase digestion chamber is placed in a flow stream that connect with the circular chamber having the multiple  
10 inlets tot he reaction channels. 20 inlets will lead to reaction channels having a different one of the synthetases and additional inlets will be employed for control channels or capillaries. The digestion chamber may be a sealed hollow fiber having a molecular weight cutoff that allows free amino acids to pass freely and is impermeable to macromolecules. Alternatively, the protein substrates may be coimmobilized with the exopeptidases on a surface (as described in Figs. 18A-  
15 18B) and held in the flow stream by a porous support, magnetic force, immobilized ligand or in some other way. As the amino acids are liberated from the protein's termini by the exopeptidases, they flow through the digestion chamber and past a continuous flow mixer. The amino acids flow through the 20 channels. The amino acids having a cognate synthetase in each channel will react to form proportionate amount of product. In this embodiment the flows are  
20 driven by centrifugal force and the system spins. A light source and computer linked detector are integrated into the system. The product of the synthetase reactions will be continuously monitored by the detector. The detector may be a scanning spectrophotometer, luminometer or fluorometer depending upon the assay used to follow the synthetase reactions. The amino acids will be identified from the location of the chamber in the array giving the signal as the synthetase  
25 reaction(s) are monitored in all chambers. If enzyme labels or other detection reagents are employed in the assay the enzyme substrates, buffers, and reagents will be pumped through the reaction and control channels from a reservoir(s). Any continuous optical detection method may be used to follow the synthetase reaction(s) in a spatially distinguishable manner including



chemiluminescent and bioluminescent assays by following the formation of the reaction products (e.g., PPI, AMP, AA-tRNAs, synthetase-AA-AMP).

Miniaturized fluorescence resonance energy transfer (FRET) assays in spatially resolved microfluidic reaction chambers and microwells are envisioned for ultrasensitive and ultra-high throughput amino acid analysis in the current invention. FRET assays detect binding in real time without a washing or separation step, are easily automated and miniaturized and ultrasensitive. Successful applications of FRET are highly promoted by the introduction of modern instruments in fluorescence detection systems. The advantages of fluorescent lifetime imaging results from the fact that fluorescence lifetimes are usually independent of the fluorophore concentration, photobleaching, and other artifacts that affect fluorescence intensity measurements [Scully et al. (1997) *Bioimaging* 5:9-18]. There are many reviews available on FRET and many instruments for these assays are commercially available [Clegg, R.M. (1995) *Curr. Opin. Biotechnology* 6:103-110; Clegg, R.M. (1996) *Fluorescence Resonance Energy Transfer(FRET)* In: *Fluorescence Spectroscopy and Microscopy*, Wang X.F., Hermann, B. (eds) J. Wiley and Sons, New York; Fultron et al. (1997) *Clin. Chem.* 43:1749-1756; Selvin, P. R. (1995) *Methods Enzymol.* 246:300-334; McDade, R.L.(1997) *Med. Dev. Diag. Indust.* 19:75-82; Moerner et al. (1999) *Science* 283:1670-1676; Chen et al (1999) *Genet. Anal.* 14:157-163; Mere et al. (1999) *Drug Discov. Today* 4:363-369; Nie, S. and Zare, R. (1997) *Annual Review of Biophysics and Biomolecular Structure* 26:567-96]. Spatially resolved fluorescence energy transfer has the capacity to detect, quantitatively, molecular interactions in real time over distances of microns.

Measurement of energy transfer is desirably based on fluorescence detection, thus ensuring high sensitivity. In addition to data acquisition with commercial microplate spectrophotometers, energy transfer methods can be incorporated into automated microfluidic assays for ultra-sensitive and ultra-high throughput analysis of biomolecular binding [Mere et al. (1999) *Drug Discov. Today* 4:363-369]. The reactions catalyzed by the synthetases in the microwells can be monitored in all wells at the same time using a plate reader. Depending on the detectable tag used and the configuration, the plate reader can be a spectrophotometer, a

fluorometer, a luminometer, a scintillation counter or a gamma counter.

Excitation is set at the wavelength of donor absorption, and the emission of donor is monitored. The emission wavelength of donor is selected such that no contribution from acceptor fluorescence is observed. If the tRNAs are labeled with fluorescein (fluor) and the elongation factors are labeled with rhodamine as described above, then fluorescein is the donor and rhodamine (Rh) is acceptor. Fluorescein excitation and emission wavelengths are around 490nm and 520nm, respectively. The labeled tRNA are converted into AA-tRNA-Fluor by the synthetases. Both donor AA-tRNA and acceptor EF-Tu:GTP-Rh are excited by monochromic light and then fluoresce at different wavelengths. Fluorescence energy transfer between the AA-tRNA-Flu and the EF-Tu:GTP-Rh is detected by measuring the photophysical properties of the donor fluorescence photons only. The acceptor photons may be barred from the detector by an optical filter; and therefore, the acceptor-labeled elongation factors that are not bound to the AA-tRNA-Fluor are not detected. Many donor/acceptor chromophores have been used in FRET assays and are suitable for use in the method of the present invention. For example, Wu et al. (1994) *Anal. Biochem.* 218, 1-13, lists 58 donor/acceptor pairs suitable for use in FRET assays.

A particular form of fluorescence resonance energy transfer known in the arts is homogeneous time-resolved fluorescence (HTRF) [Mathis, G. (1995) *Clin. Chem.* 41:1391-1397; Kolb et al. (1998) *Drug Discovery Today* 3:333-142]. In the HTRF format the tRNAs and elongation factors can be labeled with the europium cryptate to function as the fluorescence energy donor and the allophycocyanin acceptor, for example.

FRET assays have been extended to biosensors and microarrays [Buranda et al. (1999) *Cytometry* 37:21-31] and can be used in the microarrays and biosensors of the subject invention. FRET-based arrays are envisioned where the fluoresceinated tRNAs are converted into fluoresceinated AA-tRNA that bind rhodamine-labeled EF-Tu;GTP. Alternatively, the rhodamine, for example TRITC, (or other acceptor) may be immobilized to a surface to which the elongation factor is bound, and the FRET may be monitored when the fluoresceinated AA-

tRNA binds the surface-bound elongation factor. The surface may be a bead, microspot, or transducer. The binding of the labeled AA-tRNAs to the immobilized elongation factor brings the donor and acceptor into proximity, causing an energy transfer.

Fluorescein measurements are carried out with the excitation at or around 490 nm and  
 5 emission at 520 nm. Some fluorescent labels suitable for use in the subject invention include, but are not limited to, fluorescein (FITC, DTAF) (excitation maxima, 492nm/ emission maxima 516-525 nm); carboxy fluorescein (excitation maxima, 492nm/emission maxima, 514-518 nm; 2'-methoxy-CF (excitation maxima, 500nm/emission maxima, 534 nm); TRITC G (tetramethylrhodamine isothiocyanate, isomer G (excitation maxima, 535-545/emission maxima,  
 10 570-580); RBITC (rhodamine-B isothiocyanate (excitation maxima, 545-560/emission maxima, 585); Texas Red (excitation maxima, 595/emission maxima, 615-620); Cy-5 (Cyanine) (excitation maxima, 649/emission maxima, 670); Cy-3.5 (excitation maxima 581nm/ emission maxima, 596 nm); XRITC (rhodamine X isothiocyanate (excitation maxima, 582 nm /emission maxima, 601 nm); ethidium bromide (excitation maxima, 366nm/ emission maxima 600nm);  
 15 Thiazole orange (To-Pro) excitation maxima, 488 nm/emission maxima 530-580).

The t-RNAs can be fluorescently labeled by site-specifically conjugating a fluorescent label using thiouracil as described in Johnson et al (1982) J. Mol. Biol. 156, 113-140. Fluorescently labeled nucleotide analogs can be used to site-specifically label the t-RNAs using known methods [Chu et al. (1997) Nucl. Acids Res. 25:3944-9]. Alternatively fluorescent dyes  
 20 such as ethidium bromide can be used to label tRNAs; however, the sensitivity is relatively poor. The elongation factors may be site specifically labeled by using a fluorescently labeled GTP analog as described above. Alternatively, the elongation factors or other proteins used in this invention can be site specifically labeled . Molecular biology methods such as site-directed mutagenesis and unnatural amino acid mutagenesis [Anthony-Cahill et al. (1989) Trends  
 25 Biochem. Sci. 14:400] can be used to introduce cysteine and ketone handles for specific dye labeling of proteins [Cornish et al. (1994) Proc. Natl. Acad. Sci. USA 91: 2910-2914].

Imaging or scanning detectors including confocal scanners, charged coupled device arrays, photodiode arrays and optical fiber arrays can be used in the subject invention as reviewed in Brignac et al. (1999) *IEEE Eng. Med. Biol. Mag.* 18:120-22; Eggers et al. (1994) *Biotechniques* 17:516-525; Pang et al. (1999) *J. Biochem. Biophys. Meth.* 41:121-132; Setford et al. (2000) *J. Chromatogr. A* 867: 93-104; Kheterpal, I. and Mathies, R.A. (1999) *Anal. Chem.* 71:31A-37A; Crabtree et al. (2000) *Electrophoresis* 21:1329-35; Heiger et al. (1994) *Electrophoresis* 15:1234-1247; and Budach et al. (1999) *Anal. Chem.* 71:3347-3355.

The fluidic system allows automated calibration with known concentrations of amino acids, prewashing with equilibration buffer, incubation with the amino acids, synthetases, and elongation factors, postwashing to remove unbound material and regeneration of the sensor chip with an elution buffer all under computer control. An equilibration buffer (e.g., 30mM HEPES-KOH, pH 7.5, 10mM MgCl<sub>2</sub>, 50mM KCl, 5mM β-mercaptoethanol, 1mM ATP, 1mM GTP are automatically perfused through the reaction channels to regenerate the binding sites. The elution buffer (e.g., 100 mM sodium borate, pH 7.5, 1 M NaCl, 10 mM MgCl<sub>2</sub>, 5 mM β-mercaptoethanol, 50 μM GTP) and washing buffers (e.g., 50 mM Tris, pH 7.5, 10 mM MgCl<sub>2</sub>, 5 mM β-mercaptoethanol, 50 μM GTP) and amino acids for calibration will be transported through the reaction channels from separate reservoirs by microfluidic pumping. Fluidic handling (volumes and flow rates of the respective solutions) and data acquisition or image acquisition (series of fluorescence images) will be synchronized by means of a computer.

Alternatively, the AA-tRNAs or other molecules that reversibly bind the elongation factors can be immobilized in the flow channels. Then the immobilized molecules are saturated with fluorescent-labeled EF-Tu;GTP. The tRNAs are immobilized in such a way that they react with EF-Tu;GTP. Since the conserved s4U residues at position 8 in tRNAs can be conjugated with fluorophores while retaining their abilities to be aminoacylated by the synthetases, and the newly formed AA-tRNAs retain their specificity to interact with EF-Tu;GTP [Johnson et al. (1982) *J. Mol. Biol.* 156:113-140; Yu, Y.T. (1999) *Methods* 18:13-21; Sontheimer, E. J. (1994) *Mol. Biol. Rep.* 20:35-44), the thiouridines (s4Us) at position 8 in the tRNAs can be used to

conjugate the tRNAs to a surface using a spacer arm. Many useful nucleotide triphosphates are now available from commercial sources that can be site specifically incorporated into RNA to provide spacer arms having groups that react with heterobifunctional compounds suitable for immobilization [Hermanson, G. T. (1996) *Bioconjugate Techniques*, Academic Press, NY).

5 These immobilized molecules retain the ability to interact with both the synthetases and elongation factors. As shown, an AA-tRNA (for example met-tRNA<sup>met</sup>) is immobilized and saturated with fluorescently labeled EF-Tu;GTP. Newly formed AA-tRNAs created in the synthetase reaction displace a proportionate amount of labeled elongation factor which flows through an area illuminated by a laser beam and signal is continuously detected using a spatially  
10 specific detector, e.g. a CCD detector.

Alternatively, a ternary complex probe, a molecule that specifically binds the ternary complex, AA-tRNA-EF-Tu;GTP, is immobilized in the flow channel. Ternary complex probes include antibodies, oligonucleotides, ribosomes, ribosomal subunits, or other molecules that specifically and reversibly bind the ternary complex. For example antibodies or nucleotide  
15 aptamers that specifically bind the ternary complex can be obtained using standard methods known in the art [Jayasena, S.D (1999) *Clin. Chem.* 45:1628-1650; Diamandis, E.P and Christopoulos, T.K (eds) (1996) *Immunoassays*, Academic Press, New York). Alternatively ribosomes or ribosomal subunits may be used. A properly programmed ribosome binds the ternary complex rapidly and tightly, and using a nonhydrolyzable GTP analog, the ternary  
20 complex dissociates from the ribosome with a dissociation rate constant of  $2.7 \times 10^{-3} \text{ s}^{-1}$ . Hence, ribosomes can be used as capture probes to follow the formation of the ternary complex. Alternatively, a fluorescently labeled ternary complex having a nonhydrolyzable GTP analog can be adsorbed to the immobilized ribosome and displaced by the newly formed ternary complex. The displaced labeled ternary complex then flows past the detector. In this example the ternary  
25 complex probe is immobilized in the flow channel and saturated with fluorescently labeled ternary complex. Each flow channel contains a unique synthetase, its cognate tRNA and EF-Tu;GTP. These macromolecules can be continuously perfused through 20 channels, being transported from separate reservoirs using microfluidic pumping. Into each of the 20 channels is

pumped a different synthetase with its cognate tRNA and EF-Tu;GTP. Each reaction channel has an inlet for amino acids to flow into and an outlet. As amino acids flow into the reaction channel array, the amino acids having a cognate synthetase and tRNA in each channel are converted into a proportionate amount of ternary complex which then displaces a proportionate amount of the fluorescently labeled immobilized ternary complex in the channels.

Figs. 21A-21B illustrate a computer-controlled integrated microsystem for amino acid analysis and protein end group sequencing using affinity capture and detection of labeled AA-tRNAs on immobilized EF-Tu:GPT with automated calibration, washing, detection, elution, and regeneration of the capture site. The elongation factor is immobilized in the microflow channel as described above. Briefly, the elongation factor is expressed as a fusion protein having a C-terminal flexible tether (optimally composed of glycine residues to ensure flexibility and neutral polar amino acid residues such as serine) having an attachment site (e.g., a C-terminal cystine for thiol specific coupling to surfaces or a hexa-His tag or other ligand specific tag). The elongation factors are optionally immobilized onto transducers such as optical fibers or microelectrodes that are positioned in the microflow channel. The automated system allows automated calibration with known concentrations of amino acids pumped through the microfluidic array from calibration reservoirs by microfluidic pumping, prewashing to remove unbound or weakly bound material, detection, elution of the labeled tRNAs with an elution buffer, and regeneration of the capture sites with a regeneration buffer all under computer control. All fluidic handling (volumes and flow rate of respective solutions) and data acquisition or image acquisition (series of fluorescent images) are synchronized by means of a computer. The wash buffer reservoir, calibration reservoirs, elution buffer reservoirs, and regeneration buffer reservoirs are in fluid connection with the microfluidic array such that solutions from these reservoirs are pumped through the microfluidic reaction channel arrays as shown in cases where a labeled probe (for example fluorescently labeled oligonucleotides or peptide oligonucleotides that bind to the captures AA-tRNAs) are used for detection, additional reservoirs are added to automatically deliver the probes. An amino acid sample inlet port is also depicted. The 20 reaction solution

reservoirs joining each reaction channel in the array as described above (Fig. 5A) are not shown. The digestion chamber (not shown) is as described above, and amino acids flow continuously from the digestion chamber through the main channel. A computer controlled valve diverts the flow of the amino acid mixture pumped from the digestion chamber into a waste reservoir during the automated steps of washing, detection, elution, and capture site regeneration. An additional reaction channel can be added to serve as a control. In this case the reaction solution reservoir is the source of reaction solution buffer through the control channel without a synthetase, therefore, no AA-tRNAs are formed in the control channel. The experimental procedure for amino acid analysis using this instrument where the elongation factor is immobilized onto optical fibers is as follows:

1. Equilibrate the reaction channels with reaction buffer and determine a base line by applying excitation input light and detecting the emitted light.
2. Amino acid mixtures of known concentrations will be pumped through the reaction channels from calibration reservoirs and the signals will be determined as outlined below to establish a calibration curve.
3. Add amino acid sample to the sample inlet port or allow the sample to be pumped through the reaction channels from the exopeptidase digestion chamber as described in Fig. 5A. As amino acids enter each reaction channel they react with cognate synthetases and tRNAs forming AA-RNAs that are captured by the immobilized elongation factors.
4. In the case where the amino acid samples flow from the digestion chamber, a computer controlled micropump will stop the flow. Then the computer controlled micropump will pump wash buffer through the reaction channels.
5. If a fluorescently labeled AA-tRNA probe is employed, the probe will be pumped through the channels automatically following the automated washing step using computer controlled micropumps.
6. Wash buffer will again be pumped through the channels.
7. Where fluorescently labeled tRNAs are employed, steps 4 and 5 will be omitted.

8. The input light will be applied at the excitation wavelength and the emitted light intensity will be measured by the detector. The fluorescence intensity will be correlated with the concentration of amino acid in the sample. Applying excitation input light at an appropriate wavelength, fluorophores generate photons of another wavelength and the emitted light intensity will be correlated with the amino acid concentration in the sample.
9. The elution buffer will be pumped through the reaction channels.
10. Regeneration buffer will be pumped through the channels to reactivate the sensor surface for another round for sampling.
11. A base line will be measured as in Step 1 and the next sample will be taken.
12. Where the amino acids flow from the digestion chamber, a computer controlled pump or valve will now start the flow of amino acids from the digestion chamber through the microchannel array for additional readings. The steps will be automatically repeated to detect the amino acids liberated by the exopeptidases continuously as a function of time. And the record of the amino acids detected will be used to computer a sequence.
13. The above steps may include an elution step after each reading of the captured fluorescent molecules. This step will be followed by reading the baseline fluorescence and regenerating the binding site by pumping regeneration buffer through the reaction channels.
14. Suitable wash, elution, and regeneration buffers may be those described in Robeiro, et al (1995) *Anal Biochem*, 228,330-335 for the affinity purification of AA-tRNAs on immobilized elongation factor Tu from *Thermus thermophilus* EF-Tu; GTP.

#### Microtiter Plate Kit for Amino Acid Analysis Using Ultrasensitive Fluorescent Assay

Microplate (or microwell plate, both generic terms for microtiter plate) formats using fluorescent labels and microplate fluorimeters enable femtomole-attomole sensitivities. Many types of microplate fluorimeters are commercially available. Molecular Device's FLIPR or LJJ



Biosystem's new Acquest have the ability to handle 1536-well plates and have a high degree of automation. Bio-Tek Instruments model FL600 microplate fluorometer can detect less than 2 femtomoles of fluorescein with a read time of 28 sec. Molecular Device's SPECTRAmax Gemini microplate fluorometer can detect 5.0 femtomoles of FITC in 96 well plates with a read  
5 time of less than 27 sec, and BMG Lab Technologies' FluoStar can detect 50 attomoles/well Eu3+ reading 384 wells in 30 sec. Instruments are also available that combine time-resolved fluorescence with fluorescence resonance energy transfer pairing. This combination requires two fluorophores emitting at different wavelengths. The first emits right away, but the second is  
10 activated only when the two are in proximity, i.e. when two labeled molecules are bound. This allows simultaneous measurement of bound and unbound analytes and thus permits internal calibration. It also means that the assay is homogenous, and therefore, it is easy to automate and miniaturize.

#### Fluorescence detection systems in microfluidic arrays.

Note the microflow system may be constructed using multiple capillaries as well as  
15 multiple microchannels. In the present context, the word channel means channel or capillary. The microchannels of the present invention can be from 1-1000  $\mu\text{m}$  in diameter.

Detection systems for capillary arrays and microchannel arrays are known in the art [Huang et al. (1992) *Anal. Chem.* 64:967-72; Mathies et al. (1992) *Anal. Chem.* 64:2149-54; Kambara et al. (1993) *Nature* 361:565-566; Takahashi et al. (1994) *Anal. Chem.* 66:1021-1026;  
20 Dovichi et al. (1994) In: *DOE Human Genome Workshop IV*, Santa Fe, NM, November 13-17 Abstract #131; Wooley et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:11348-52; Wooley et al. (1997) *Anal. Chem.* 69:2181-21866; Simpson et al. (1998) *Proc. Natl. Acad. Sci. USA* 95:2256-2261; Schmalzing et al. (1998) *Anal. Chem.* 70:2303-10; Ueno, K. (1994) 66:1424-31; Lu et al. (1995) *Appl. Spectrosc.* 49:825-833].

25

#### Fluorescence detection system

The microfluidic system can use side-entry laser irradiation and irradiate all the

microflow channels simultaneously. Detection can be achieved with a highly sensitive camera system from a direction perpendicular to the incident laser beam. The fluorescence from the irradiated region produces a line image on the CCD detector, which may be a cooled CCD camera coupled with a cooled image intensifier and this detector is connected to a computer. The excitation light source may be a He-Ne laser. The excitation wavelength will depend on the assay type and fluorophore(s) used. The laser beam can be focused at the outlet of the parallel channels to excite the fluor(s) as they flow out of the channel array. A light emitting diode can also be used as a light source for exciting a fluorescent detectable tag. A photomultiplier tube can be used in the detection system or the excitation light source.

10 Any of the transducers used in biosensors can be engineered in an array format and used to monitoring the binding of AA-tRNAs to the elongation factor Tu;GTP for amino acid analysis in the subject invention. Recent developments in engineering have improved transducer piezoelectric technology, leading to a new generation of sensor devices based on planar microfabrication techniques. Piezoelectric biosensors [see, e.g., Ghidilis et al. (1998) Biosens. Bioelectron. 13:113-31; Suleiman et al. (1994) Analyst 119:2279-82; Karube et al. (1988) United States Patent No. 4,786,804] are well suited to miniaturization and detect femtomole levels of analyte. In addition, labeling of the analyte is not necessary. Surface plasmon resonance biosensors are commercially available and can monitor biomolecular interactions in real time during continuous flow.

20 Piezoelectric biosensors and surface plasmon-based biosensors for amino acids are within the scope of detectors useful in the practice of the present invention. Piezoelectric crystals and surface plasmon resonance biosensor formats are envisaged for amino acid analysis in the subject invention. The elongation factors, synthetases, tRNAs, or ternary complex probes can be immobilized onto transducers using known methods. The biorecognition elements can be immobilized onto piezoelectric crystals for example, according to the methods of Storri et al. (1998) Biosens. Bioelectron. 13:347-57 and Lu H.C. et al. (2000) Biotechnol. Prog. 13: 347-57. Piezoelectric array biosensors have been described, for example, Wu, T.Z. (1999) Biosens.

Bioelectron. 14:9-18.

In general, any object that acts as a waveguide can be engineered into an evanescent wave biosensor. Planar waveguide biosensor arrays have been described [Rowe-Taitt et al. (2000) Anal. Biochem. 231:123-133; Rowe et al. (1999) Anal. Chem. 71:3846-52; Rowe et al. (1999) Anal Chem. 71:433-9; Flora et al. (1999) Analyst 124:1455-62; Herron et al (1999) United States Patent No. 5,919,712].

#### Scintillation Proximity Assay

Scintillation proximity assays are envisaged for amino acid analysis. In scintillation proximity assays a radioisotope is used as an energy donor and a scintillant-coated surface (e.g. a bead) is used as an energy acceptor. Scintillation proximity assays (SPA) are described in United States Patent No. 4, 568, 649 which is incorporated herein by reference. The elongation factor:GTP can be bound to SPA beads (commercially available from Amersham Corp., Amersham Place, Little Chalfont, England). For example, biotinylated elongation factor may be conjugated to avidin or streptavidin coated SPA beads. Biotin in the form of N-hydroxysuccinimide-biotin is available from Pierce Chemical Co., Rockford, IL. This embodiment comprises an acceptor SPA beads and quantitation of radiolabeled AA-tRNA on a scintillation counter (for example, a microchip or microplate scintillation counter).

Amino acid analysis can be performed with tRNA arrays of the present invention by introducing a solution of the 20 synthetases in amino acylation buffer along with the amino acid mixture to be analyzed. The AA-tRNAs formed on the array can be detected by binding a fluorescently labeled elongation factor, as illustrated in Fig. 2A. Alternatively, each synthetase can be co-immobilized to a different microspot with EF-Tu;GTP to construct synthetase microarrays, as shown in Fig. 2B. Bound ternary complexes can then be detected by capturing fluorescently labeled tRNAs.

25

Various methods known to the art for arraying both proteins and nucleic acids are used in the arraying of the synthetases and tRNAs on microchips. For example, attachment of cDNA targets to a glass chip can be carried out using non-covalent charge interactions. In such a method, glass slides are coated with poly-L-lysine onto which cDNA clones or other nucleic acid molecules are printed [De Risi et al. (1997) *Science* 278:680-686]. This method of attachment relies on the electrostatic interactions between the positively charged lysine residues and the negatively charged phosphate backbone of the nucleic acids and hence should also be suitable to pattern the 20 specific tRNAs on a chip. Several methods of constructing protein microarrays have also been developed and are known to those skilled in the art. For example, Berringer Mannheim (Indianapolis, IN) developed a method that deposits arrays of 100-200 spots (~80 um in diameter) on the flat bottom of wells [Ekins, R.P. (1998) *Clin. Chem.* 44:2015-2030].

In addition to the requirement of high sensitivity, the enzymatic amino acid analyzers of the present invention provide spatially resolved detection. Highly resolved optical detection of fluorescent labels enables the quantitation of captured ligands on arrays of many thousands of bioaffinity molecules simultaneously. Spatially resolved fluorescence detection in miniaturized systems can be achieved by direct imaging through a microscope, through optical fibers, or through optical waveguides. Fluorescent signals pass through spatial and spectral filters to the detection system. Ultrasensitive, spatially resolved detection on high-density arrays may be achieved with confocal laser scanners or imaging CCD cameras, which are known in the art. A schematic illustration of an amino acid analyzer microarray detection system of the present invention is shown in Fig. 3. Upon excitation by a laser, a CCD camera detects the pattern of fluorescent molecules on the surface, and image analysis software correlates the position of the fluorescent signal with the identity of the amino acids. Formation of the ternary complex on the surface can be measured quantitatively using a modified epifluorescence microscope equipped with a CCD camera. Real-time ultrasensitive measurements can be made, for example, by using evanescent wave excitation and an intensified video CCD camera. Many tiny spatially resolved wells or microflow channels can house the patterned array of recognition elements. Thousands of

these chambers can be constructed on a single chip or plate. Fluorescent array detectors can quantitate end-labeled nucleotides at subattomole levels. Label-free probes can be detected using surface plasmon resonance [Thiel et al. (1997) *Anal. Chem.* 69:4948-4956], although array density and sensitivity do not match those of the fluorescence systems.

5           The delivery of microliter to nanoliter volumes of samples to the arrays of the present invention can be achieved using recently developed micropipetting systems [Rose and Lammo (1997) *Automat. New* 2:12-19]. Using these tools, carboxypeptidase or aminopeptidase digestions can be performed in nanowells and tiny samples (microliter or nanoliter volumes) aliquoted into amino acid analysis nanowells by, e.g., robots during the time course of the  
10 digestion. These automated systems are useful for end-group sequencing of many samples in parallel at sub picomole levels. Due to the nonlinear rate of hydrolysis by these enzymes, however, continuous assays are preferable, as explained below.

          Carboxypeptidases and aminopeptidases have been used for sequencing proteins for many years [Light, A. (1967) *Meth. Enzymol.* 11:426-444; Breddam and Ottesen (1987)  
15 *Carlsberg Res. Comm.* 52:55-63; Royer, G. (1972) *J. Biol. Chem.* 218:1807-1812]. Carboxypeptidases and aminopeptidases have been used for end group protein and peptide sequencing [Martin et al. (1971) *Carlsberg Res. Comm.* 44:99-102; Klarskow et al. (1989) *Anal. Biochem.* 180:28-37; Thiede et al. (1995) *FEBS Letts* 357:65-9; Bonetto et al. (1997) *Anal. Chem.* 69:1315-1319; Bonetto et al. (1997) *J. Protein Chem.* 16:371-374; Light, A. (1968)  
20 *Methods Enzymol.* 11:426-444]. These enzymes are ideal for removing amino acids sequentially from the N- termini of proteins (aminopeptidases) or the C-termini of proteins (carboxypeptidases). During the course of a digestion, samples are taken and analyzed later using an amino acid analyzer. Inherent in the use of these discontinuous assays is the assumption that the amino acids are being released linearly with time over the period chosen, however, the  
25 rate of cleavage is sequence dependent and varies unpredictably. When an analyte varies unpredictably a continuous (real-time) assay is needed. A continuous assay is not possible using existing methods of amino acid analysis. This problem is solved by the continuous amino acid

biosensor arrays of the present method which utilize the method of amino acid analysis described herein.

Carboxypeptidases and aminopeptidases for use in sequencing reactions described herein  
5 are commercially available from numerous suppliers (e.g., Pierce Chemical Co., Rockford, IL).  
Examples of carboxypeptidases suitable for use in the subject invention for end group  
sequencing and end group analysis include carboxypeptidase Y, carboxypeptidase P,  
carboxypeptidase A, and carboxypeptidase B. Aminopeptidases, including aminopeptidases L  
and M, which are commercially available, are used for amino terminal sequence analysis. The  
10 exopeptidases can be immobilized using spacer arms for use in end group sequencing.  
Immobilized aminopeptidases and carboxypeptidases suitable for protein end group sequencing  
are commercially available. Mixtures of different carboxypeptidases for C-terminal sequencing  
and different aminopeptidases for – terminal sequencing are often used to give suitable digestion  
rates.

#### 15 The Digestion Chamber

The digestion chamber is traversed by a microdialysis or ultrafiltration probe having a  
molecular weight cutoff that allows free passage of amino acids but is impermeable to  
macromolecules. If the molecular weight cutoff is 6,000 Da, for example, then amino acids  
should pass freely across the membrane but polypeptides longer than about 50 amino acids  
20 should not. Peptides to be analyzed may furthermore be conjugated to other molecules to prevent  
passage through the membrane. For example, peptides may be conjugated to dextran or other  
polymers or substances. Peptides to be used for C-terminal sequencing can be conjugated to  
molecules using amino-terminal coupling chemistries, and peptides to be used for N-terminal  
sequencing can be conjugated to polymers using carboxy-terminal coupling chemistries. If  
25 immobilized exopeptidases are to be used, the peptides to be sequenced are coupled to small  
polymers (e.g. MW 50-100 kDA) rather than beads to prevent mass transport problems.

As provided herein, a biosensor is a self-contained integrated device that is capable of

providing quantitative or semi-quantitative analytical information using a biological recognition element which is in direct contact with a transduction element. For a review of real time, miniaturized sensors; see, e.g., Rogers and Mulchandani (1998) *Affinity Biosensors: Techniques and Protocols*, Humana Press, Totawa, NJ. Biosensors can be classified according to their

5 transduction mechanisms and include microelectrodes, surface acoustic wave sensors, and fiber optic sensors. A commercially available biosensor system called BIAcore (Pharmacia Biosensor, Uppsala, Sweden) contains a sensor microchip, a laser light source emitting polarized light, an automated fluid handling system, and a diode-array position sensitive detector [Raghavan and Bjorkman (1995) *Structure* 3:331-333]. This system uses a surface plasmon resonance assay, an

10 optical technique that measures changes in the refractive index at the sensor chip surface. These systems can monitor biological interaction phenomena at surfaces in real-time under continuous flow conditions. Any of the usual energy transduction modes can be fabricated in an array format and used to construct amino acid analysis biosensor arrays. Each biorecognition element can be placed on transducers which monitor mass changes, the formation of electrochemical products,

15 or the presence of fluorescence. Optical and electrochemical transducers, however, provide the most sensitive biosensors and are well suited for miniaturization and are thus advantageous in the practice of the present invention.

#### Optical Fiber Amino Acid Biosensor Array

Fiber optics can be used as thin flexible pipes to transport light to and from tiny volumes

20 of immobilized chemistry at the probe end. Optical fibers offer several advantages for the construction of amino acid analysis biosensor arrays of the present invention. For example, optical fiber bundles for combining sensing and imaging and creating optical sensing arrays may be utilized [See, e.g., Healy et al. (1995) *Science* 269:1178-1180]. Optical fibers provide a highly miniaturizable transduction format and thus allow monitoring in real-time [Healey and Walt

25 (1997) *Anal. Chem.* 69:2213-2215]. The tips of optical fibers can be of submicron dimensions. Optical fiber sensors can use the evanescent field to excite and collect the fluorescence of molecules bound to the surface. The evanescent wave excites only fluorophores that are bound to the surface. This allows real-time detection of the captured probe on microarrays even in the

presence of high concentrations of fluorophores in the bulk solution without washing. Specific synthetases or tRNAs cognate for a different amino acid can thus be immobilized to a separate fiber. Each fiber tip signals the amino acid cognate to the immobilized synthetase or tRNA. For example, each synthetase can be co-immobilized with EF-Tu;GTP to a different fiber, and the formation of the ternary complex on each fiber can be monitored using fluorescently labeled tRNAs. When coupled to aminopeptidase or carboxypeptidase digestions these ultrasmall sensors may allow real-time protein end-group sequencing in tiny volumes (<1 microliter).

Preferably, the systems of the present invention are designed so that each liberated amino acid will diffuse (or be transported) to its immobilized cognate synthetase (or tRNA) and be detected before the next amino acid is released. Diffusion is rapid over distances of a few micrometers. Diffusion times increase with the size of the molecule and the square of the distance traveled. For amino acids in water at 37 C diffusion times are approximately 1msec for 1  $\mu\text{m}$ , 0.1 sec for 10  $\mu\text{m}$  and 17 min for 1 mm. For systems larger than a few micrometers, transport must take place by convection for real-time sequential monitoring.

A C-terminal enzymatic protein sequenator of the present invention with an evanescent wave optical fiber amino acid analyzer biosensor array is illustrated in Fig. 4. As shown the carboxypeptidases are immobilized to a separate zone within the nanochamber. The carboxypeptidase digestion chamber can be separated from the biosensor array by a semipermeable membrane having a molecular weight cutoff such that amino acids pass freely, but it is impermeable to macromolecules. As amino acids are released into solution by the carboxypeptidases, they cross the membrane and are detected by the biosensor array. Amino acid analyzer arrays can be incorporated into microdialysis or ultrafiltration probes and coupled to amino- or carboxypeptidase digestions for end-group sequencing.

## 25 Microflow Systems

Because diffusion in liquids is random and slow over distances greater than a few micrometers, the incorporation of arrays into flow systems for automated processing facilitates



high throughput analysis and permit sequential monitoring. Solid-phase ligand assays are currently performed in microtiter plates; however, this technique requires long incubation times to achieve equilibrium conditions and is difficult to miniaturize and automate. By contrast, flow systems are easily automated and miniaturized and allow fine control of reagent additions and rapid chemistries by reducing diffusional limitations. In addition, reproducibility is extremely high and calibrations are easy to perform [Scheller et al. (1997) *Frontiers in Biosensors. 1. Fundamental Aspects*, Birkhauser Verlag, Basel, Switzerland] . When coupled with microdialysis and flow injection systems, biosensors have become available for on-line, real-time monitoring [Freaney et al. (1997) *Ann. Clin. Biochem.* 34:291-302; Cook, J. (1997) *Nat. Biotech.* 15:467-471; Steele and Lunte (1995) *J. Pharm. Biomed. Anal.* 13:149-154; Kaptein et al. (1997) *Biosens. Bioelectron.* 12:967-976; Nima et al. (1996) *Anal Chem.* 68:1865-1870]. In one preferred embodiment, the present invention couples amino acid detecting arrays to microdialysis or miniaturized ultrafiltration systems to achieve real-time and ultrasensitive detection of amino acids as they are released into solution by carboxypeptidases or aminopeptidases.

The microsystem can be divided into two parts: the mechanical portion with the biochemistry and microfluidic pumps and the electronic portion which has the laser, detector, and the computer interface.

In one preferred embodiment, the computer interface will be approached by building a custom circuit which will connect to 20 light detectors and other input timing signals. The custom circuit will be a stand alone microprocessor which will collect all of the timing and light intensity information and send the resulting data out to a computer, for example, via a USB or serial port. The computer will be programmed for data analysis.

Chip enzymatic nanosequencers are a still further aspect of the present invention. Certain components of such systems such as continuous flow mixers, pumps, microreactors, microdialysis and miniaturized ultrafiltration systems are known [Kricka et al. (1998) *Clin.*

Chem. 44:2008-2014]. A C-terminal nanosequencer of the present invention is illustrated in Fig. 5A. The carboxypeptidase digestion takes place in a tiny chamber traversed by a microdialysis probe or miniaturized ultrafiltration probe. The membrane has a molecular weight cutoff such that it allows free passage of amino acids but is impermeable to the protein substrate and carboxypeptidases. As the amino acids are liberated from the protein's termini, they cross the membrane and enter a flow stream which carries them to the biosensor array for continuous detection. The sequential record of the continuous detection of the 20 amino acids by the biosensor array provides the terminal sequence of the protein.

As illustrated in Fig. 5A, the biosensor array consists of 20 reaction channels. Each reaction channel has an inlet for amino acids to flow into and an outlet and is connected to a different reservoir by a microchannel. Each reservoir contains the 20 synthetases and a fluorescently labeled tRNA specific for a different amino acid. (Alternatively, each reservoir can contain one synthetase and a fluorescently labeled cognate tRNA). The 20 reaction solutions are continuously transported through the reaction channels (e.g. by continuous-flow micropumps). EF-Tu;GTP is immobilized downstream in each reaction channel and used to capture and detect the fluorescently labeled AA-tRNAs. A laser beam is focused on the immobilized EF-Tu;GTP. For example, EF-Tu;GTP can be immobilized to optical fibers placed in each reaction channel. It is possible to selectively excite and detect fluorescence from the immobilized AA-tRNA-EF-Tu;GTP complexes even in the presence of high concentrations of fluorescently labeled tRNAs in the bulk solution (e.g. using evanescent wave excitation). As amino acids cognate for the fluorescently labeled tRNA in each channel flow through, a proportionate amount of fluorescently labeled AA-tRNA is formed. The newly formed AA-tRNAs are captured downstream by the immobilized elongation factor. When AA-tRNAs bind to the immobilized elongation factor, laser light in the evanescent wave excites the fluorophore, generating a signal. The GTP bound elongation factor binds AA-tRNAs but the GDP bound elongation factor does not. Accordingly, the AA-tRNAs may be eluted with a buffer containing GDP in order to reactivate the immobilized elongation factor-based sensor with a regeneration buffer containing GTP. This elution method has been used to affinity purify AA-tRNAs on immobilized EF-Tu;GTP [Chinali, G. (1997) *J. Biochem. Biophys. Meth.* 34:1-10]. Other suitable elution

systems contain high salt, e.g., 1 M NaCl.

Additional microflow formats are also within the scope of the present invention. For example, a different synthetase (see Fig. 5B) or tRNA (one specific for a different amino acid) can be immobilized in the 20 reaction channels. Alternatively, an amino acid biosensor array (e.g. optical fiber or microelectrode) can be placed in a flow stream and detect amino acids as they flow through the array. (see Fig. 5C). Other methods for following the reaction of each amino acid with its cognate synthetase in spatially resolved microflow or microwell formats are suggested. Fluorescent tags may be attached to the synthetases and the change in fluorescence as the synthetase binds its cognate amino acid is monitored. In other embodiments, the molecule that specifically binds AA-tRNAs (e.g. EF-Tu:GTP ) is fluorescently labeled and the change in fluorescence caused by the binding to the AA-tRNA is monitored to follow the reaction. The elongation factor may be conveniently fluorescently labeled, for example, by using a fluorescently labeled GTP analog.

The high-throughput amino acid analysis microchips described herein are useful in the developing field of proteomics. These microarrays allow for the amino acid analysis of all proteins separated by a 2D electrophoresis gel on a single chip or plate simultaneously. In proteomics, it is especially important to determine the N- and C-terminal sequence of an intact protein. End-group sequencing can be used to identify the start and stop point of a protein or gene; provides sequence information necessary for PCR cloning of the intact gene; identifies limited proteolytic products, which are common to many important regulatory mechanism; and provides a powerful method to identify proteins separated by 2D gels. Microfabricated end-group sequencers such as those described can be constructed as massively paralleled, computer controlled and integrated systems, where both N- and C-terminal sequencing of many proteins can be performed on a single chip platform simultaneously. The terminal sequence tags generated can be processed on-line and the proteins identified by database searching. Among the most outstanding deficiencies in the current set of methods in protein chemistry are the ones for C-terminal sequencing. Since no sensitive and reliable method for C-terminal sequencing is

available, the C-terminus of proteins is a protein region that is often not analyzed. Hence, the C-terminal sequenators of the present invention are especially useful in proteome projects.

High throughput methods for protein end group amino acid analysis methods to identify the C-terminus of proteins are further aspects of this invention. The methods for C-terminus analysis prior to the present invention are inadequate for analysis of minute quantities of protein. These inadequate methods include hydrazinolysis [Steydon, D.J. (1988) *Anal. Biochem.* 174:677-686] and tritium incorporation using tritiated water after treatment of the protein with acetic anhydride to form the oxazolone [Matsuo et al. (1966) *Biochem. Biophys. Res. Commun.* 22:69-74]. Both methods are relatively insensitive and prone to problems. Most researchers have been forced to identify the C-terminus of a protein by peptide mapping strategies. This method is not quantitative and may miss the correct C-terminal peptide or minor but important C-terminal peptides. The present invention couples carboxypeptidase digestion with high throughput microarrays and microsystems for rapid C-terminal analysis. The method described herein is suitable for protein end group amino acid compositional analysis, which is used to identify proteins in conjunction with sequence database searching.

Another important consideration in proteome studies is sensitivity. Proteins, unlike genes, have no amplification methods, so sensitivity is even more important than for gene analysis. In order to analyze minor proteins separated from 2D gels we must work at the femtomole level. Current amino acid analyzers and sequenators fall short of this level by three orders of magnitude. By converting the amino acids into macromolecules (AA-tRNAs) that are specifically bound by another macromolecule (e.g. EF-Tu;GTP) with a high affinity ultrasensitive ligand assays and automated microarrays for amino acid analysis of the present invention are thus particularly useful. With laser induced fluorescence as a detection method, simple and sensitive amino acid analyzers and end-group sequenators thus form an aspect of the present invention.

Using genetic engineering techniques well known to the art, one of ordinary skill can

express the elongation factor and synthetases as fusion proteins which comprise a polypeptide tether. The tether is composed of polar amino acids. For example, tethers composed of serine and glycine are flexible as well as polar. The polypeptide tether desirably contains binding motifs, domains or epitope tags recognized by antibodies for the immobilization of the fusion protein. Alternatively, the synthetases and elongation factors can be joined using bifunctional crosslinking reagents, such as those with long polar spacer arms.

In one embodiment of the invention, the synthetase and the elongation factor are joined to a flexible polypeptide tether, in the central portion of which is an epitope recognized by an antibody. The synthetase and the elongation factor can be immobilized to the epitope's cognate antibody which itself is affixed to the surface of a well, array locus or microchannel used in the present invention. Alternatively, the epitope within the polypeptide tether chain can be unique for each particular synthetase, where there are twenty epitopic specificities and twenty antibodies specific thereto. Desirably, antibodies used for immobilization (or capture) are monoclonal antibodies. This embodiment is exemplified in Fig. 6A.

In an alternative embodiment, each synthetase and the elongation factor are linked to one end of a tether or spacer arm, and there is a ligand at the other end of the tether or spacer arm. In a particular embodiment, the ligand at the end of the tether or spacer arm is biotin. The synthetase and elongation factor then bind via the ligand to the ligand binding partner (avidin or streptavidin in the case where the ligand is biotin). The ligand binding partner can be bound to an array locus, the interior of a microchannel or the bottom of a microwell. Alternatively, there can be an antibody affixed to the array locus, microchannel or microwell and to the antibody is bound a ligand-bearing polypeptide which also bears an epitope recognized by the antibody bound to the surface of the assay device. The ligand can again be biotin if the ligand binding partner is avidin or streptavidin or other biotin-binding molecule. See Fig. 6B for a diagrammatic representation of this embodiment. Avidin or streptavidin or desirable because there each molecule can bind more than one molecule of biotin.

In another embodiment of the invention, each synthetase and the elongation factor are separately bound to one end of a tether or spacer arm, which is bound to a ligand at the end opposite the protein.. The surface of the array locus, the microchannel or microwell contains a bound oligonucleotide. Binding of the synthetase and elongation factors to the array locus,  
5 microchannel or microwell is mediated by an oligonucleotide complementary to the surface bound oligonucleotide to which is bound a ligand. A ligand binding partner which can bind the ligands attached to the synthetase, elongation factor and oligonucleotide completes the attachment system. In a specific embodiment the ligand is biotin and the ligand binding partner is avidin or streptavidin. This system is shown in Fig. 6C.

10 Proteins and nucleic acids have been immobilized onto solid supports in many ways. Methods used for immobilizing proteins and nucleic acids are described in the following references, and others [Mosbach (1976) *Meth. Enzymol.* 44:2015-2030; Weetall (1975) *Immobilized Enzymes, Antigens, Antibodies and Peptides*; Hermanson, G.T. (1996) *Bioconjugate Techniques*, Academic Press, NY; Bickerstaff, G. (ed.) (1997) *Immobilization of Enzymes and*  
15 *Cells*, Humana Press, NJ; Cass and Ligler (eds.) *Immobilized Biomolecules in Analysis*, Oxford University Press; Watson et al. (1990) *Curr. Opin. Biotech.* 609:614; Ekins, R.P. (1998) *Clin. Chem.* 44:2105-2030; Roda et al. (2000) *Biotechniques* 28:492-496; Schena et al. (1998) *Trends in Biotechnol.* 16:301-306; Ramsay, G. (1998) *Nat. Biotechnol.* 16:40-44; Sabanayagam et al. (2000) *Nucl. Acids Res.* 28:E33; United States Patent No. 5,700,637 (Southern, 1997); United  
20 States Patent No. 5,736,330 (Fulton, 1998); United States Patent No. 5,770,151 (Roach and Jonston, 1998); United States Patent No. 5,474,796 (Brenman, 1995); United States Patent No. 5,667,667 (Southern, 1997); all of which are incorporated by reference herein].

For optical biosensors solid supports such as fused silica and quartz are appropriate substrates for immobilization. Adsorption, entrapment and covalent attachment are among the  
25 techniques employed for immobilization of biomolecules onto solid supports.

Electrochemical-based enzyme immobilization methods are convenient for enzymes on

microelectrodes; however, this method is restricted use with amperometric sensors. This method allows each enzyme or nucleic acid to be located at one electrode (the working electrode). There are several situations in which conventional crosslinking based immobilization is inadequate in the construction of microelectrodes, for example, when on-wafer deposition (i.e., immobilization on the whole wafer before it is cut into smaller segments for use in individual devices) is required, leading to many localized immobilizations or during fabrication of multianalyte sensors requiring several distinct membrane sensors. The three main types of immobilization developed to overcome these problems are based on photochemistry, electrochemistry and printing [see, e.g., Bickerstaff, G.F. (ed.) (1997) *supra*].

The proteins can be adsorbed, embedded or entrapped or covalently linked to surfaces. the proteins can be adsorbed or attached to nanoparticles, for example, and these nanoparticles can be position in microflow channels. The nanoparticles can be held in position using magnetic nanoparticles and magnetic force or by a filter, grid or other support. Alternatively, the proteins can be adsorbed or covalently attached to the surfaces within the microflow channels or wells.

The biomolecules can be immobilized on the surfaces within the microflow channels, wells or membranes, or the biomolecules can be immobilized onto the surfaces of beads, membranes or transducers or other surfaces placed in the flow channels, chambers or wells. Suitable beads for immobilization of proteins or nucleic acids (especially tRNAs) include chemically or physically crosslinked gels and porous or nonporous resins such as polymeric or silica based resins. Suitable media for adsorption include, without limitation, ions exchange resins, hydrophobic interaction compounds, sulfhydryls and inherently active surfaces and molecules such as plastics or activated plastics, aromatic dye compounds, antibodies, antibody fragments, aptamers, oligonucleotides, metals or peptides. Examples of some suitable commercially available, polymeric supports include, but are not limited to, polyvinyl, polyacrylic and polymethacrylate resins. Steric hindrance arising from these supports should be minimal. Free sulfhydryls are used in site specific conjugation of proteins and nucleic acids to surfaces and labels.

Many coupling agents are known in the art and can be used to immobilize biomolecules in the methods and devices of the present invention. Coupling agents are exemplified by bifunctional crosslinking reagents, i.e., those which contain two reactive groups which may be separated or tethered by a spacer. These reactive ends can be of any of a number of  
5 functionalities including, without limitation, amino reactive ends such as N-hydroxysuccinamide, active esters, imidoesters, aldehydes, epoxides, sulfonyl halides, isocyanate, isothiocyanate, nitroaryl halides, and thiol reactive ends such as pyridyl disulfide, maleimides, thiophthalimides and active halogens.

Enzymes with quaternary structure include the amino acyl tRNA synthetases used in the  
10 present invention. These enzymes can undergo inactivation by dissociation of subunits and stabilization of these enzymes can be achieved by crosslinking the subunits as taught, for example, in Torchilin et al. (1983) *J. Molec. Catalysis* 19:291-301.

Another method of immobilizing proteins uses proteins with N or C terminal spacers or tethers. The immobilized reagent or factor is a specific biomolecular recognition reagent, e.g.,  
15 synthetase, tRNA, elongation factor or probe and is attached covalently or noncovalently on a surface. This can be used to form a capture site. In preferred embodiments, the immobilized reagent can be chosen to directly bind the analyte (e.g) the amino acid(s)) or indirectly bind the analyte by means of an ancillary specific binding member that is bound to the analyte. The immobilized reagent can be immobilized on the structure before or during the performance of the  
20 assay by means of a suitable attachment method.

When macromolecular ligands are used, the biomolecules must be immobilized in such a way as to reduce steric hindrances generated by the support. A variety of methods for achieving this are known in the art. For example, the active site or other binding region of the biomolecule  
25 can be orientated away from the surface [Reviewed in Bickerstaff, GF (ed.) *Immobilization of Enzymes and Cells*, pp 261-275).



When it is necessary to reduce steric problems or an immobilized biomolecule reacting with a macromolecule, as in some of the preferred embodiments of the current invention, a suitable spacer arm is used to immobilize the biomolecule to a surface. The spacer arm distances the biomolecule from the support surface. The spacer arm should be long enough to promote efficient separation of the biomolecule from the support; the spacer arm should be very flexible to provide high mobility to the immobilized biomolecule and thereby allow maximum interaction with the macromolecule ligand. Suitable spacer arms include but are not limited to dextrans, particularly those oxidized by periodate, polypeptides, protein, nucleic acids, and peptide nucleic acids.

Printing methods for making microarrays in the current art can be used to deliver nucleic acid or proteins to surfaces in predetermined locations. For example, aminophenyl-trimethoxysilane treated glass surfaces can bind 5' amino-modified oligonucleotides nucleic acids using a homo bifunctional crosslinker to attach the aminated oligonucleotide to the aminated glass as taught in Guo et al. (1994) *Nucleic Acids Research* 22:5456-5465. Another known method for arraying nucleic acids is to react the nucleic acid with succinic anhydride and attach the resulting carboxylate group via an ethyldimethylaminopropylcarbodiimide-mediated coupling reaction [Joos et al. (1997) *Anal. Biochem.* 247: 96-101]. In another method 5' phosphate modified nucleic acids react with imidazole to produce a 5'-phosphoimidazolide that can bind to surface amino groups via a phosphoramidate linkage [Chu et al. (1983) *Nucleic Acids Research* 11:6513-6529]. The linker must be long enough to eliminate steric hindrance caused by the solid surface to ensure efficiency of the following binding reactions. For example, Shchepinov et al. (1997) *Nucleic Acids Research* 25:1155-1161, reported that an optimal spacer length is at least 40 atoms long will increase binding yields by 150-fold in nucleic acid hybridization experiments on microarrays.

Over the past two decades, the avidin-biotin system has been developed for the immobilization of proteins and nucleic acids. For a review, see Wilchek, M, and Bauer EA (ed) *Avidin-Biotin Technology*, Academic Press, San Diego, CA. Proteins or nucleic acids can be

immobilized using avidin-biotin technology where a biotin labeled molecule can be bound irreversibly to avidin, which is attached to the solid support. The extraordinary affinity of avidin (or its bacterial relative streptavidin) for biotin forms the basis of this system. Since avidin, streptavidin, their analogues, and their derivatives are very stable, their immobilization is usually  
5 advantageous compared to other proteins. Because one avidin molecule can bind four biotin molecules, it is possible to co-immobilize each synthetase and elongation factor Tu:GTP using avidin-biotin technology. In this embodiment of the invention, the synthetases and elongation factor are attached to long flexible biotinylated spacer arms. The spacer arm can be a commercially available polymer such as dextran, a polypeptide, a nucleic acid, or some other  
10 tether. The streptavidin (or avidin or a derivative thereof) bearing the co-immobilized synthetase and elongation factor can be immobilized as a microspot in a microarray. This can be accomplished, for example, by binding a biotinylated oligonucleotide or nucleic acid probe having a unique sequence to each of the 20 synthetase-elongation factor pairs co-immobilized using avidin molecules. A nucleic acid or oligonucleotide probe having a sequence  
15 complementary to each of the 20 synthetase-elongation factor-oligonucleotide conjugates can be arrayed onto a surface in known positions. In this way each of the 20 synthetases are immobilized to the surface in known positions as the nucleic acid (or probe nucleic acid) conjugated to avidin molecules binds its complementary sequence by Watson-Crick base pairing.

In another embodiment, the synthetase-elongation factor-avidin molecule can be  
20 conjugated to a different biotinylated peptide or peptide bearing a strep-tag sequence. The Strep-tag constitutes a nine amino acid-peptide that specifically binds to streptavidin occupying the same binding site where biotin normally binds. Strep-tag fusion proteins can be constructed and used to immobilize proteins to streptavidin. Any Affinity-Tag sequences such as Hexa-histidine for metal chelate immobilization and epitope sequence for specific binding by an  
25 immobilized antibody can be used. In one embodiment of the subject microarray, 20 different epitope sequences and 20 different antibodies, one that specifically binds each epitope sequence is used to array the biorecognition molecules. These conjugates are thereby arrayed onto surfaces bearing 20 different antibodies, one specific for a unique epitope on each peptide on the

20 synthetase-elongation factor-avidin conjugates. The antibodies may be arrayed to the surface using any of the microarray technologies known in the art.

Recombinant DNA methodologies are commonly used to generate fusion proteins having N- or C-terminal extensions that provide either a tether or spacer arm and binding sites for the immobilization of proteins. Such methods will be suitable for the immobilization of proteins and nucleic acids in the subject invention. Examples of these methods are given in the following references: Nilsson et al. (1997) *Protein Expr. Purif.* 11:1-16; Shpigel et al. (1999) *Biotechnol. Bioeng.* 65:17-23; Kroger et al. (1999) *Biosens. Bioelectron.* 14:155-161; Piervincenzi et al. (1998) *Biosens. Bioelectron.* 13:305-312; Airene et al. (1999) *Biomol. Eng.* 16:87-92; Skerra, A. and Schmidt, T.G. (1999) *Biomol. Eng.* 16:151-156; and Jones et al. (1995) *J. Chromatogr. A*, 707, 3-22.

In methods for the immobilization of the synthetases, tRNAs, or elongation factor, a spacer arm or tether is preferred. Elongation factor Tu from *Thermus thermophilus* containing six histidine residues on its C-terminus has been immobilized on Ni(2+)-nitriloacetic acid agarose and used to affinity purify amino acyl-tRNA [Ribeiro et al. (1995) *Anal. Biochem.* 228:330-335]. Importantly, the immobilized EF-Tu;GTP does not lose its affinity for AA-tRNAs.

For t-RNA arrays the 20 different t-RNAs can be immobilized by covalent or noncovalent attachment. The t-RNAs may be arrayed by electrostatic binding to basic molecules such as poly-L-lysine, poly-L- arginine, protamine or other basic proteins or polypeptides. Immobilization of nucleic acids on microarrays by electrostatic binding to immobilized poly-L-Lysine has resulted in the detection of mRNA species present at a ratio of 1:100,000 [DeRisi et al. (1997) *Science* 278:680-686]. Alternatively t-RNAs can be bound to complementary sequences of single stranded nucleic acids or to RNA binding proteins. Oligonucleotides can be synthesized directly on the surfaces of chips, or can be pre-synthesized and then deposited onto the chips. tRNAs can bind to these arrayed nucleotides by complementary base pairing.

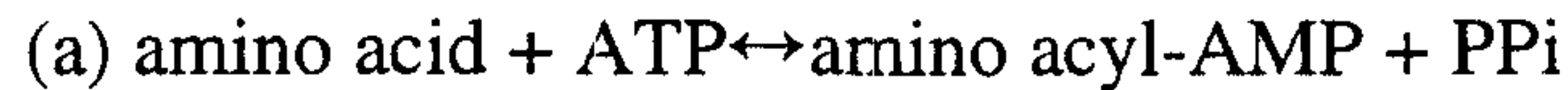
Controlled electric fields have been used to immobilize nucleic acids on microelectrodes [Sosnowski et al. (1997) Proc. Natl. Acad. Sci. USA 94:111-1123].

Array encoding technologies other than the positional encoding used for microarrays are known in the arts. For example, Luminex (Austin, TX) describes a method for encoding  
5 microspheres according to their fluorescence [Fulton et al. (1997) Clin. Chim. 43:1749-1756; Fulton, RJ US Patent No. 5,736,330] A different nucleic acid can be attached to each type of microsphere, and the microspheres can be mixed and bound to a fluorescently labeled sample. Reading in a flow cytometer then allows each microsphere to be identified and the corresponding fluorescently labeled molecule to be identified. Other methods for the synthesis and  
10 hybridization of nucleic acids on microspheres are taught in Hakala et al. (1997) Bioconj. Chem. 8:378-384. Single microspheres have been immobilized in wells etched from optical fiber bundles. These biosensor arrays allow the elements to be in the sub-micrometer size range [Michael et al. (1998) Anal. Chem. 70:1242-1248]. Any of the biosensor technology can be engineered in an array format and used to make amino acid biosensors as described in the current  
15 invention.

The microarrays, microsystems, or kits of the present invention can be readily incorporated into the technologies of the current art, such as those disclosed in the prior art section above, for example. The proteins and nucleic acids of the subject invention may be immobilized in any number of ways. The methods for array construction or biomolecule  
20 immobilization are not important in the subject invention, as a vast number of methods known in the art are suitable.

Any one of a number of particular assays can be used to follow the biospecific recognition of each amino acid by its cognate amino acyl tRNA synthetase. A number of assays have been developed by the art that are suitable for monitoring the reactions in the subject  
25 invention.

The synthetase catalyzes the two step reaction [reviewed in Schimmel, P. (1987) *Annu. Rev. Biochem.* 56:125-158; Friest, W. (1989) *Biochemistry* 28:6787-6785]:



5           The recognition of each amino acid by its cognate synthetase is monitored by following the formation of amino acyl-tRNA or PPi in reaction a or by following the formation of amino acyl-tRNA or AMP in reaction b.

10           Real-time methods for following the formation of PPi (reaction a) are known in the art and are suitable for use in the some embodiments of the current invention. For example, a continuous spectrophotometric assay for measurement of inorganic phosphate that is released from PPi upon hydrolysis by inorganic pyrophosphatase can be measured [Webb, M.R, (1992) *Proc. Natl. Acad. Sci. USA* 89:4884-4887]. This method has been applied to monitor reaction a [Lloyd et al. (1995) *Nucleic Acids Res.* 23:2886-2892].

15           A fluorescent probe has been described that can rapidly measure inorganic phosphate (Pi) as released by enzymes such as inorganic pyrophosphatase in real time in biological systems [Brume et al. (1994) *Biochemistry* 33:8262-8271]. This method may be employed to follow reaction a.

            The reactions catalyzed by the AA-tRNA synthetase can also be followed by monitoring the formation of AMP or amino acyl-tRNAs in reaction b.

20           For example, the formation of AMP in reaction b has been followed in a continuous spectrophotometric assay. [Wu, M.X. and Hill, K.A.W. (1994) *Anal. Biochem.* 211:320-323]. The amino acyl-tRNA synthetase-dependent formation of AMP is enzymatically coupled to the lactate dehydrogenase oxidation of NADH. Oxidation of NADH is monitored at 340 nm in the presence of excess coupling enzymes, ATP, and phosphoenolpyruvate. This assay is applicable

to all the AA-tRNA synthetases. Multienzyme coupling assays of this type have been successfully applied to the kinetic study of a vast number of enzymes over the years [see, e.g. Oliver, I.T. (1955) *Biochem. J.* 61:116-122] and this approach allows the production of ultrasensitive assays for following the synthetase reactions.

5            In another assay to follow the reactions catalyzed by the synthetases, the formation of AA-tRNAs in reaction b is monitored. The current inventor has described a novel assay for following the formation of all AA-tRNAs using a binding assay. Elongation factor 1 a (called elongation factor Tu in bacteria), when complexed to GTP, binds all AA-tRNAs with high affinity and specificity, forming a stable ternary complex. The elongation factor GTP complex  
10 binds all AA-tRNAs with high affinity but not the free tRNA. Therefore, by following the binding of AA-tRNAs to the elongation factor, the overall reaction catalyzed by the synthetases can be monitored. This assay is especially advantageous. Since both AA-tRNAs and the elongation are macromolecules, ultrasensitive assays may be devised that employ, for example, high specific activity labels. This assay is applicable to microchannel and microarray systems as  
15 well as affinity biosensors.

The development of these miniaturized array-based systems improves the throughput and sensitivity of amino acid analysis and protein end-group sequencing by orders of magnitude and allow proteins, like DNA and RNA, to be subjected to mass screening.

20            Monoclonal or polyclonal antibodies, preferably monoclonal, specifically reacting with a particular protein or epitope of interest, such as an AA-tRNA-EF:Tu-GTP complex may be made by methods known in the art. See, e.g., Harlow and Lane (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratories; Goding (1986) *Monoclonal Antibodies: Principles and Practice*, 2d ed., Academic Press, New York; and Ausubel et al. (1992) *Current Protocols in Molecular Biology*, Green Wiley Interscience, New York, NY.

25            Standard techniques for cloning, DNA isolation, amplification and purification, for

enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like, and various separation techniques are those known and commonly employed by those skilled in the art. A number of standard techniques are described in Sambrook et al. (1989) *Molecular Cloning*, Second Edition, Cold Spring Harbor Laboratory, Plainview, New York; 5 Maniatis et al. (1982) *Molecular Cloning*, Cold Spring Harbor Laboratory, Plainview, New York; Wu (ed.) (1993) *Meth. Enzymol.* 218, Part In; Wu (ed.) (1979) *Meth. Enzymol.* 68; Wu et al. (eds.) (1983) *Meth. Enzymol.* 100 and 101; Grossman and Moldave (eds.) *Meth. Enzymol.* 65; Miller (ed.) (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Old and Primrose (1981) *Principles of Gene Manipulation*, 10 University of California Press, Berkeley; Schleif and Wensink (1982) *Practical Methods in Molecular Biology*; Glover (ed.) (1985) *DNA Cloning* Vol. In and II, IRL Press, Oxford, UK; Hames and Higgins (eds.) (1985) *Nucleic Acid Hybridization*, IRL Press, Oxford, UK; Setlow and Hollaender (1979) *Genetic Engineering: Principles and Methods*, Vols. 1-4, Plenum Press, New York; and Ausubel et al. (1992) *Current Protocols in Molecular Biology*, Greene/Wiley 15 Interscience, New York, NY. Abbreviations and nomenclature, where employed, are deemed standard in the field and commonly used in professional journals such as those cited herein.

The following examples are provided for illustrative purposes, and are not intended to limit the scope of the invention as claimed herein. Any variations in the exemplified articles and/or methods which occur to the skilled artisan are intended to fall within the scope of the 20 present invention.

All references cited herein are incorporated by reference in their entireties to the extent that there is no inconsistency with the present disclosure.

## EXAMPLES

### Example 1. Spatially Resolved Homogenous Binding Assays

25 Either a microflow channel or a microwell format can be used. The protein of interest is

sequentially degraded, either from the amino terminus or the carboxy terminus. Alternatively, the protein can be totally digested, and one simultaneous measurement of all twenty primary amino acids can be made. Where the total digestion of the protein is carried out for the analysis of the amino acid composition of the protein, the proteases utilized can include those including  
5 but not limited to one or more of the following: trypsin, chymotrypsin, staphylococcal V8 protease, aminopeptidase and a carboxypeptidase. Preferably, for a total digestion, there are a combination of proteases used. Where the carboxy terminus is the target, carboxypeptidase is used. The degradation can be carried out in a digestion chamber which is separated from the flow channel by a molecular sieve (e.g., a selectively permeable membrane, microdialysis  
10 membrane or microdialysis probe, through which free amino acids but not polypeptides of greater than about 6000 molecular weight can pass in an unhindered fashion. The exopeptidase can be immobilized in the digestion (or reaction) chamber, either to a surface within the chamber or on a solid support such as a microbead within the chamber. The free amino acids diffuse into a flowstream that enters a microfluidic array of reaction channels. Through each reaction channel  
15 is pumped a reaction buffer having a different tRNA and its cognate synthetase along with EF-Tu:GTP. When AA-tRNAs are formed and bound by the EF-Tu:GTP, the fluorescence changes. The fluor can be bound to the tRNA or to the EF-Tu:GTP provided that the specificity and the ability to bind ligands are not changed.

A fluor can be bound to the tRNA or to the EF-Tu:GTP, provided that the specificity and  
20 the ability to bind ligands are not changed. Both tRNAs and EF-Tu:GTP have been fluorescently labeled and found to retain their binding specificities.

Reactive groups such as thiol, amine, or phosphorothioate can be introduced in nucleic acids for coupling of chromophores. For example, see Johnson et al. (1982) *J. Mol. Biol.* 156:113-140. Labeling of tRNAs can be achieved by hybridizing a fluorescently labeled  
25 oligonucleotide or peptide nucleic acid to the single stranded regions of the tRNAs. Alternatively, fluors can be covalently conjugated to the tRNAs [Janiak et al. (1990) *Biochemistry* 29; 4268-4277] labeled tRNAs by attaching fluorescein covalently to the



thiouridine (s4U) at position 8 which is a conserved residue (U or s4U) in all tRNAs. The labeled tRNAs retained their abilities to be amino acylated by the synthetases and recognized by the elongation factors. Upon binding of EF-Tu:GTP with the fluorescent AA-tRNA, the emission intensities increased. The emission intensity was nearly the same for all AA-tRNAs when bound to EF-Tu:GTP. The method of Janiak et al. can be used to label the t-RNA with fluorescein. Synthetic or enzymatic procedures have been established, allowing site-specific incorporation of thionucleotide(s) such as 4-thiouracil within RNA, and structural and biological activities remain intact [reviewed in Favre et al. (1998) *J. Photochem. Photobiol. B* 42:109-124].

Alternatively, the elongation factors may be labeled with fluors. Many references can be found on modification of various groups in proteins or peptides with fluors. These are summarized in reviews or monographs; for example, see Haugland, R.P. (1992) *Handbook of Fluorescent Probes and Research Chemicals*, 5<sup>th</sup> ed., Molecular Probes, Eugene, OR. Although several groups can be used to couple to a chromophore, the thiol group is thought to be the best candidate in that many groups are thiol specific or selective, and thus unique labeling is possible. With site-directed mutagenesis a thiol group can be added to or deleted from a desired position. Since the three dimensional structure of the ternary complex of aminoacylated tRNA and EF-Tu-GTP has been solved [reviewed in Nissen et al. (1996) *Biochimie* 78:921-933], the contact sites between the elongation factor and AA-tRNAs are known. From the structure many amino acid residues on the protein's surface are available that do not interact with AA-tRNAs and are hence good candidates for labeling. The elongation factor can be conveniently labeled using a fluorescent GTP analog. Elongation factor Tu has been labeled with fluorescent GTP analogs, and it has been found to retain its specificity for AA-tRNAs. For example, the ribose of GTP was covalently modified with the dye rhodamine (Rh) to form GTP-Rh, and the GTP-Rh was used to label EF-Tu. The EF-Tu:GTP-Rh retained its specificity for AA-tRNA [Watson et al. (1995) *Biochemistry* 34:7904-12]. GTP has been labeled with other fluors and used to label EF-Tu [Giovane et al. (1995) *Eur. J. Biochem.* 227:428-432; Eccleston et al. (1987) *Biochemistry* 26:3902-3907]. EF-Tu complexed with fluorescent GTP analogues retains its specificity for AA-tRNAs.

Another way to fluorescently tag the elongation factor is to fuse it with the green fluorescent protein [Iwane et al. (1997) FEBS Letts. 407:235].

5 Since rhodamines absorb where fluorescein emits, they can be used as energy acceptors in fluorescence energy transfer assays. Watson (1995) et al. have shown that AA-tRNAs labeled with fluorescein specifically bind to EF-Tu;GTP labeled with rhodamine. They used fluorescence energy transfer to determine the macromolecular arrangement in the amino acyl-tRNA-EF-Tu complex.

10 For the analysis of free amino acids in a sample, the sample liquid is placed in the reaction chamber, so that the amino acids pass through the molecular sieve and into the microchannels or microwells or into contact with the microspots for quantitation. In this embodiment, there is one measurement made for each amino acid, rather than a series of measurements for each amino acid over time.

15 In the microwell format, the sequential proteolysis is carried out as above. The released amino acids are dispensed in parallel into microwells each specific for a particular amino acid and containing the reagents necessary for the amino acid react with the cognate synthetase to form AA-tRNAs, which are bound by the EF-Tu;GTP. In one embodiment, the tRNA contains a bound fluor, for example as taught in Janiak et al. (1990) Biochemistry 29:4268-4277. The wells contain immobilized EF-Tu;GTP which will bind the AA-tRNA. The wells are emptied and  
20 desirably washed prior to excitation of the fluor and detection of the signal. In this embodiment, the twenty wells are repeated multiple times, and there is a sequential dispensing of exopeptidase digest into each set. A micropipettor can dispense the amino acid-containing dialysate from the digestion chamber. Detection of those wells in which there is AA-tRNA EF-Tu;GTP complex successively over time allows the determination of the terminal amino acid sequence of the  
25 protein. Where the exopeptidase is an amino peptidase, the N-terminal sequence of the protein is deduced; and where the exopeptidase is a carboxypeptidase, the C-terminal sequence of the protein is deduced. See Fig. 20 for a diagrammatic illustration of a microplate to be used in this

embodiment of the invention. The wells can alternatively comprise labeled tRNAs which can be immobilized to the bottoms of the microwells by binding to immobilized EF-Tu;GTP or a labeled oligonucleotide capable of binding to each particular AA-tRNA can be used to generate a signal in the presence of each particular AA-tRNA, in which case a washing step precedes signal  
5 determination. Samples can be taken every 5 min as described using reaction conditions as described in Patterson, D.M. (1995) *Anal. Chem.* 67:3971-3978, with carboxypeptidase Y.

Although the release of amino acids by the exopeptidase is nonlinear, by taking frequent time points one can generate rate curve information and the terminal sequence can be deduced.

To detect the presence of a particular amino acid covalently bound to its cognate tRNA,  
10 the well or microchannel is illuminated with light at the excitation wavelength and fluorescence is detected according to the wavelength of the emitted light. A laser of the desired wavelength of light can be used for the excitation.

In another embodiment, the free amino acids which leave the digestion chamber across the selectively permeable microdialysis membrane can be channeled to amino acid specific  
15 channels, each of which are specific for a particular amino acid due to the presence of a particular amino acyl tRNA synthetase and tRNA. The AA-tRNA can then be detected on an array containing the detectors with positional information related to a particular amino acid specificity of each channel and its particular synthetase and tRNA. Information from the detectors is transmitted to a computer where it is decoded to provide amino acid sequence information. The  
20 change in amino acyl tRNA formed with time is directly related to the sequential endwise degradation of the protein of interest in the digestion chamber. See Figs. 21A-21B for a diagrammatic representation of such a microfluidic system for endgroup analysis (in this diagram, C-terminal amino acid sequence analysis) and sequence. As the amino acids are liberated from the protein substrate in the digestion chamber and cross a microdialysis  
25 membrane, they enter a flowstream that carries them into the amino acid detector microfluidic array. The amino acid mixture, which over time is sequentially enriched for a particular amino

acid according to its position within the protein of interest, flows into a central chamber and then the flowstream is split between twenty reaction channels, each specific for a particular amino acid. Each reaction channel is in fluid connection with a unique reservoir containing a particular tRNA and synthetase. The amino acids react with their cognate tRNAs and synthetases, and they are detected in a spatially resolved manner. An optical fiber can be coupled to the detector portion of the channel or channel accepting reacted material from the reaction channels, with the result that signals generated in response to laser excitation are fed to a computer which then translates the data into amino acid sequence information. Desirably the channels are in fluid communication with waste receptacle(s) so that continuous flow can be accomplished as the protein of interest is sequentially degraded.

Alternatively, the microdialysis probe is attached to microchannels that are perfused with a solution containing the twenty amino acyl tRNA synthetases, EF-Tu;GTP and a tRNA species specific for one amino acid. As the amino acids diffuse across the microdialysis membrane and enter the detection chamber, they are converted to a proportionate amount of the ternary complex with EF-Tu;GTP, which complex displaces a corresponding amount of labeled ternary complex within the detection chamber. The labeled complex then flows to the detector and is quantitated. From the record of the passage of labeled molecules past the detector in the twenty channels each specific for a particular amino acid, the sequence of the protein of interest is deduced.

A further specific embodiment of the microwell format is one in which within each microwell or nanowell, there is a central spot in which the protein of interest is present together with an immobilized exopeptidase, for example, a carboxypeptidase. The amino acids freed by carboxypeptidase digestion of the protein of interest, diffuse to the twenty individual spots within the well, each of which is specific for a particular amino acid. Within the cognate spot, the amino acid is converted to the AA-tRNA by the action of the particular amino acid tRNA synthetase present. Hundreds to thousands of wells on a single plate or microchip allow the simultaneous analysis of many minute samples. In this embodiment, the synthetase and elongation factor are immobilized to a unique microspot. If evanescent excitation and/or

detection are used, real time amino acid analysis is possible. As shown the tRNAs are fluorescently labeled, and the formation of the ternary complex is monitored by the change in fluorescence as the ternary complex forms. The amino acids are being released continuously so that the concentration in solution is highest for amino acids closest to the terminus where digestion occurs and they are detected first.

#### Example 2. Continuous Displacement Format

The protein of interest is sequentially degraded using an aminopeptidase or a carboxypeptidase in a reaction chamber separated from the flow channel by a selectively permeable membrane as described above. Amino acids pass, but polypeptides greater than 6 kDa do not. The amino acid joins a flow stream which contains the twenty amino acyl tRNA synthetases, a specific tRNA, EF-Tu;GTP and reaction buffer. A ternary complex of synthetase/amino acid/tRNA forms, and then the synthetase leaves the EF-Tu;GTP-AA-tRNA complex. Then the flow channel contains a ligand (such as a monoclonal antibody, polyclonal antibody or single chain antibody fragment which retains the binding specificity of a AA-tRNA-specific antibody or an EF-Tu-GTP-specific antibody) which is bound to a enzyme-conjugated EF-Tu;GTP-AA-tRNA complex. The unlabeled complex produced in the flow channel from degradation product amino acids displaces the bound, enzyme-conjugated complex. Then a flow stream containing enzyme substrate (and if necessary a suitable buffer) is inputted into the flow channel, and detectable product is made. Alternatively, the flow channel walls can contain EF-Tu;GTP-AA-tRNA complex which contains a detectable label such as a radioisotope or a fluor. Detection of the displaced detectably labeled complex is accomplished using an appropriate detector and excitation device, if necessary. See Figs. 7A-7B.

Fig. 18 schematically illustrates a computer-controlled integrated microfluidic system for amino acid analysis and end group sequencing. Twenty reservoir arrays, each specific for one of the naturally occurring amino acids, are each in fluid connection with a unique reaction channel. The laser and detector are connected to the reaction channels. The laser can be guided to the reaction channel array using optical fibers or waveguides., Alternatively, the detectors can be

built in. Proximal CCD or other proximal detection systems such as a scintillation proximity system can also be utilized.

### Example 3. Microchip Array Format

In the microarray embodiment of the invention, each of the twenty amino acyl tRNA  
5 synthetases or the twenty tRNAs are spotted at a known position on a microchip as part of a high  
density array. EF-Tu;GTP, tethered biotin and streptavidin are bound with the synthetases on the  
chip. Then sample containing amino acid from one stage of exoprotease digestion, together with  
the tRNA or synthetase, is applied to the chip and the free amino acid is converted to the amino  
acyl tRNA and then to the AA-tRNA-EF-Tu;GTP complex on the surface of the chip at the spot  
10 on the array wherein the cognate tRNA is located. The array is observed using a CCD-camera  
mounted on an epifluorescence microscope. Signals from the CCD-camera are decoded by a  
computer to which the readout is coupled. See Figs. 8A-8C. The carboxypeptidases can be  
arrayed to the wells as separate microspots. Alternatively, the protein substrates that are  
coimmobilized to the exopeptidase on particles can be placed into the microwells.

### 15 Example 4. Continuous Elution-Microarray Format

In the continuous flow elution embodiment, a displacement assay is used to measure the  
fluorescent signal of a displaced labeled molecule. See Fig. 9. An array of twenty capillary  
chambers, one per amino acid, is used to detect and quantitate the twenty amino acids. For  
simplicity Fig. 9 shows only one of the capillary chambers which are run in parallel. In each  
20 chamber a known density of bioaffinity molecules which bind the EF-Tu;GTP ternary complex is  
immobilized and saturated with the labeled bioaffinity molecule. Each detection flow chamber is  
perfused with amino acylation buffer containing the twenty amino acyl tRNA synthetases, EF-  
Tu;GTP and a tRNA specific for one particular amino acid. Amino acids enter the chamber  
under pressure from a mixed chamber. An amino acid having a cognate tRNA in the chamber is  
25 converted to a proportionate amount of the ternary complex which then flows to the microaffinity  
column and causes displacement of a proportionate amount of labeled ternary complex from the  
solid support within the column. The displaced, labeled ternary complexes are carried by

flowing buffer and detected downstream by a fluorescence detector, or other detector as appropriate to the label used. Depending on the instrumentation, for example, by using multiple fluorescent tags and multiple rounds of laser-induced fluorescence, it is possible to detect a single molecule. The record of the labeled ternary complexes using known concentrations of unlabeled antigen is established so that unknown concentrations of newly formed ternary complex can be quantitated. Displacement efficiencies are established and optimized by using known concentrations of the ternary complex. Bioaffinity molecules that bind that ternary complex with high specificity and have a high dissociation rate constant are selected to create rapid, ultrasensitive biosensors. Highly sensitive, continuous flow immunosensors can detect analytes concentrations in the picomole range in less than one minute [Kusterbeck et al. (1990) J. Immunol. Meth. 135:191-197].

A particular ELISA format and only one of the twenty parallel microchannels is illustrated in Fig. 16. The microflow channel contains amino acids released by the sequential proteolytic digestion of the protein of interest and the channel is at that point perfused with cognate synthetase and tRNA.

#### Example 5. CCD Detector as Solid Support in Amino Acid Sequence Analysis

In this embodiment a CCD detector serves as an active solid support which quantitatively detects and images the distribution of labeled target molecules bound to the immobilized biorecognition elements on the spatially addressable pixels. This format facilitates ultrasensitive, spatially specific detection with integrated data acquisition and computation. The microchip detector is suitable for detecting luminescent and radioisotope reporter groups with high sensitivity in a spatially resolved manner. Fig. 10 provides a diagram of a computer-controlled and system for amino acid analysis and end-group sequencing using a CCD detector as an active solid support. Each locus in the array contains bound molecules necessary for the immobilization and detection of a particular AA-tRNA-EF-Tu;GTP ternary complex at a particular spot. The address within the array conveys the identity of the particular amino acid.

Fig. 17 illustrates direct CCD molecule detection on a microchip. Biomolecular probes including, without limitation, elongation factors, synthetases or tRNAs, are immobilized directly onto the pixels of a CCD or they can be attached to a cover slip placed on the CCD surface. As shown twenty different tRNAs, each with a different amino acid specificity, are immobilized and the elongation factor is captured by binding to the AA-tRNA. Alternatively, the elongation factor can be immobilized and the tRNA labeled. Each amino acid is attached to its cognate tRNA by its cognate synthetase. The newly formed AA-tRNA binds to the immobilized elongation factor and is detected. The binding molecule (e.g., the elongation factor) can be labeled with a radioisotope, a chemiluminescent molecule or a fluorescent tag. After binding of the labeled molecule to the CCD array, photons or radioisotope decay products are emitted at the pixel locations where the labeled molecule has bound. Electron-hole pairs are generated in the silicon where the charged particles or radiation from the labeled molecule are incident on the CCD gates. Electrons are collected beneath adjacent CCD gates and are sequentially read out on a display module. The number of photoelectrons generated at each pixel is directly proportional to the number of labeled molecules bound and hence the amino acid cognate to the synthetases and tRNAs in each channel or well. Other proximity assays such as scintillation proximity assays are also within the scope of the present invention.

#### Example 6. Confocal Scanner as Detector in Amino Acid Sequence Analysis

In this embodiment for protein end group and amino acid sequence analysis a confocal scanner serves as the detector for the signal associated with the AA-tRNA-EF-Tu;GTP ternary complex. The change in fluorescence is monitored in each of the twenty microflow channels, after laser beam excitation, in the case of a fluorescent label, to follow the reactions catalyzed by the synthetases in a spatially resolved manner. This embodiment is represented diagrammatically in Fig. 11.

Figs. 12A provides a schematic of the confocal scanner system, and 12B illustrates the connections of a single microchannel within the twenty microchannel array, together with the inlets for the samples containing the amino acids, the wash buffer and the reaction solution.



Fig. 12C shows a twenty reaction microchannel array. The sample is delivered in parallel to each of the twenty microchannels, each engineered for the detection of a single amino acid, the relative location of reaction solution input and fiber optic detection optics, with coupling to a CCD detector, then a computer where the signals are decoded to provide the sequence information or the quantity data.

#### Example 7. Scintillator Surface Detection of radiolabeled AA-tRNA-EF-TU;GTP Complexes

Spatially resolved scintillation proximity assays are illustrated in Figs. 13A-13B. Each flow channel is surfaced with a scintillator. A particular radiolabeled tRNA and its cognate synthetase flow through each channel. EF-Tu;GTP is immobilized on the scintillator surface within the channel. As the amino acids enter the channel, the amino acid cognate to the tRNA is coupled to the tRNA to produce the corresponding AA-tRNA, which binds the immobilized elongation factor of the channel surface. There is continuous detection due to the interaction of the radioactive decay with the scintillator also bound to the channel surface. When a beta particle crosses the scintillator surface, part of its energy is converted to light. The light spot is intensified. The real time signal produced by the light produced is detected after amplification of the light is detected by a CCD camera where it has generated a cluster of charges. The spot of electrons is serially processed, localized, stored and displayed by the electronic system. The electronic detection system may be composed of a camera, an interface board and a computer. During the data acquisition time the successive binding events are accumulated in real time and displaced on a computer monitor if desired. The summation of the events constitutes the final digital image, which can be stored in a computer-readable form and/or transferred to a processor for generation of amino acid sequence or quantity data.

Fig. 13B shows a microchannel with a scintillator surface. Miniaturized light detectors are placed in the bottom of the flow channels. The light generated when the radiolabeled molecule binds to the immobilized ligand is continuously monitored and recorded, and optionally processed.

### Example 8. Flowing Immobilized Detection

Fig. 14 shows a amino acid sequence analytical tool in which the twenty labeled tRNAs or the twenty synthetases are each arrayed onto separate beads. These beads are introduced into a mixing chamber together with a sample flowstream which contains the amino acids. The components react and bind generally as described above and the reacted components flow past a laser emitting light at the excitation wavelength and a detector. The signal is transferred to a computer for recording and data analysis.

### Example 9. Amino Acid Analytic Device with Optical Fiber Biosensor

Fig. 15 is a schematic illustration of an amino acid optical fiber biosensor. This amino acid optical fiber biosensor uses immobilized EF-Tu;GTP to bind and detect the fluorescently labeled AA-tRNAs. The elongation factor is immobilized on an optical fiber placed in a microchannel. When complexed with GTP the elongation factor selectively binds all AA-tRNAs with high affinity. The amino acids are converted to the cognate AA-tRNAs by the synthetases in amounts proportional to their concentration in the sample flow, and hence the concentration of an AA-tRNA is directly related to the amino acid concentration in the sample stream. The sample containing the amino acids flows into a reaction microchannel. The reaction channel is joined by a microchannel delivering a continuous flow of reaction solution containing a synthetase and fluorescently labeled tRNA specific for a particular amino acid. The amino acid in the sample is converted to a proportionate amount of the corresponding AA-tRNA. Optical fibers can be used to both excite and detect fluorescence in the evanescent field so that only the surface-bound fluorescent molecules are detected. This allows the continuous and real time monitoring without a wash step. The elongation factor binds the AA-tRNAs only when also bound to GTP but not GDP. Hence, the bound AA-tRNAs can be eluted using a buffer containing GDP. The immobilized elongation factor can be then reactivated by washing with a regeneration buffer containing GTP and the fiber can then be reused. The binding of the fluorescently labeled AA-tRNAs can be monitored in arrays of microchannels run simultaneously. In this way all twenty amino acids can be analyzed simultaneously, continuously and using very small sample volumes (picoliters).

#### Example 10. Hollow Fiber Embodiment

Figs. 13A-13B present a schematic representation of a continuous flow amino acid analysis system in which the sequential proteolytic digestion of the target protein takes place within a sealed hollow fiber having a molecular weight cutoff such that amino acids pass freely but macromolecules do not. As amino acids are released by the exopeptidase, they diffuse through the pores of the fiber and enter the flowstream (Fig. 13A) and are carried to the microfluidic reaction and detection arrays. As shown in Fig. 13B, a laser beam excites the fluor bound to the AA-tRNA in the ternary complex, and the emitted light is detected by a detector, for example, a CCD detector. In this embodiment, the twenty microchannels are in a circular array (carousel) and as the carousel rotates, the scanning detection systems detects whether there is labeled ternary complex in one or more microchannels.

#### Example 11. Nanoparticle digestion chamber

The polypeptide targeted for sequence analysis and the carboxypeptidase enzyme can both be tethered (i.e., immobilized) on the same surface in the digestion chamber, which can be particularly tiny. See Fig. 18. Nanoparticles, especially magnetic nanoparticles, can be used in a chamber in which there is an especially high concentration of enzyme and substrate. The volume can be as small as 1  $\mu$ l. The nanoparticles are immobilized, and an effluent stream of liberated amino acids flows into the detector arrays. The carboxypeptidases such as carboxypeptidase P or carboxypeptidase Y can be tethered to a surface by its C-terminus. The protein target of sequencing can be immobilized using a long and flexible tether so that it has a free carboxy terminus, is confined to the surface but it has freedom to move about by diffusion and collide with the immobilized enzyme. Even though the absolute amount of target protein may be low, the concentration on the surface is high. In this way, the immobilized substrate does not have to diffuse very far before it collides with enzyme; thus the net reaction rate will be increased as compared with reaction rate for enzyme and substrate both in solution. In addition, the immobilized carboxypeptidase cannot serve as a substrate, which could complicate data analysis if amino acids were released from the immobilized carboxypeptidase as well as from the protein for which sequence data is sought.

### Example 12. Electrochemical Detection of Amino Acid Sequence Information

Microarray systems are provided which use electrochemical detection methods. Both microfluidic and microwell array formats can be adapted to this detection method. A labeled ternary complex is reversibly immobilized inside a flow channel or microwell. The label can be an electrogenic label such as an enzyme. Alternatively the label can be an enzyme that catalyzes a chemiluminescent reaction. The substrate is introduced into the flow channel via a microchannel in fluid connection with the channel. Alternatively, the substrate for the enzyme conjugate can be immobilized within the well or on the inside surface of the flow channel. As the amino acid sample enters the chamber, each amino acid is converted into a proportionate amount of the ternary complex (AA-tRNA-EF-Tu;GTP) which then displaces a proportionate amount of the reversibly bound labeled ternary complex. The substrate is converted to product by the enzyme conjugate and the product is detected by a microelectrode positions within the channel or well. In another format the labeled ternary complex is reversibly immobilized on the microelectrode surface. The newly formed ternary complex (due to amino acid release from the target protein within the digestion chamber) displaced the labeled ternary complexes and results in a proportionate decrease in signal at the microelectrode. Figs. 19A-19B illustrate this aspect of the invention. For example, horseradish peroxidase can be used with an electrogenic substrate to generate a detectable signal. When carried out in very small volumes, ultrasensitive electrochemical assays are achieved.

## I CLAIM:

1. A method for analyzing at least one primary amino acid , said method comprising the steps of
  - (a) providing a sample comprising at least one primary amino acid;
  - (b) contacting said sample with an amino acyl tRNA synthetase and a tRNA cognate for the at least one primary amino acid to form at least one amino acyl-tRNA (AA-tRNA);
  - (c) contacting the at least one AA-tRNA with an elongation factor to form an AA-tRNA-elongation factor ternary complex;
  - (d) contacting the AA-tRNA-elongation factor ternary complex with a biorecognition element; and
  - (e) detecting the interaction of the AA-tRNA-elongation factor ternary complex with the biorecognition element of step (d).
2. The method of claim 1 wherein said sample is a biological sample.
3. The method of claim 2 wherein said sample is a blood or serum sample.
4. The method of claim 3 wherein the amino acid detected is phenylalanine and wherein the amino acyl tRNA synthetase and the tRNA are cognate for phenylalanine.
5. The method of claim 1 wherein the sample provided is subjected to molecular sieving, wherein compounds of less than about 6 kDa molecular weight can pass through and compounds of greater than about 6 kDa cannot pass, prior to the step of contacting with amino acyl tRNA synthetase and tRNA.

6. The method of claim 1 wherein all twenty primary amino acids are detected.
7. The method of claim 6 wherein the sample is a protein, said protein being completely digested to all twenty primary amino acids in a digestion chamber prior to the step of contacting the at least one primary amino acid with at least one primary amino acyl tRNA synthetase and the at least one tRNA.
8. The method of claim 7 wherein the digestion chamber comprises at least one endoprotease and at least one exopeptidase, wherein the endoprotease is selected from the group consisting of trypsin, chymotrypsin, thrombin, subtilisin, proteinase K, pepsin, papain, factor Xa, enterokinase, endoproteinase Glu-C, endoproteinase Asp-N, endoproteinase Arg-C, elastase, collagenase and cathepsin C and wherein the exopeptidase is selected from the group consisting of aminopeptidase A, aminopeptidase M, carboxypeptidase A, carboxypeptidase B, carboxypeptidase C, carboxypeptidase Y, carboxypeptidase N and aminoacyl peptidase.
9. The method of claim 7 wherein the degradation of the protein of interest is carried out in a digestion chamber having a molecular sieve such that amino acids but not molecules of greater than 6.0 kDa can pass through the molecular sieve and wherein said digestion chamber is in fluid communication with the tRNA, amino acyl tRNA synthetase and elongation factor.
10. The method of claim 6 wherein all twenty primary amino acids are detected after contact with an elongation factor simultaneously.
11. The method of claim 6 wherein the step of contacting the amino acids with cognate amino acyl tRNA synthetases and tRNAs utilizes a spatially resolved combination of amino acyl tRNA synthetase and cognate tRNA, with each cognate synthetase and tRNA

pair being at a specific location.

12. The method of claim 11 wherein the specific location for each of the twenty primary amino acids is within a microchannel or microcapillary into which tRNA and amino acyl tRNA synthetase have flowed from a reservoir comprising a reaction liquid, said reservoir being in fluid communication with said microcapillary or microchannel.
13. The method of claim 11 wherein the specific location for each of the twenty primary amino acids is a surface of a microchannel or a microcapillary or a microwell or a flow channel or a known locus on a surface of a chip.
14. The method of claim 13 wherein the specific location is within a microwell of a microtiter plate.
15. The method of claim 14 wherein a AA-tRNA-elongation factor ternary complex is detected using a plate reader which is a spectrophotometer, a luminometer, a scintillation counter, a Raman spectrophotometer, a charge coupled device camera or a gamma counter.
16. The method of claim 1 wherein the identity of each amino acyl tRNA is determined by mass spectrometry.
17. The method of claim 1 wherein each tRNA specific for each of the at least one primary amino acid comprises a unique distinguishing label for detection.
18. The method of claim 1 wherein the AA-tRNA-elongation factor ternary complex is detected using a biosensor selected from the group consisting of a piezoelectric crystal, a surface plasmon resonance system, an acoustic wave sensor device, a fluorescence detector or a proximity scintillation surface.

19. The method of claim 1 wherein the biorecognition element is bound to a transducer to create an amino acid biosensor.
20. The method of claim 19 wherein the elongation factor is immobilized to the amino acid biosensor.
21. The method of claim 1 wherein the biorecognition element is a ternary complex probe immobilized to a transducer.
22. The method of claim 20 wherein the transducer is an optical fiber, an electrode, a piezoelectric crystal, a thermistor or a planar wave guide.
23. The method of claim 9 wherein the molecular sieve is a diafiltration membrane or a selectively permeable membrane.
24. The method of claim 1 wherein the tRNA is labeled with a detectable tag.
25. The method of claim 24 wherein the detectable tag is a fluorophore, a chromophore, a nanoparticle, a metal, an enzyme, a liposome-based label, an electrogenic label, ferrocene, biotin or a radioisotope.
26. The method of claim 1 wherein the elongation factor is labeled with a detectable tag.
27. The method of claim 26 wherein the detectable tag is a fluorophore, a chromophore, a nanoparticle, a metal, an enzyme, a liposome-based label, an electrogenic label, ferrocene, biotin or a radioisotope.
28. The method of claim 1 wherein the AA-tRNA-elongation factor ternary complex is detected using a ternary complex probe.



29. The method of claim 28 wherein the ternary complex probe is an antibody or an antibody fragment specific for the ternary complex.
30. The method of claim 28 wherein the ternary complex probe is a nucleic acid.
31. The method of claim 29 wherein the ternary complex is a ribosome or a ribosomal fragment which binds the ternary complex.
32. The method of claim 1 wherein the at least one amino acyl tRNA synthetase is immobilized to a surface.
33. The method of claim 6, wherein amino acid sequence information from a terminus of interest of said protein is determined, wherein after the step of providing the sample there is the step of carrying out sequential degradation from a terminus of a protein of interest using an exopeptidase specific for the terminus of interest of said protein to release at least one released primary amino acid; and after steps (b) through (e) of claim 1, there are subsequent steps of sequentially detecting the ternary complexes formed during the time course of degradation; and decoding data obtained in step (d) to produce amino acid information.
34. The method of claim 33 wherein the terminus is the C-terminus of the protein and wherein the exoprotease is a carboxypeptidase.
35. The method of claim 34 wherein the carboxypeptidase is carboxypeptidase N, carboxypeptidase P, carboxypeptidase Y, carboxypeptidase A or carboxypeptidase B.
36. The method of claim 31 wherein the terminus is the N-terminus of the protein and wherein the exoprotease is an aminopeptidase.

37. The method of claim 31 wherein the aminopeptidase is aminopeptidase M or aminopeptidase L.
38. The method of claim 33 wherein the degradation of the protein of interest is carried out in a reaction chamber having a selectively permeable membrane such that amino acids but not polypeptides of greater than 6 kDa can pass through the membrane and wherein said digestion chamber is in fluid communication with the tRNA, amino acyl tRNA synthetase and elongation factor.
39. The method of claim 38 wherein the degradation of the protein of interest is carried out within a hollow core fiber, said hollow core fiber allowing the passage through of amino acids but not polypeptides of greater than 6 kDa.
40. The method of claim 39 wherein the hollow core fiber is in fluid communication with a microchannel in which a flowstream containing tRNA, amino acyl synthetase and an elongation factor is moving.
41. The method of claim 33 wherein the tRNA is covalently bound to a fluorescent label.
42. The method of claim 1 wherein the elongation factor is bacterial EF-Tu;GTP.
43. The method of claim 33 wherein the protein of interest and the exopeptidase are immobilized on a solid support.
44. The method of claim 43 wherein the solid support is a nanoparticle.
44. The method of claim 33 wherein the at least one released amino acid is in fluid communication with a plurality of microchannels, in each of which a reaction fluid comprising a different tRNA and cognate amino acyl tRNA synthetase and elongation

factor is flowing.

45. The method of claim 44 wherein the a portion of the microchannel has elongation factor immobilized on at least one interior surface of each microchannel and wherein there is a detection system for amino acyl-tRNA bound to the elongation factor on the interior surface of the microchannel.
46. The method of claim 45 wherein there is a laser for excitation of the fluorescently labeled ternary complex and a detector for emitted light, said detector being in electronic communication with a computer.
47. The method of claim 46 wherein the detection system comprises a confocal scanner, a charge-coupled device (CCD) camera or a surface plasmon resonance detector.
48. The method of claim 45 wherein the detection system comprises a proximity scintillation surface and the label detected is a radioactive label associated with a tRNA.
49. The method of claim 33 wherein there is a washing step after the ternary complex formation step to remove amino acyl tRNA from the ternary complex.
50. The method of claim 47 wherein there is a reactivation step after the washing step so that the elongation factor can bind amino acyl tRNA.
51. A miniaturized integrated microfluidic amino acid analysis system for performing amino acid analysis, said system comprising
  - (a) a plurality of reaction microchannels, wherein each microchannel is formed in a planar substrate and wherein each microchannel has a sample inlet for receiving a sample and a discharge outlet,

- (b) at least one reservoir for input to said reaction microchannels, wherein said reservoir is in fluid connection to at least one reaction microchannel;
  - (c) a means for inputting fluid from the reservoir to each reaction channel;
  - (e) a waste reservoir in fluid connection with said discharge outlet;
  - (f) a detection system in each of reaction microchannels, said detection system detecting a product of an amino acyl tRNA synthetase reaction in the microchannels during use of the system;
  - (g) a main flow channel in fluid connection to said reaction channels joining at said sample inlets, said main flow channel being in fluid connection to one or more reservoirs and having a continuous flow mixer; and
  - (h) a means for transporting fluid from said reservoir(s) through the main channel and through the reaction channels
52. The system of claim 51 comprising a digestion chamber, wherein said digestion chamber is in fluid communication with the reaction microchannels via the sample inputs of said reaction microchannels.
53. The system of claim 51 further comprising a molecular sieve upstream of the sample inlets of the reaction microchannels.
54. The system of claim 53 wherein the molecular sieve is a microdialysis probe or a selectively permeable membrane through which molecules less than about 6 kDa pass freely.

55. A syringe interface in fluid connection to said main channel.
56. The miniaturized total analysis system of claim 51 for performing protein end group amino acid sequencing and end group amino acid compositional analysis according to the method of claim 1, wherein said system further comprises a molecular sieve between the digestion chamber and the reaction channel, and a means for moving fluid from a reservoir or syringe through the molecular sieve and into said main channel.
57. The system of claim 51 wherein said the sample inlet of the reaction chamber is a syringe port.
58. The system of claim 51 wherein said digestion chamber comprises at least one exopeptidase and wherein each reaction channel comprises at least one amino acyl tRNA synthetase and at least one tRNA cognate to the amino acyl tRNA synthetase.
59. The system of claim 51 having a plurality of reaction channels, wherein each reaction channels comprises an affinity zone having immobilized biomolecular recognition molecules, wherein each reaction channel is integrated with a detector system to detect a signal from an AA-tRNA bound to said biomolecular recognition molecules immobilized in the affinity zone.
60. The system of claim 59 wherein the signal detected is a mass change, fluorescence, chromophore, radioactive decay, an electrical signal or chemiluminescence.
62. The system of claim 59 wherein the detection system comprises a biosensor selected from the group consisting of a piezoelectric crystal, a surface plasmon resonance system, an acoustic wave sensor device, a fluorescence detector or a proximity scintillation surface.
63. The system of claim 53 further comprising a digestion chamber within which is at least

one immobilized protease, wherein said molecular sieve is between the digestion chamber and the sample inlet of the reaction microchannels.

64. The system of claim 51 wherein each reaction channel is integrated with a detection system for characterizing the properties of the sample, and wherein each reaction channel is integrated with computer controlled valves that control passage of fluids from the digestion chamber into each reaction channel.
65. The system of claim 51 wherein each reaction channel comprises an amino acyl tRNA synthetase immobilized on an interior surface of the channel.
66. The system of claim 65 wherein each reaction channel further comprises a biomolecular recognition molecule immobilized to a detection region of said microchannel.
67. A microtiter plate kit for amino acid analysis said kit comprising:
  - (a) a microtiter plate, said plate having a multiplicity of wells;
  - (b) a set of twenty containers, wherein each container comprises a different amino acyl-tRNA synthetase such that together the set of container includes amino acyl tRNA synthetases specific for twenty primary amino acids;
  - (c) a second set of containers, wherein each container contains a tRNA specific for a different one of the 20 primary protein amino acids;
  - (d) additional containers comprising reagents for following the reactions catalyzed by the aminoacyl-tRNA synthetases.
68. The microtiter plate kit of claim 67 wherein the tRNA molecules comprise a detectable tag.

69. The microtiter plate kit of claim 68 wherein the detectable tag is a fluorophore, a chromophore, a chemiluminescent molecule, an enzyme, a metal, a nanoparticle or a radioisotope.
70. A method for detecting at least one amino acid using the kit of claim 67, said method comprising the steps of:
- (a) dispensing an amino acyl-tRNA synthetase specific for at least one amino acid is dispensed into at least one microplate well in a known location;
  - (b) dispensing a tRNA cognate to the amino acyl tRNA synthetase dispensed into a well in step (a);
  - (c) dispensing reagents for following the reactions catalyzed by the amino acyl-tRNA synthetases are added to each well;
  - (d) dispensing a sample into at least one microplate well and allowing formation of an amino acyl-tRNA in the microplate well and binding of said amino acyl-tRNA to a biomolecular recognition element immobilized to the surface of the well; and
  - (e) detecting binding of said amino acyl-tRNA to a biomolecular recognition element.
71. The method of claim 70 wherein the sample is a biological sample.
72. The method of claim 70 wherein the biological sample is a blood or a serum sample.
73. The method of claim 70 wherein each amino acyl-tRNA synthetase and cognate tRNA are dispensed to a well of known location such that formation of amino acyl-tRNA at a particular location provides amino acid identity information.

74. The method of claim 73 wherein the sample is dispensed concurrently into twenty wells.
75. The method of claim 74 wherein the sample is repeatedly dispensed concurrently into twenty wells, such that each dispensing takes place sequentially over time with the result that the change in detection of the amino acyl tRNAs over time reflects a change in distribution of amino acid content over time.
76. The method of claim 75 wherein data are collected from the microwells over time, and wherein the data are transmitted to a computer or microprocessor.
77. A kit for amino acid analysis according to claim 67 wherein amino acyl tRNA synthetase reactions are monitored by following the formation of PPi.
78. A kit for amino acid analysis according to claim 67 wherein amino acyl-tRNA synthetase reactions are followed by the formation of AMP.
79. A kit according to claim 67 wherein AA-tRNAs formed by reactions of aminoacyl-tRNA synthetases, tRNAs, and at least one amino acid are followed by binding them to bacterial elongation factor, EF-Tu;GTP employing a ligand assay comprising the following steps:
- (a) dispensing at least one unique tRNA specific for a cognate amino acid and its cognate aminoacyl-tRNA synthetase into at least one well in a known location, wherein the well comprises a bound biorecognition element specific for an amino acyl tRNA;
  - (b) dispensing a sample into the at least one well of step (a) with the result that an amino acyl tRNA is formed when an amino acid cognate to the tRNA and the amino acyl tRNA synthetase are present; and



- (c) detecting binding of an amino acyl tRNA to the biorecognition element.
80. The kit of claim 79 wherein the biorecognition element is elongation factor Tu;GTP.
81. The kit of claim 79 wherein at least one of the tRNA and the biorecognition element is labeled with a detectable tag.
82. The method of claim 70 further comprising a step of washing the at least one well to remove any tRNA is not bound to the biorecognition element.
83. The method of claim 82 wherein a labeled probe is employed to bind to and detect the captured AA-tRNAs after a wash step, wherein the probe is an oligonucleotide with a detectable tag or peptide nucleic acid with a detectable tag, wherein the oligonucleotide or peptide nucleic acid hybridizes to the captured AA-tRNAs.
84. A kit according to claim 80 wherein the elongation factor is labeled with a fluor and its binding to AA-tRNA is monitored by a fluorescent assay.
85. A kit according to claim 80 wherein the tRNAs are labeled with a fluor and the binding of the EF-Tu;GTP to AA-tRNAs is monitored by fluorescent assay.
86. A kit according to claim 80 where the elongation factor is immobilized to scintillation proximity assay beads and used to capture and detect radiolabeled AA-tRNAs.
87. The method of claim 1 where the biorecognition elements are arrayed as zones on a membrane.
88. The method of according to claim 1 wherein the biorecognition elements are arrayed as microdots on a chip.

89. The method according to claim 1 where the biorecognition elements are arrayed on a planar waveguide
90. The system according to claim 51 where the biorecognition elements are arrayed into microchannels.
91. The system according to claim 51 wherein the biorecognition elements are arrayed into microcapillaries.
92. The system of claim 51 wherein the biorecognition elements are arrayed to the bottom of flow channels.
93. A method according to claim 1 wherein the biorecognition elements are arrayed on a film or scintillator sheet.
94. The method claim 1 wherein the formation of the ternary complex employs dual distinguishable fluorescent labels, wherein the elongation factor is labeled with one detectable label and the tRNA is labeled with a second detectable label.
96. The method of claim 94 wherein the first label is Texas Red and the second label is fluorescein, and after formation of the ternary complex, and the ratio of bound fluorescein and Texas Red labels is determined using a dual-channel laser scanning confocal microscope as a detection system.
96. A method according to claim 1 wherein the biorecognition element is an antibody specific for the ternary complex and wherein the ternary complex is detected using competitive, noncompetitive, displacement, and sandwich type immunoassays for amino acid analysis.

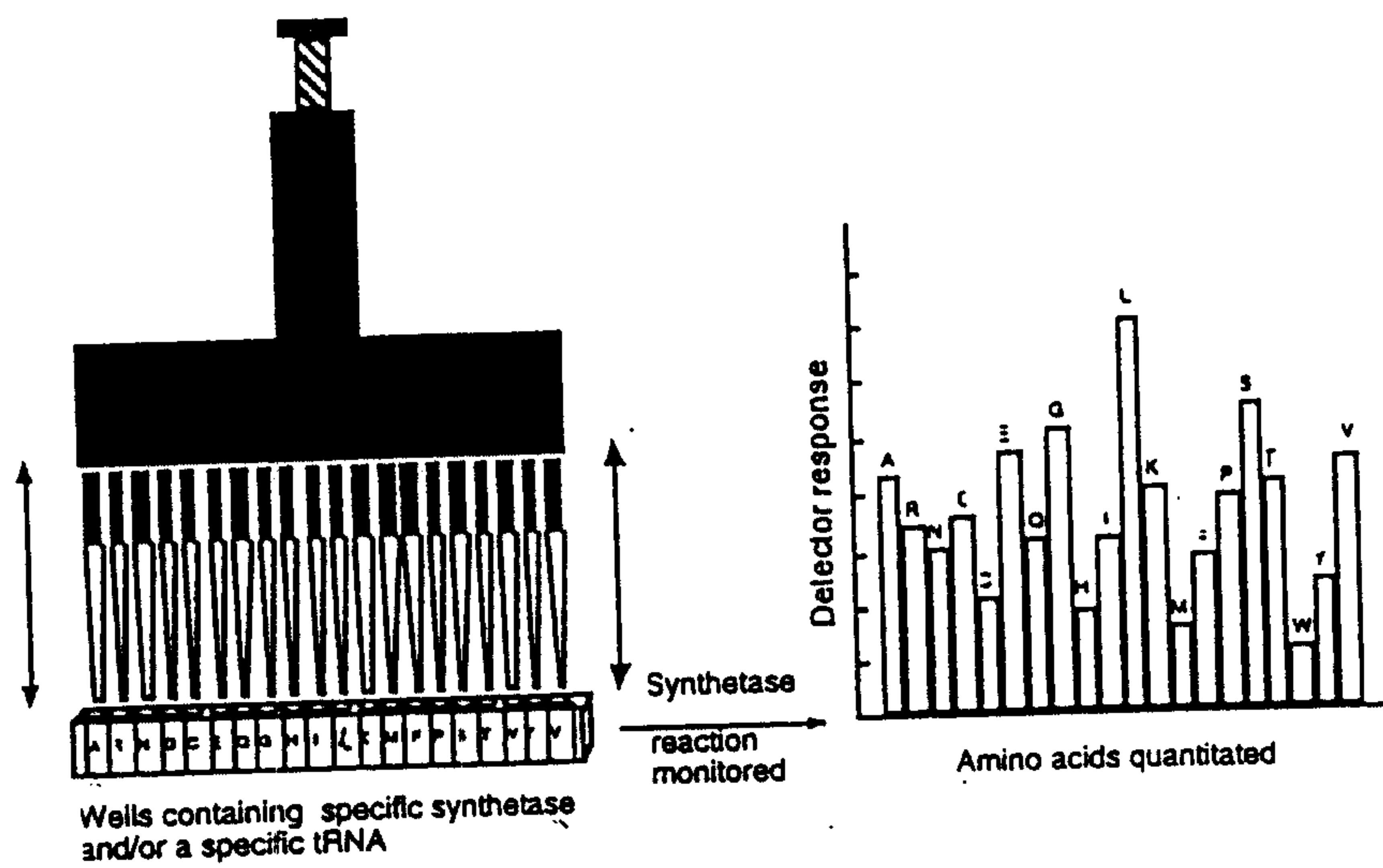


FIG. 1A

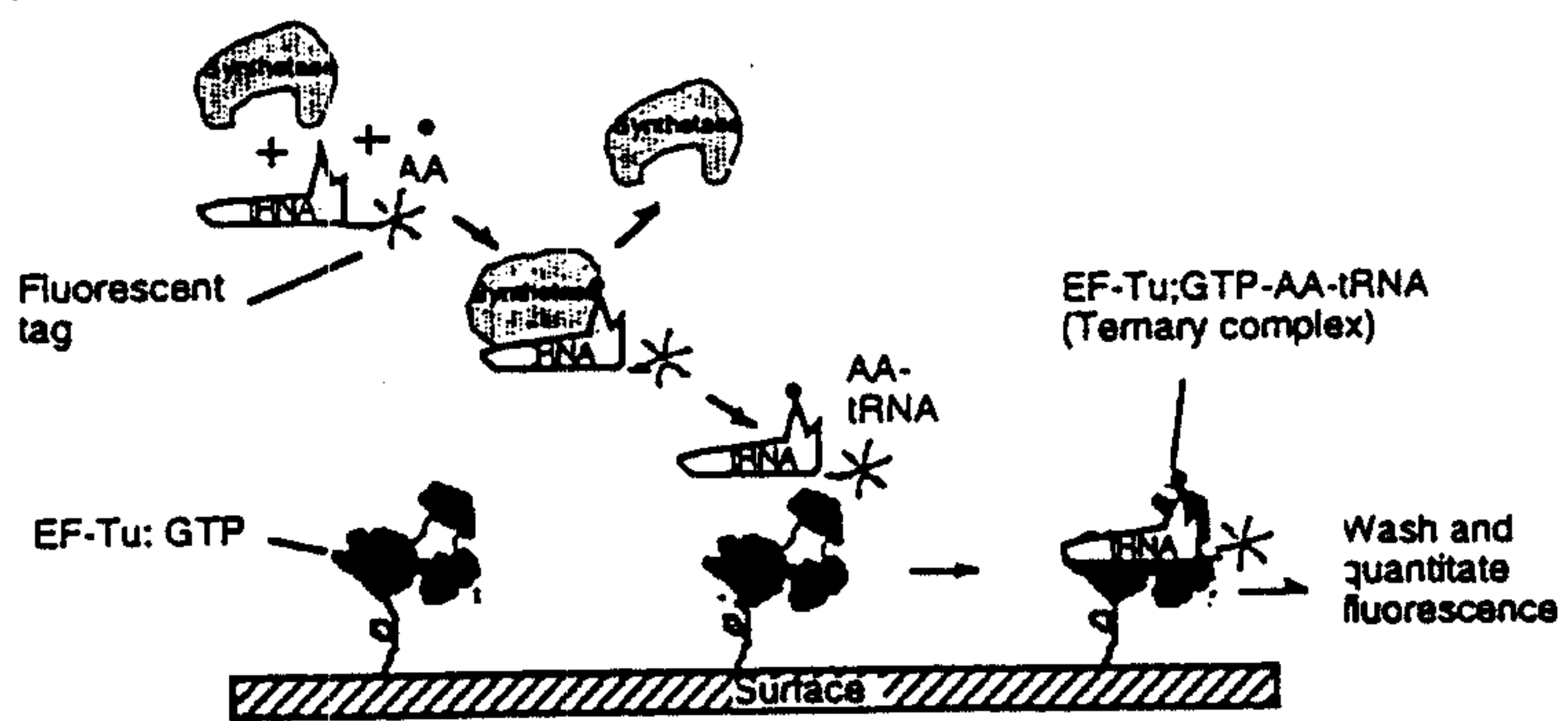


FIG. 1B

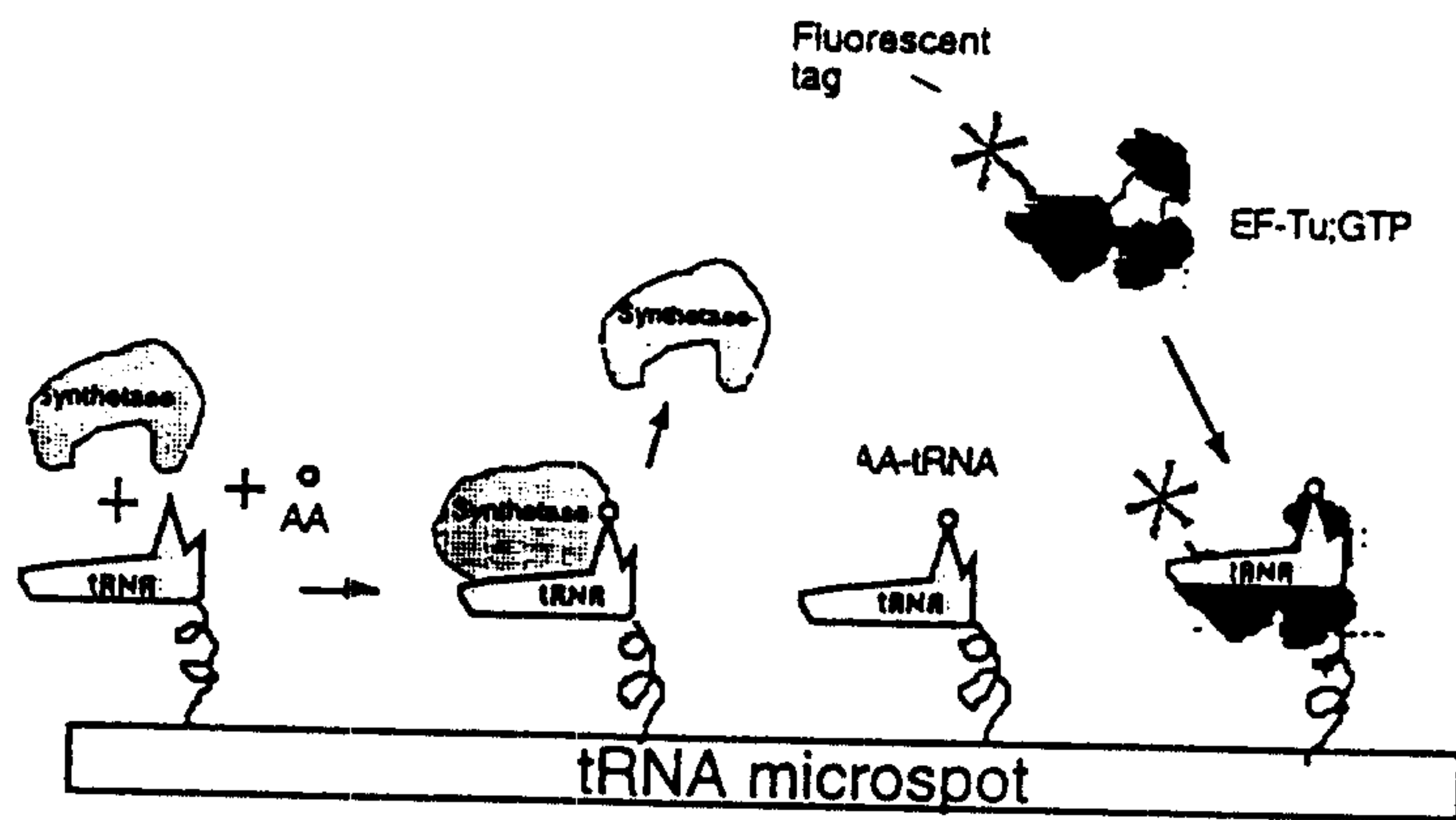


FIG. 2A

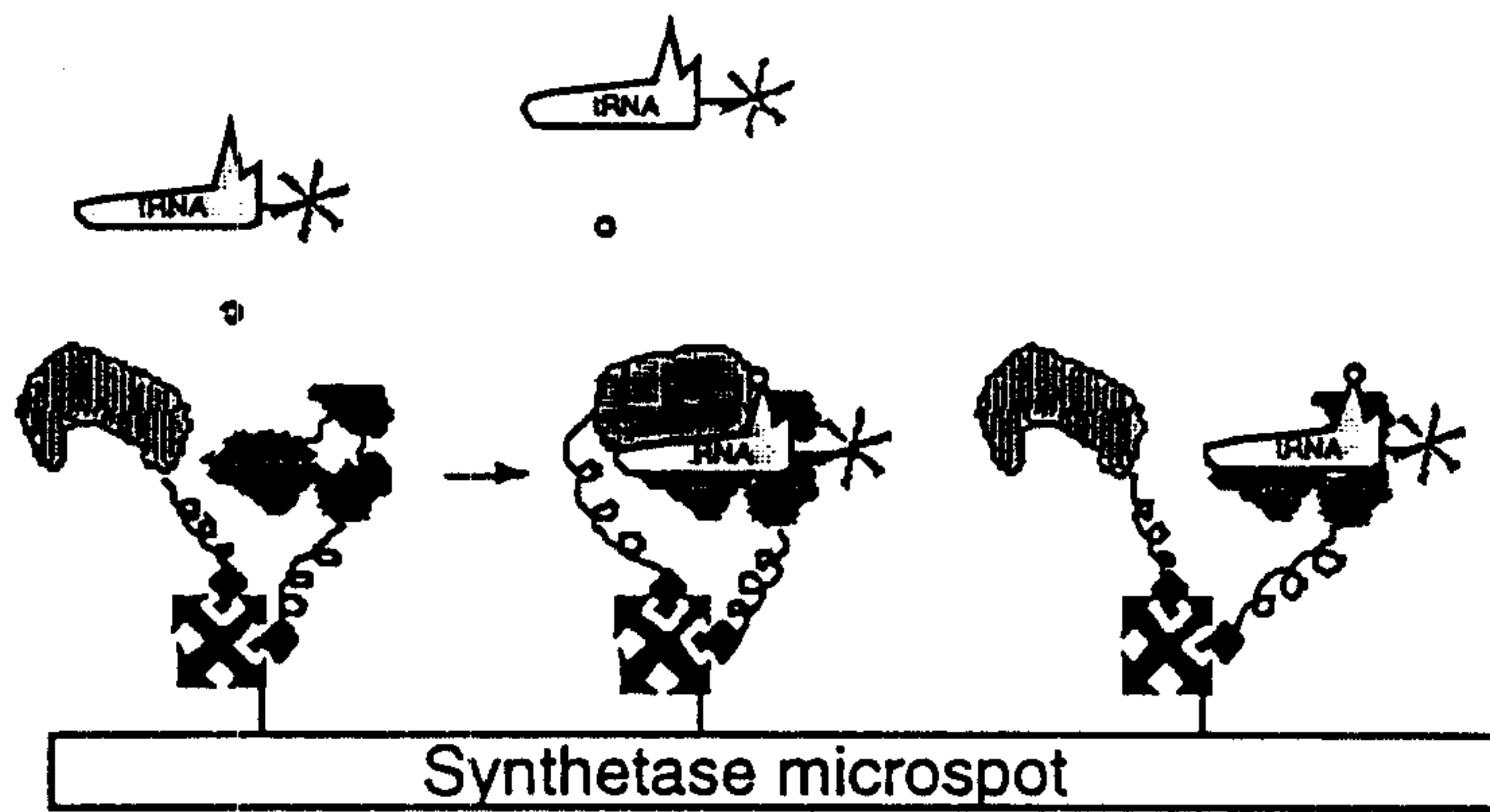
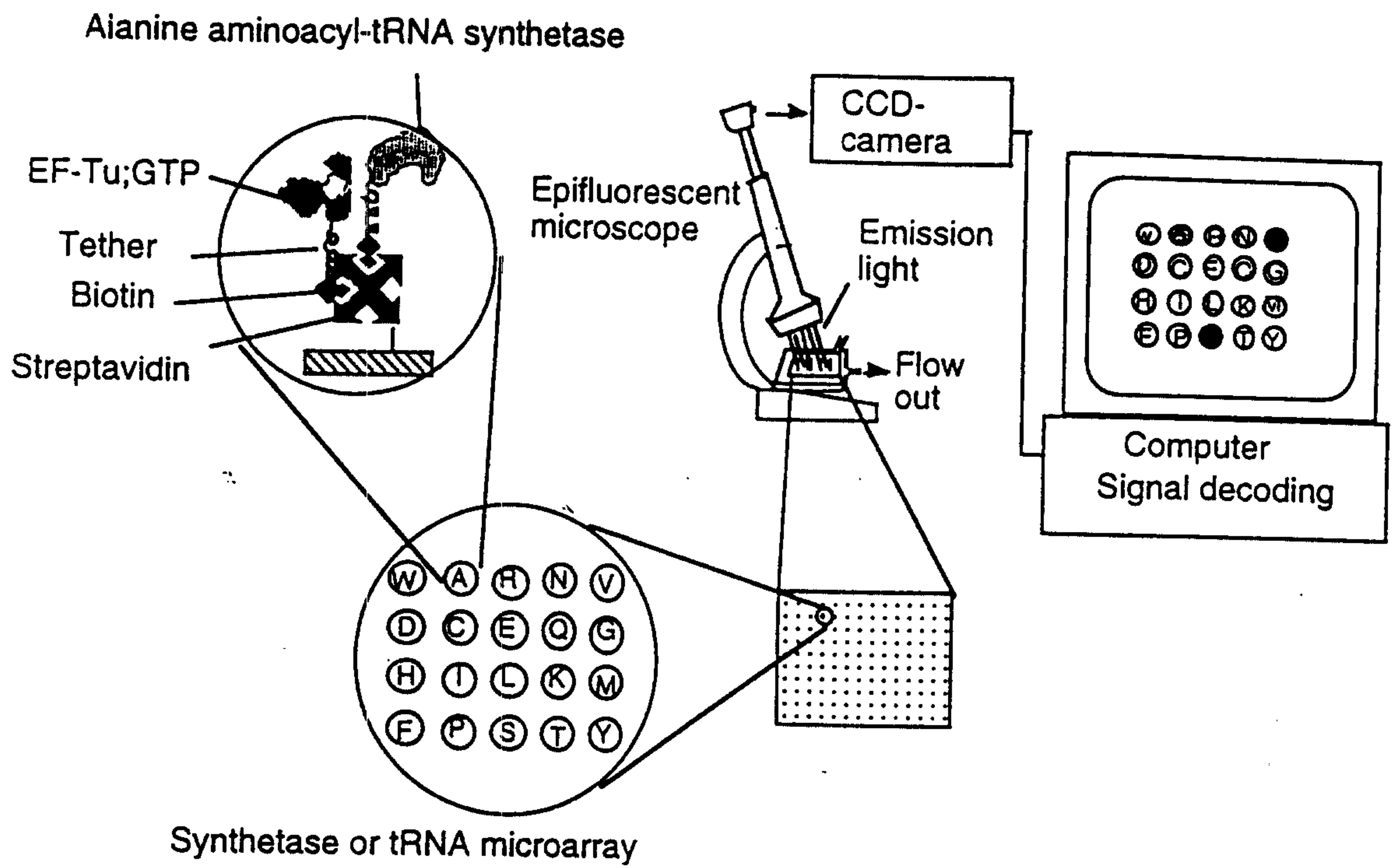
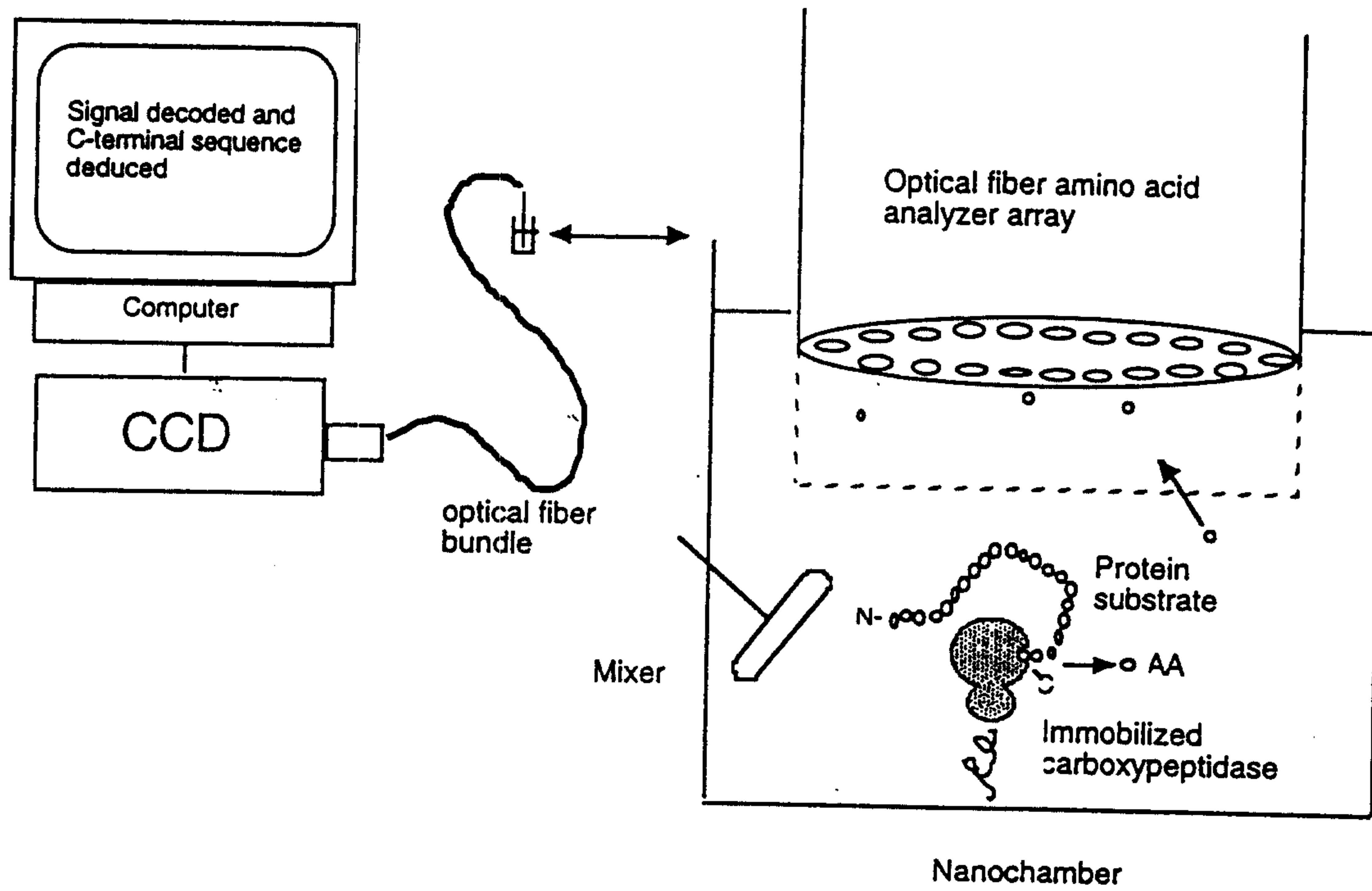


FIG. 2B



**FIG. 3**



**FIG. 4**

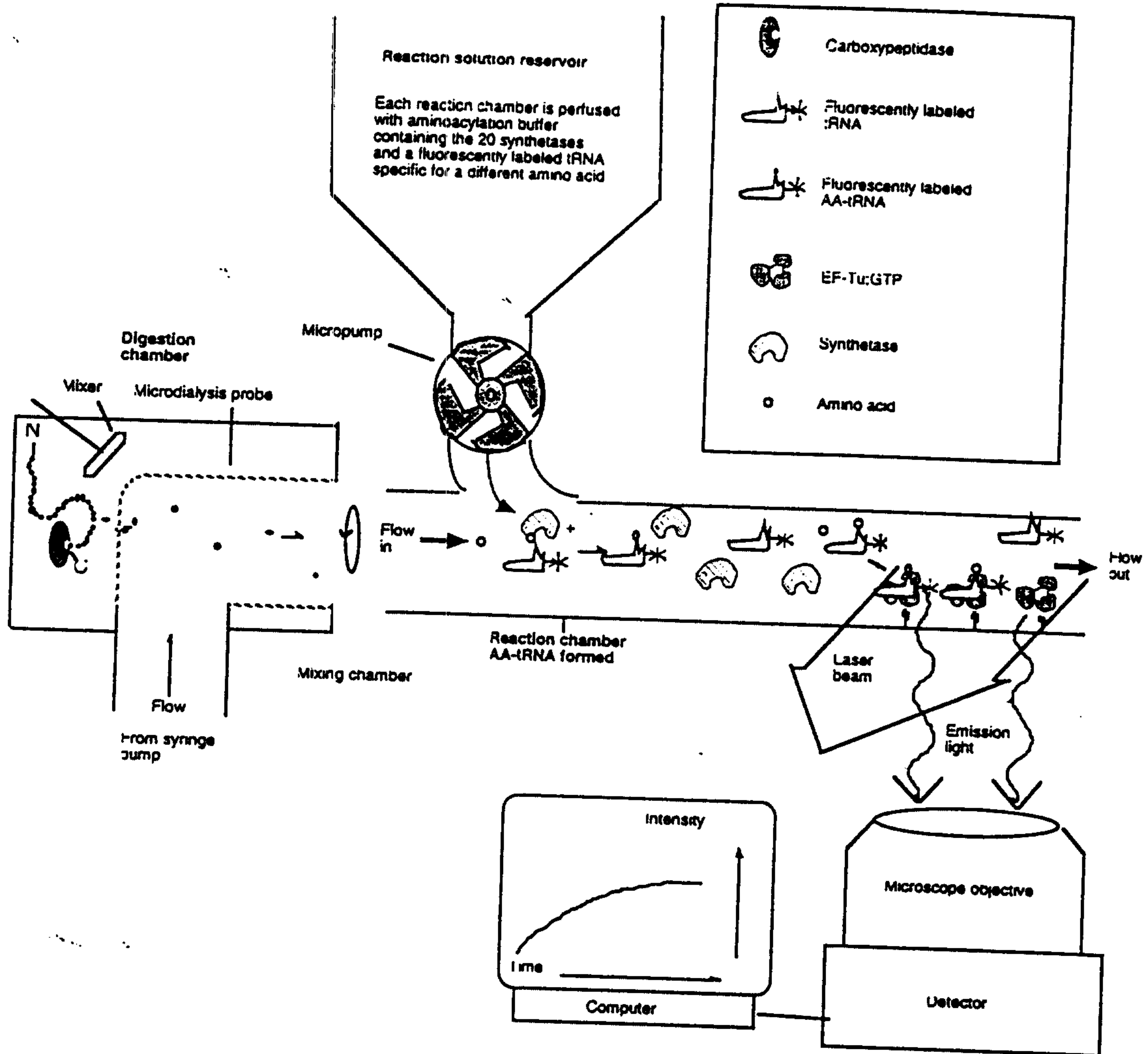
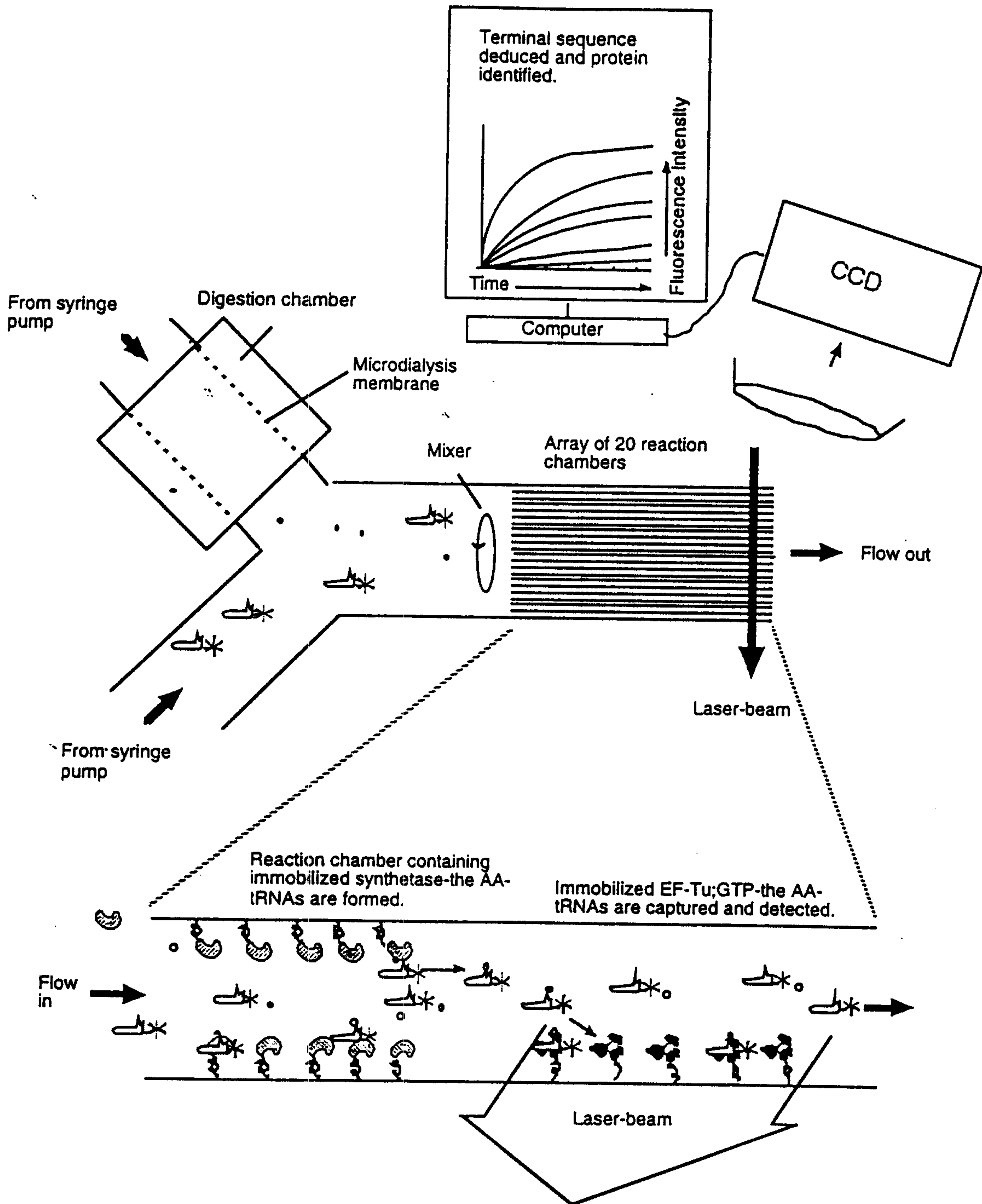
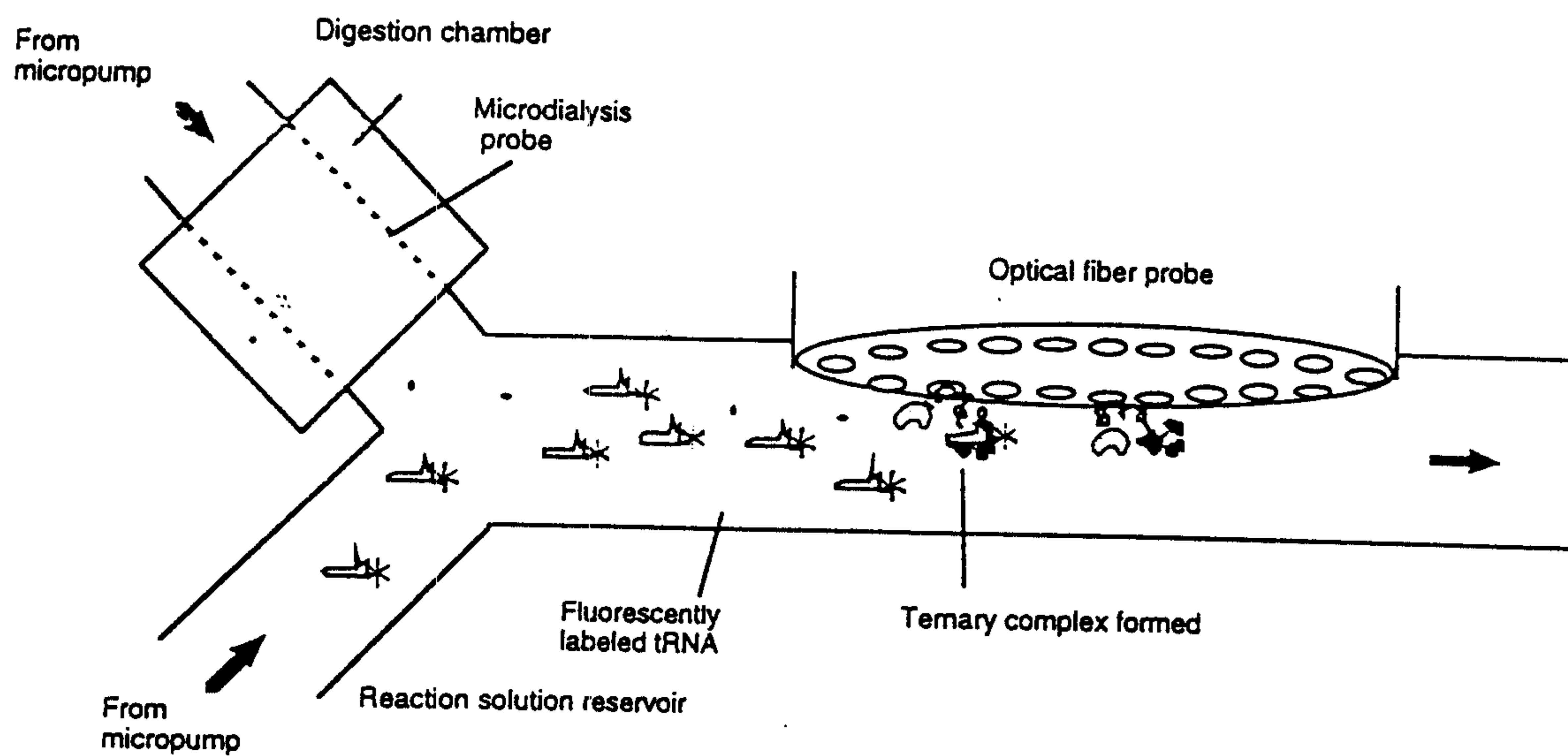


FIG. 5A



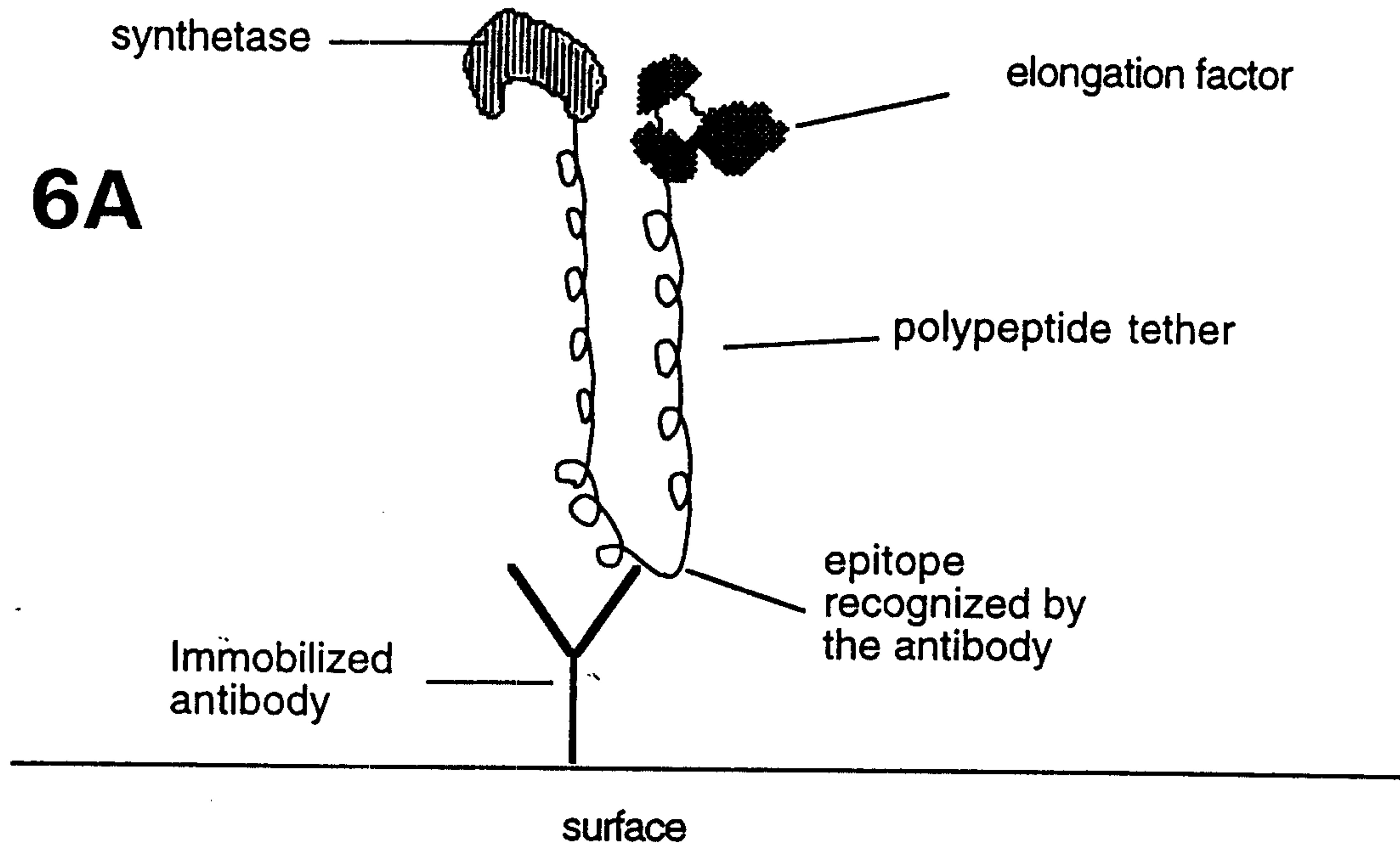
**FIG. 5B**



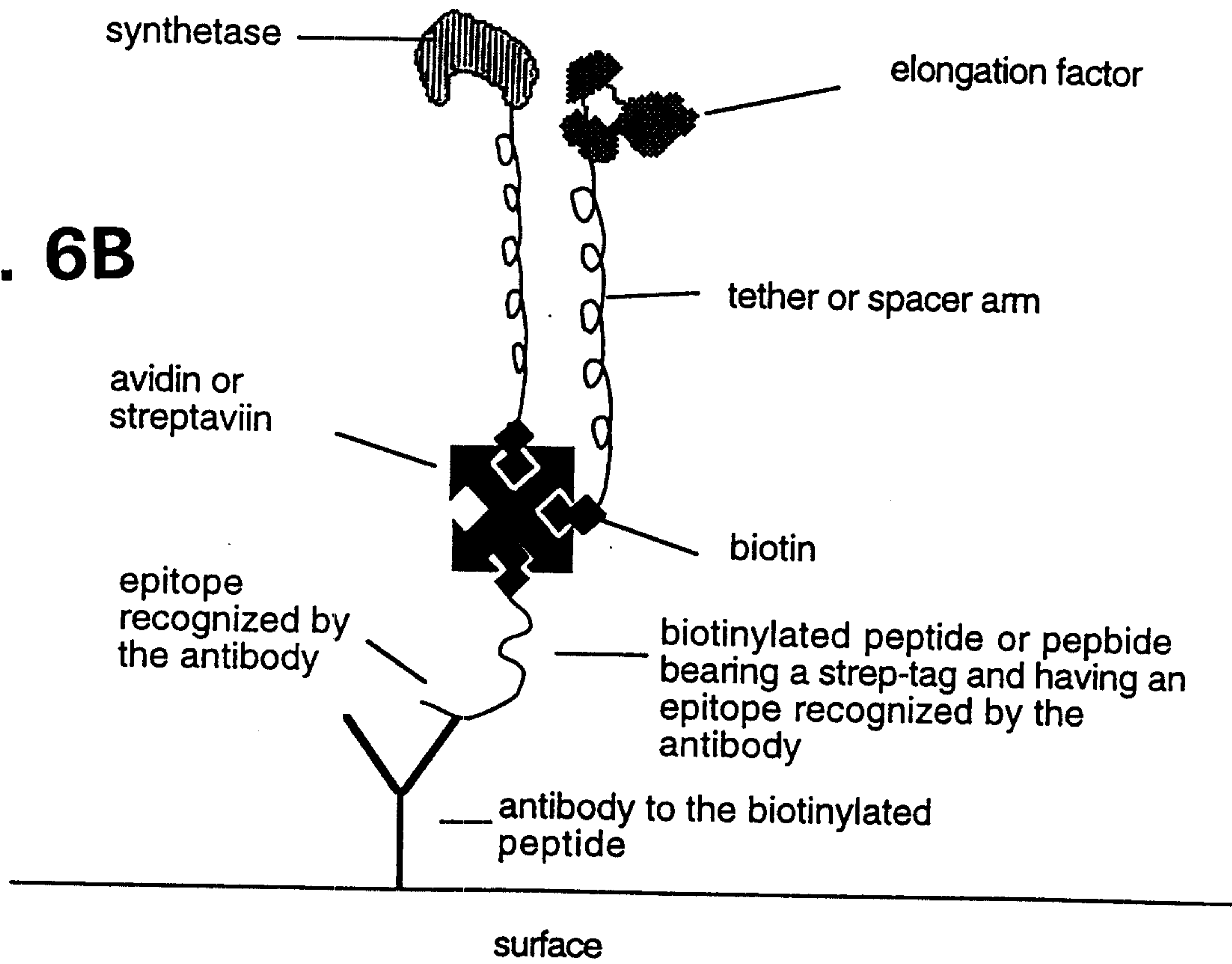


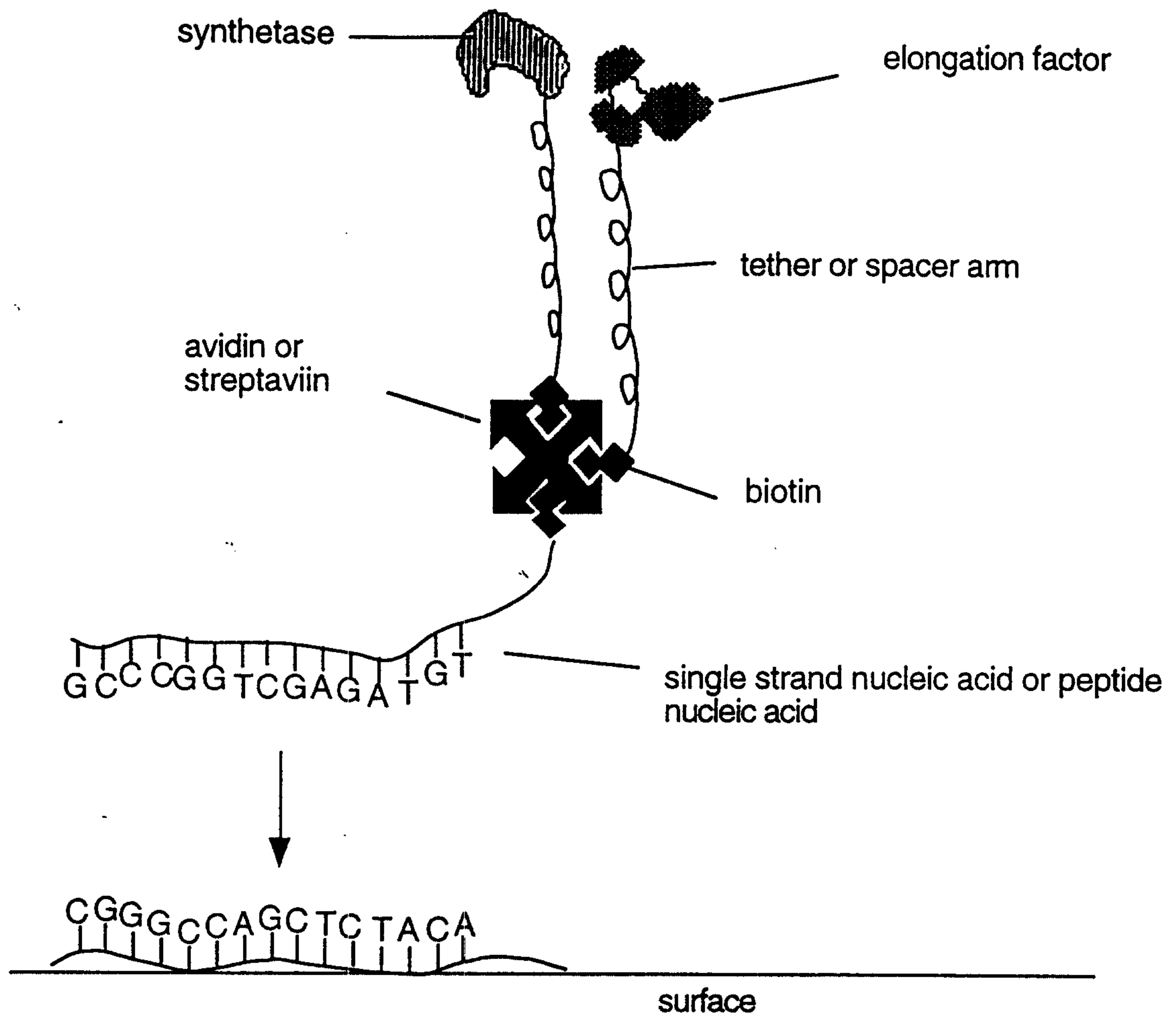
**FIG. 5C**

**FIG. 6A**



**FIG. 6B**





**FIG. 6C**

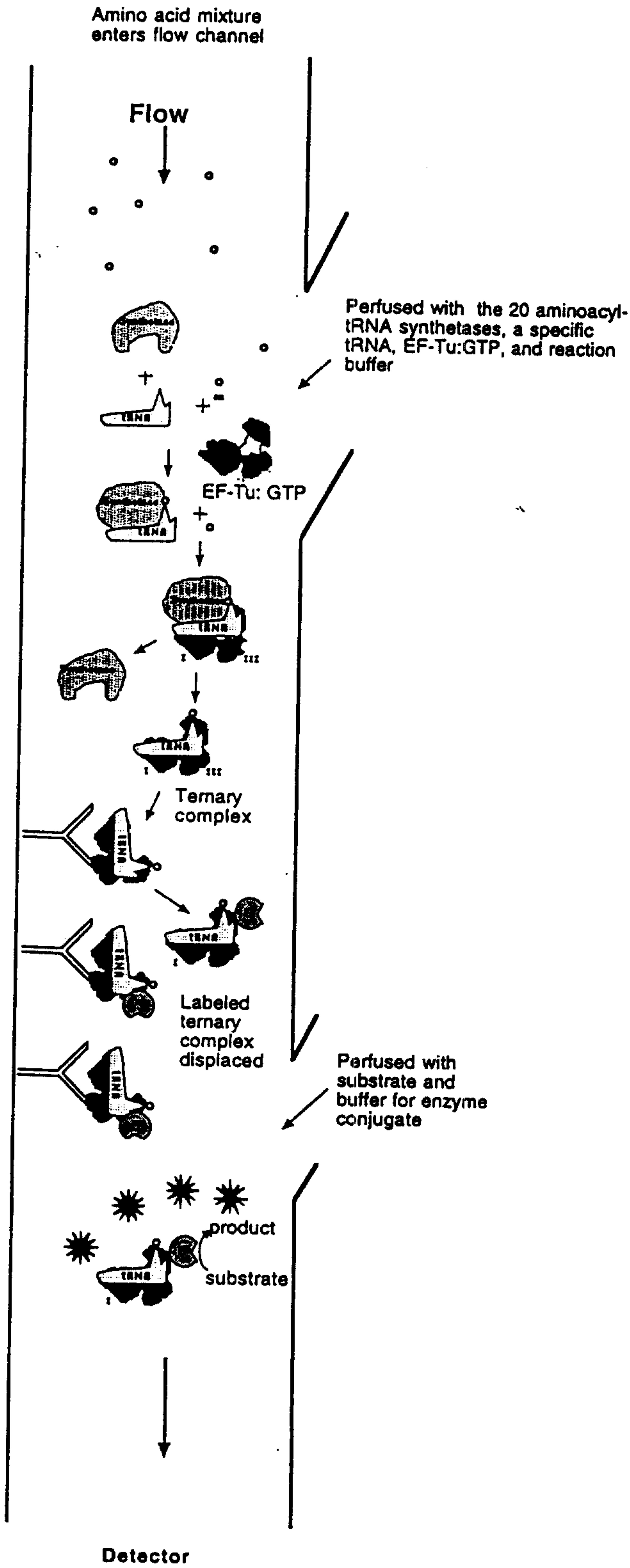


FIG. 7A

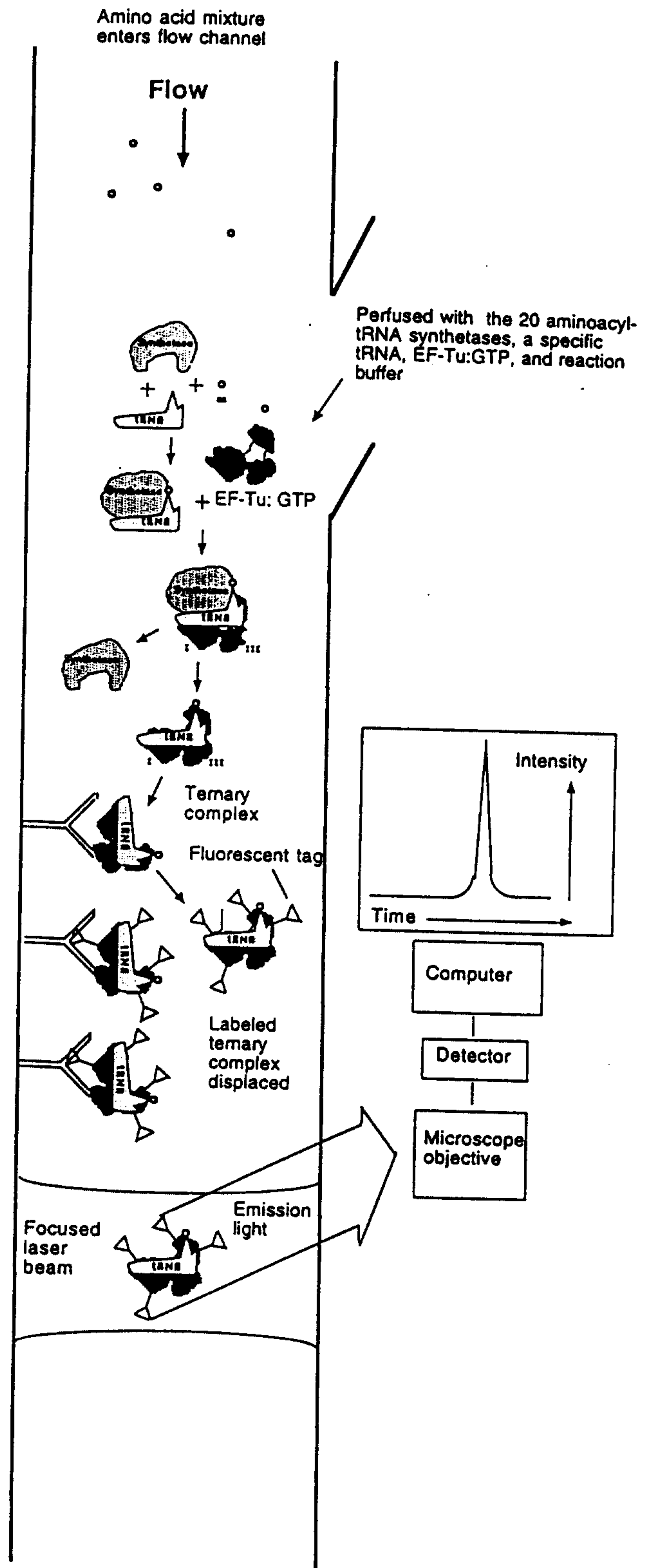


FIG. 7B

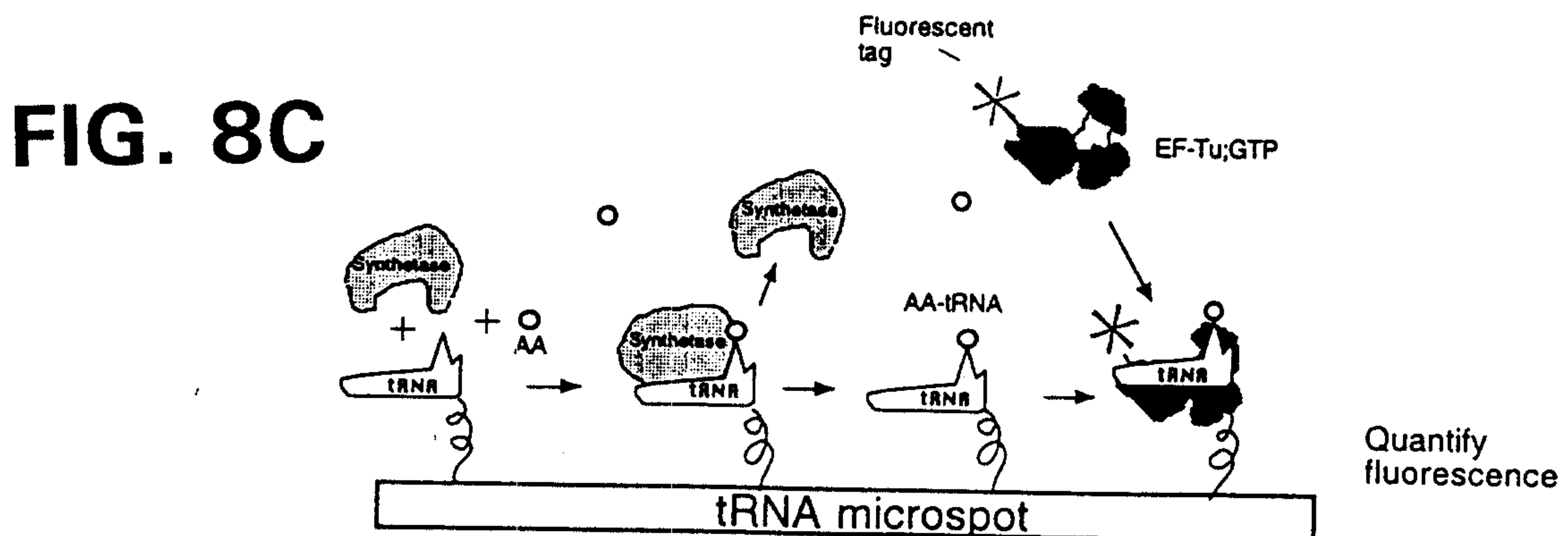
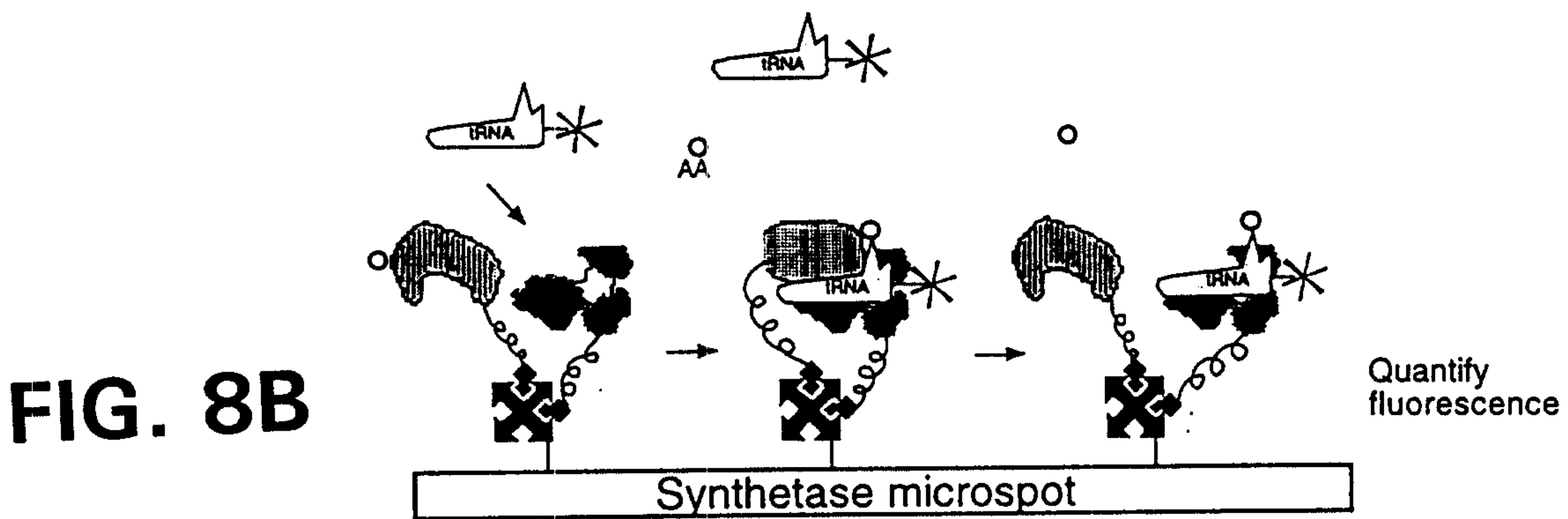
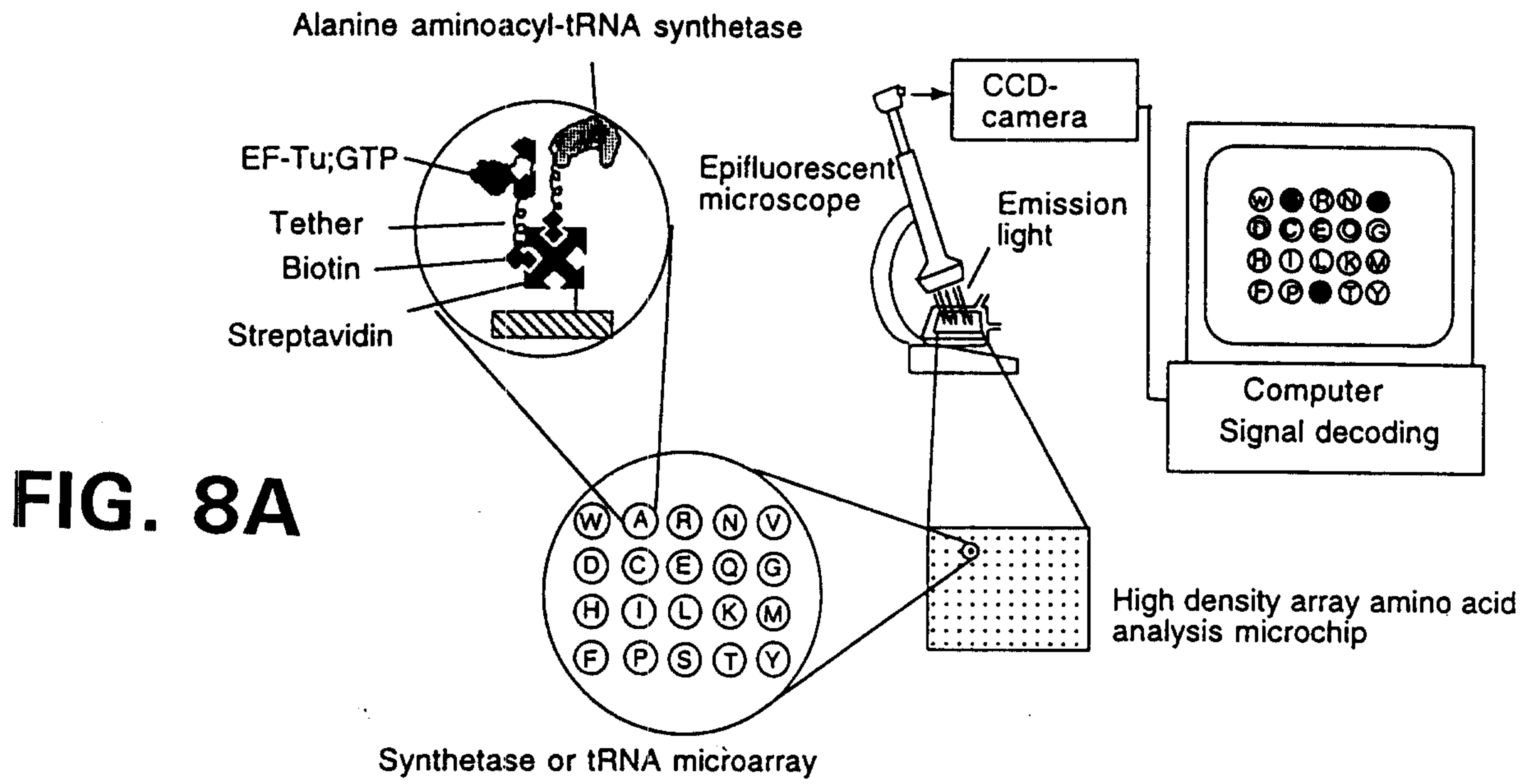
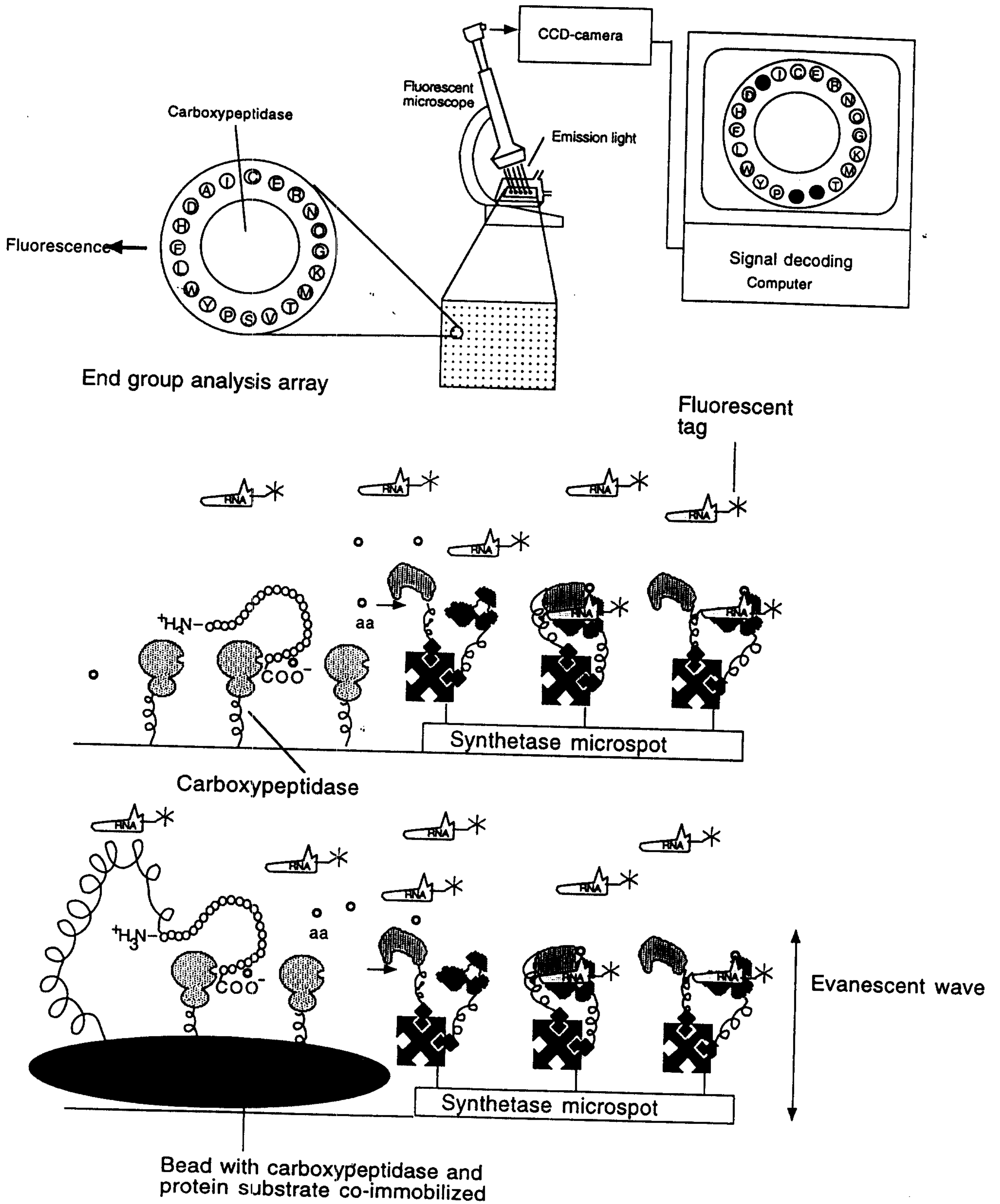
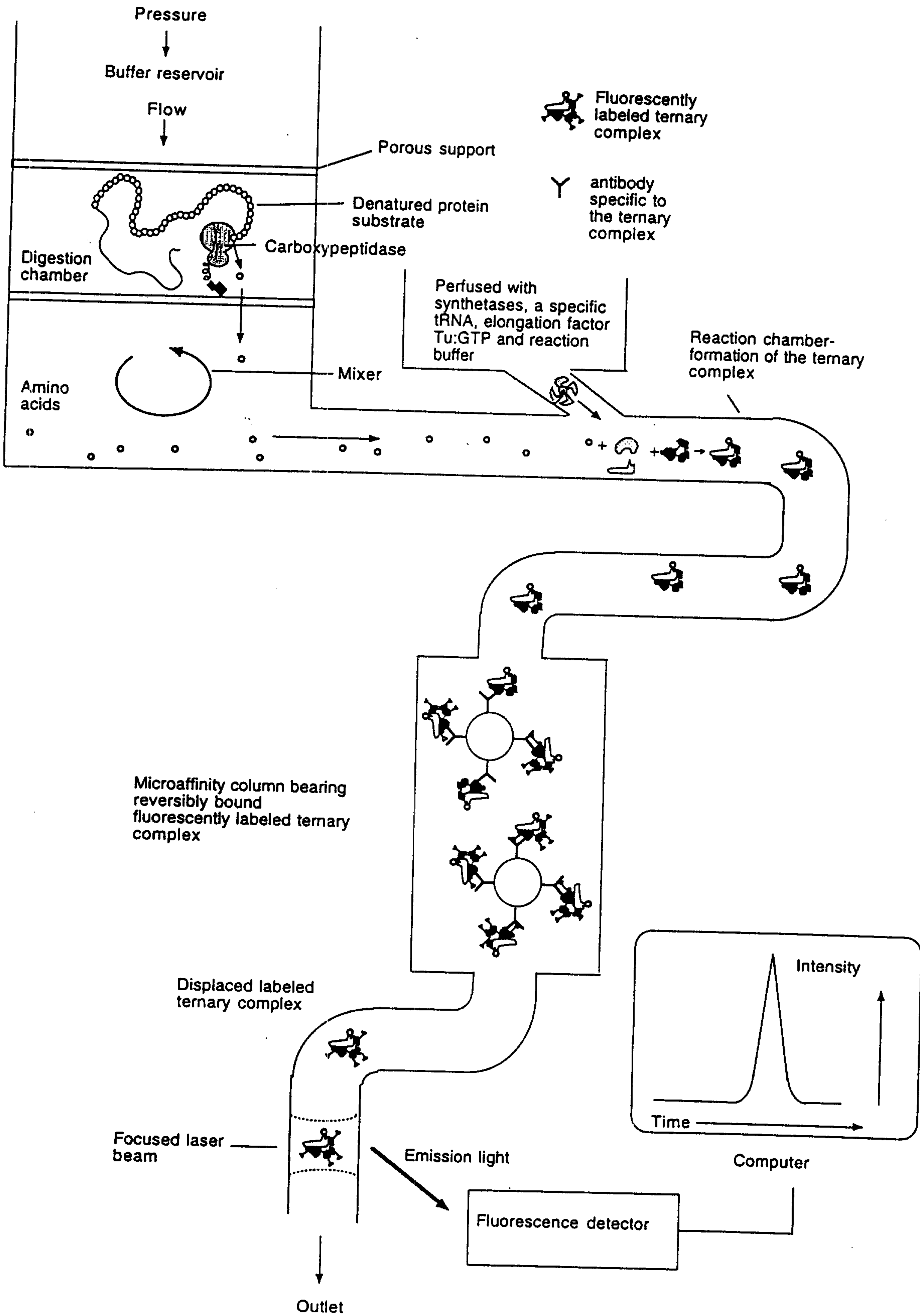
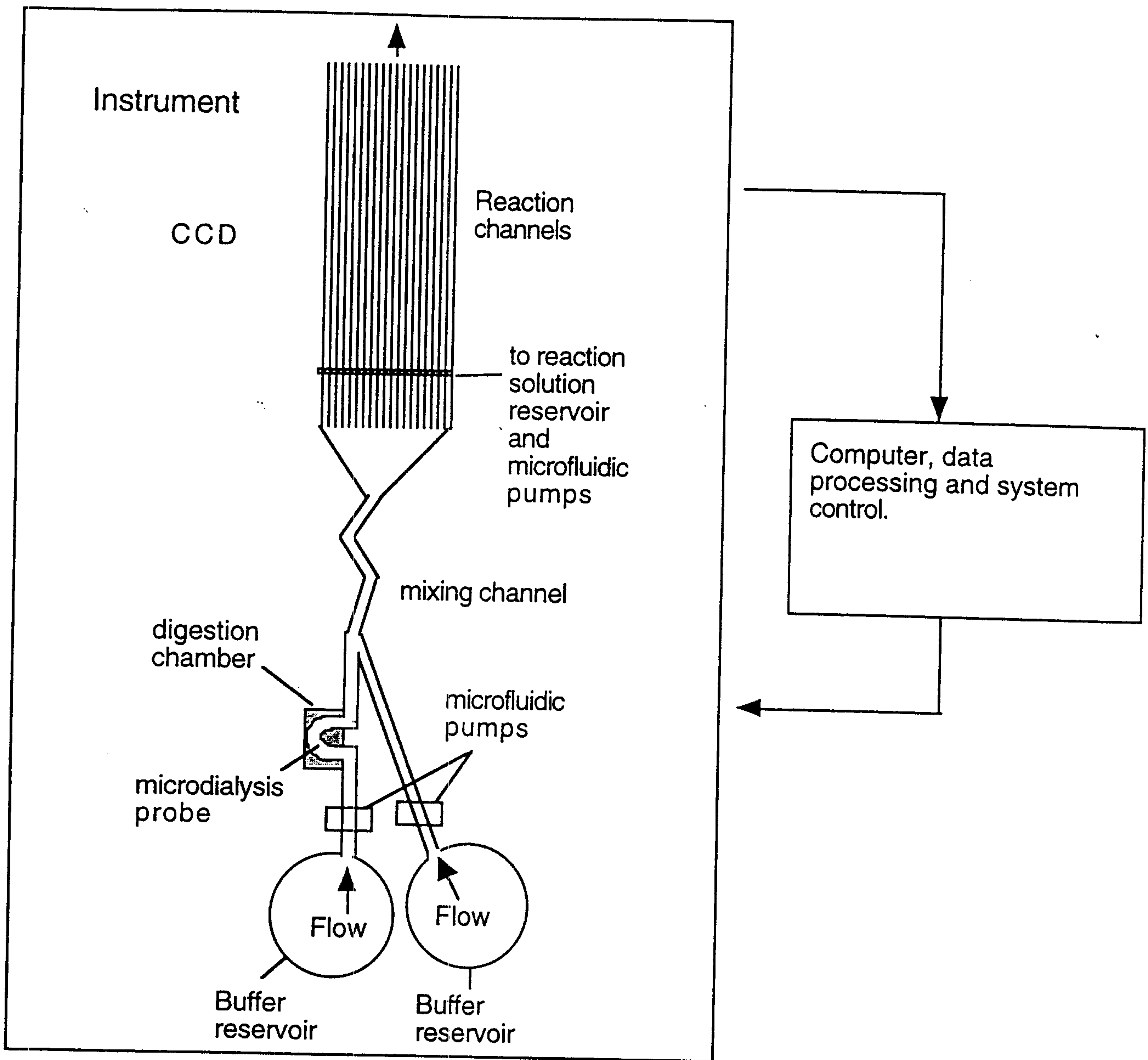


Fig.8D





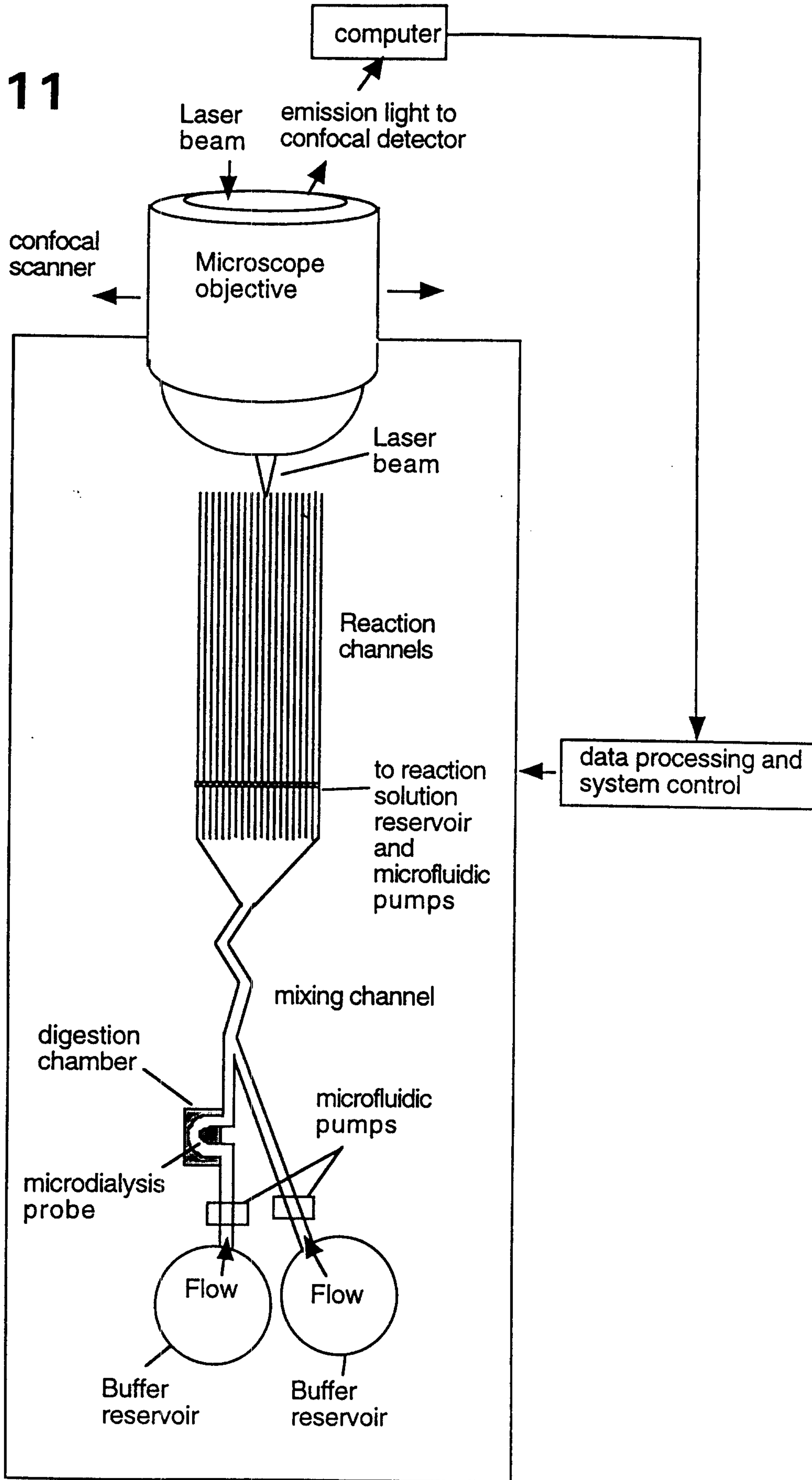
**FIG. 9**

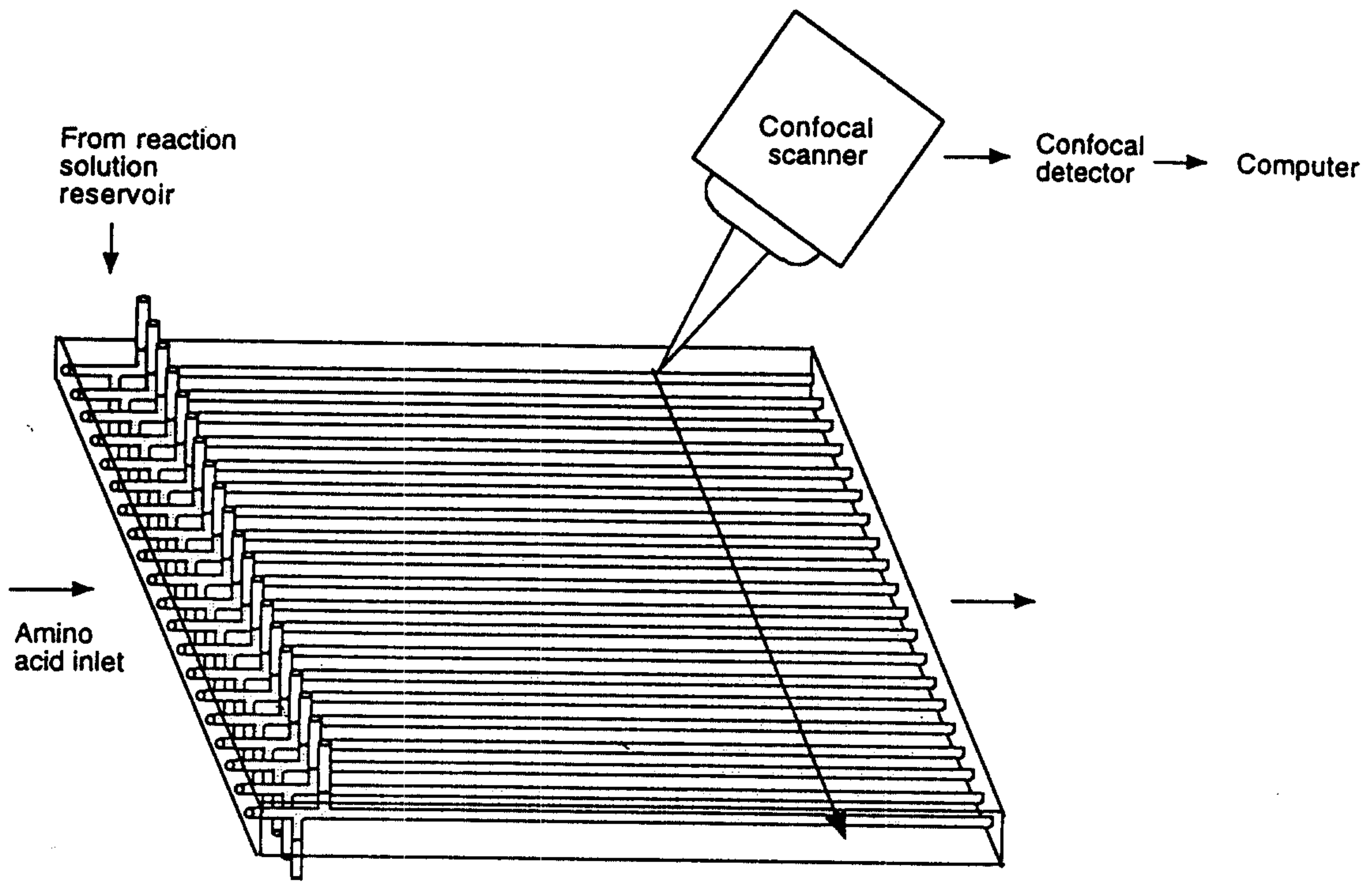


**FIG. 10**

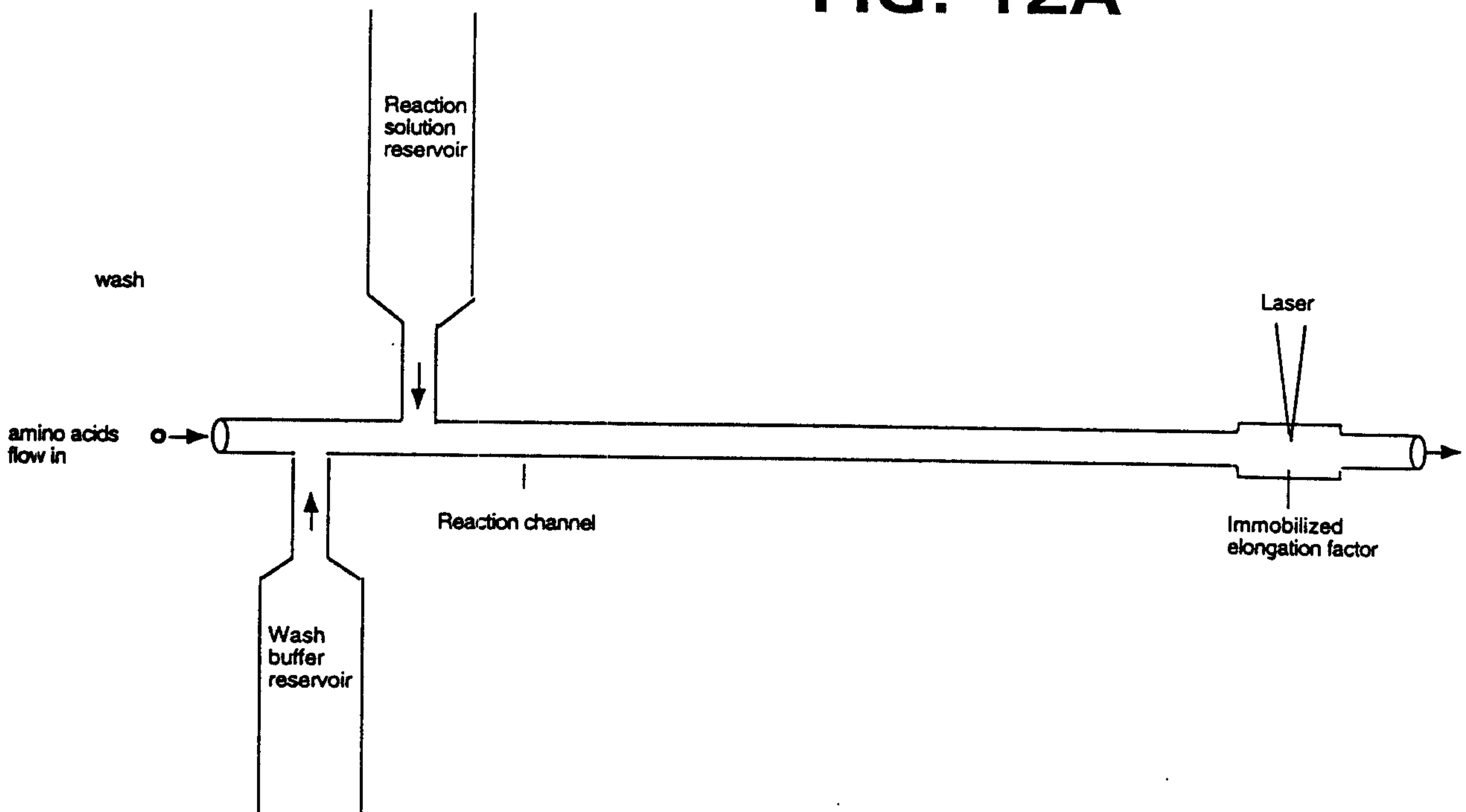


FIG. 11

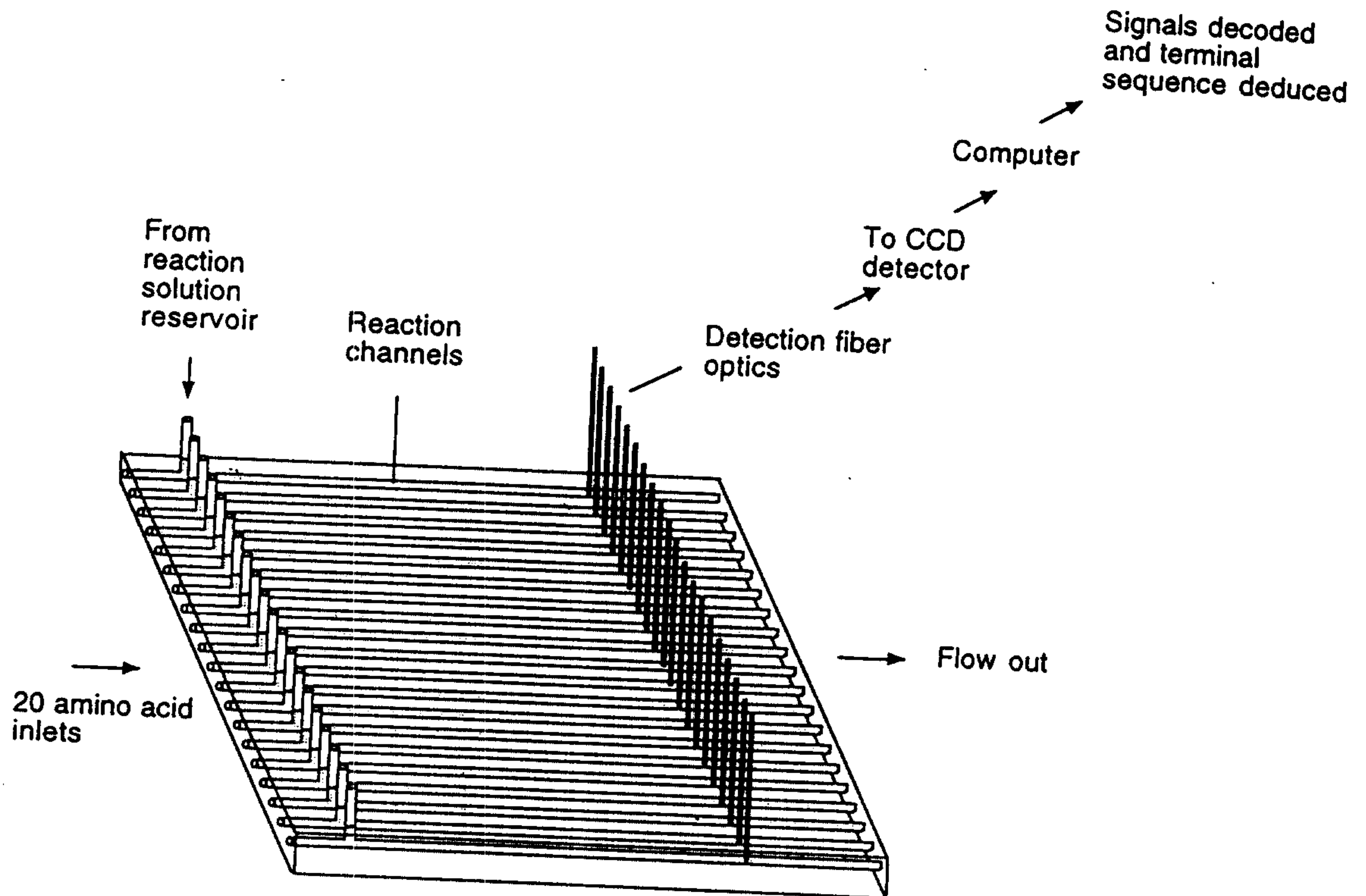




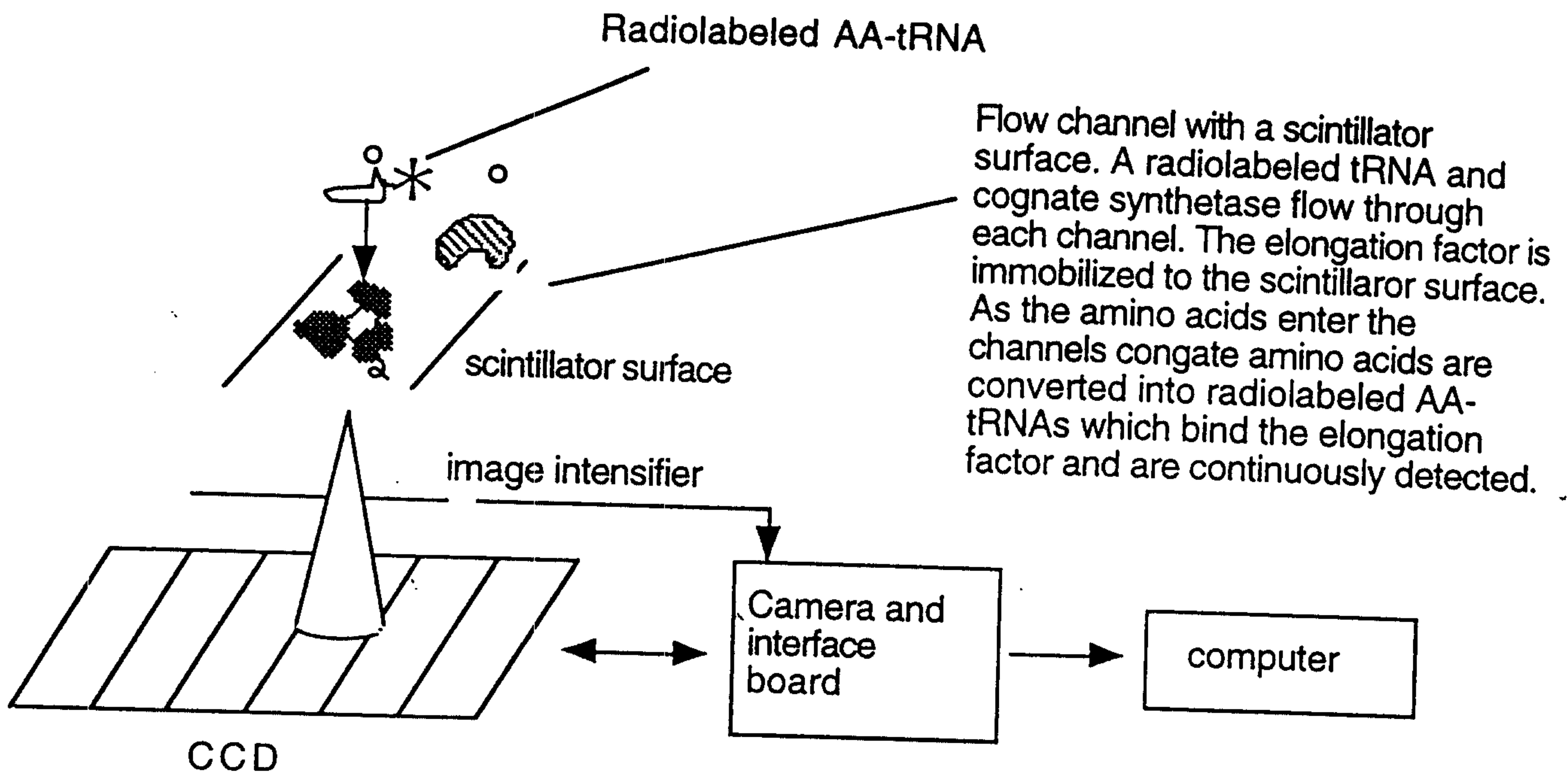
**FIG. 12A**



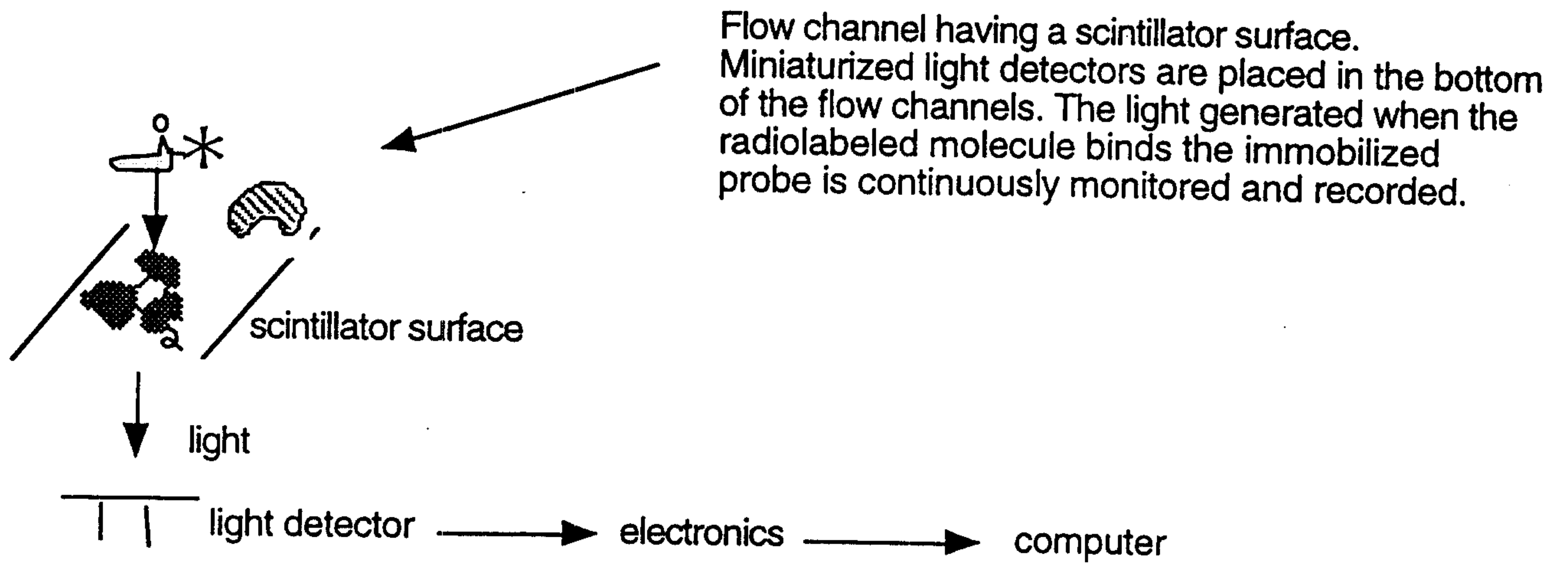
**FIG. 12B**



**FIG. 12C**



**FIG. 13A**



**FIG. 13B**

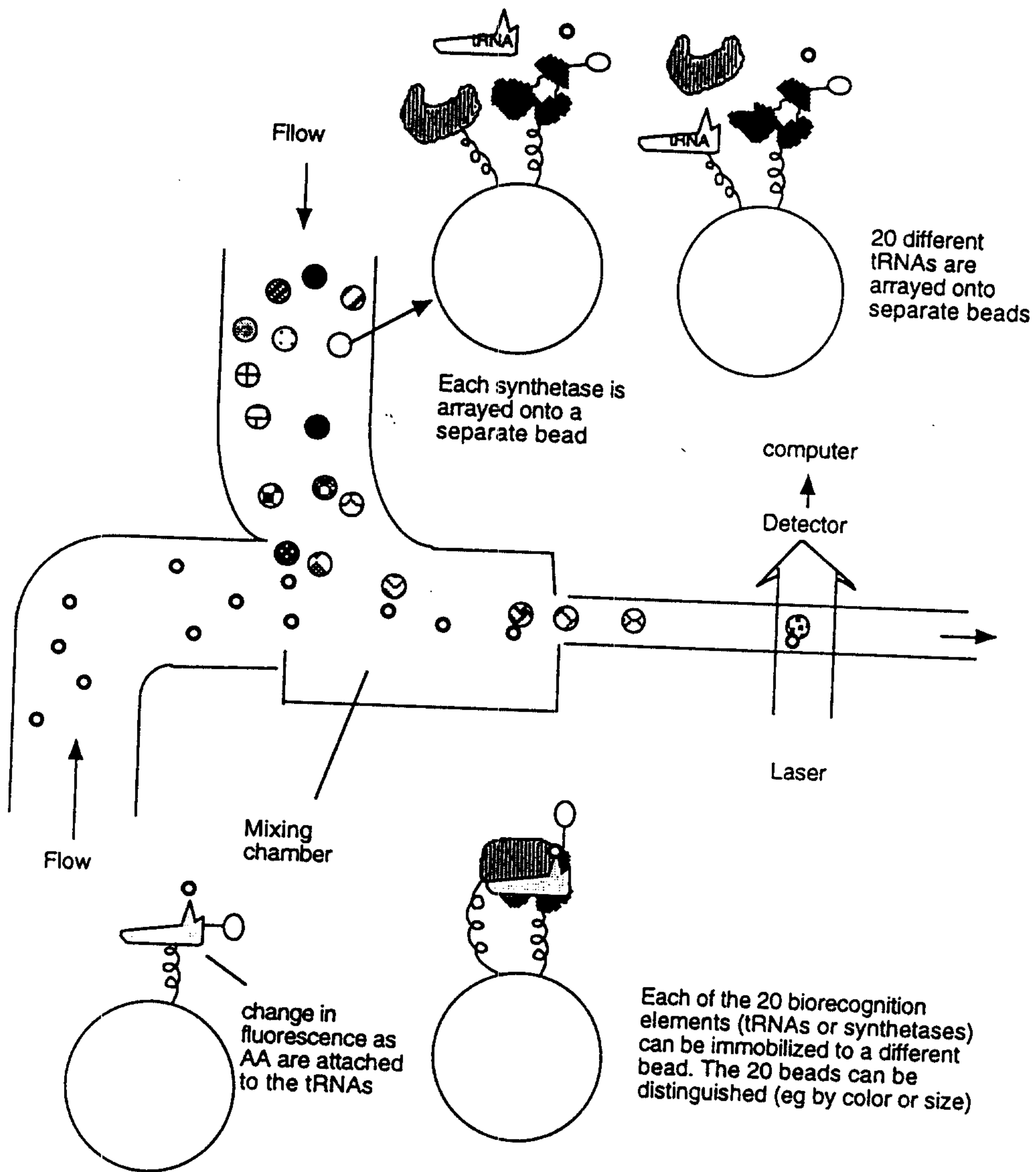


FIG. 14

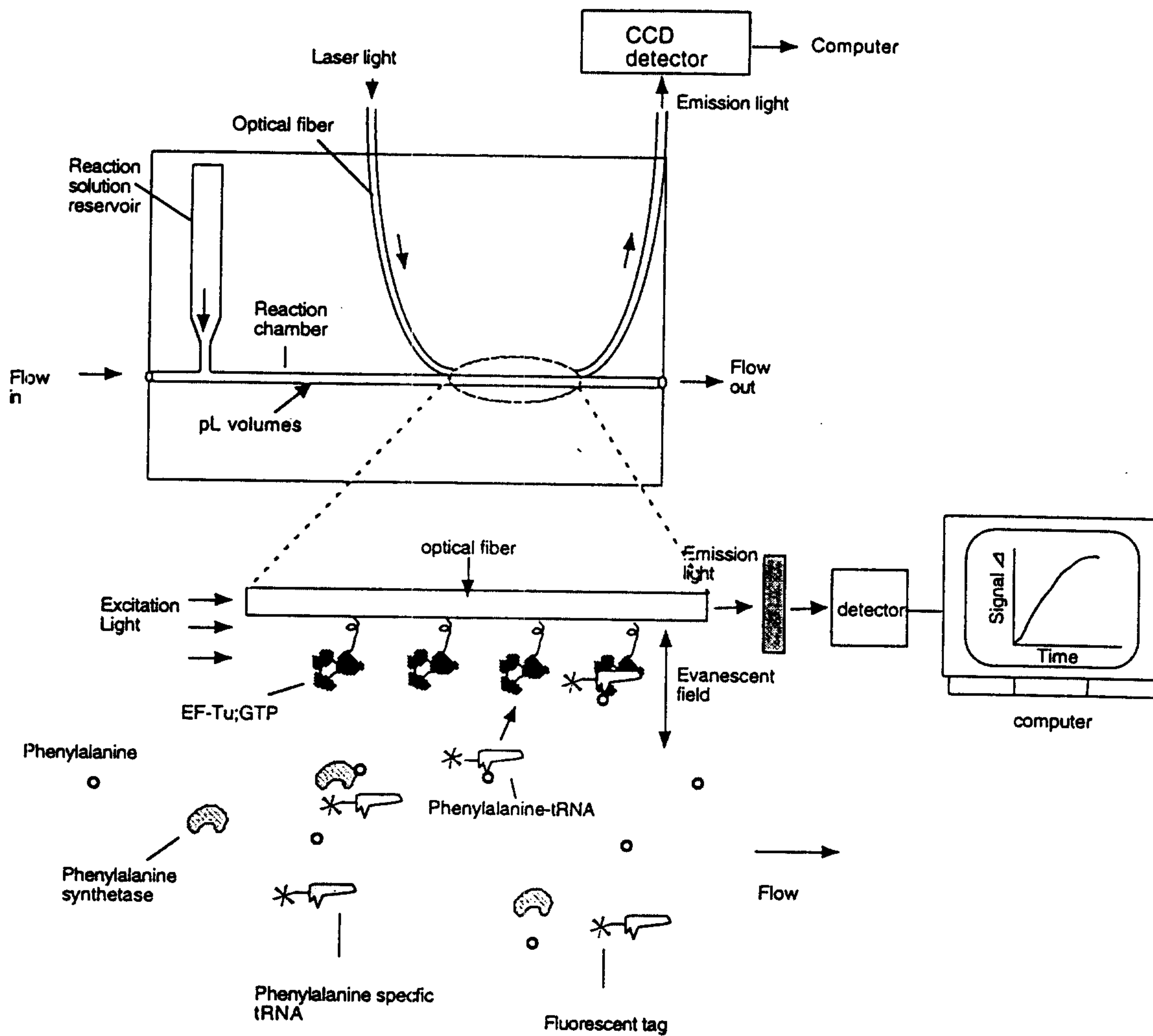


FIG. 15

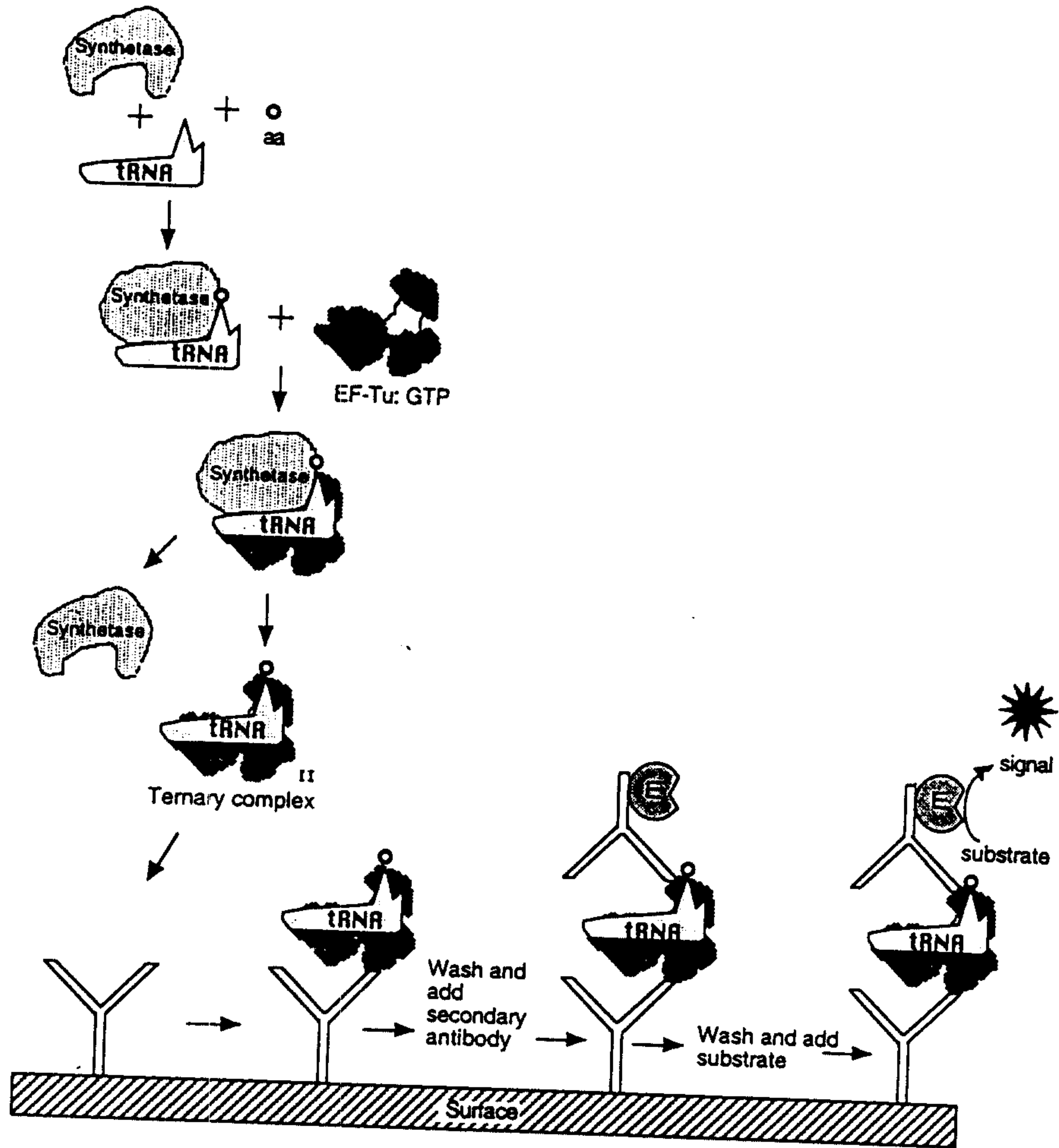
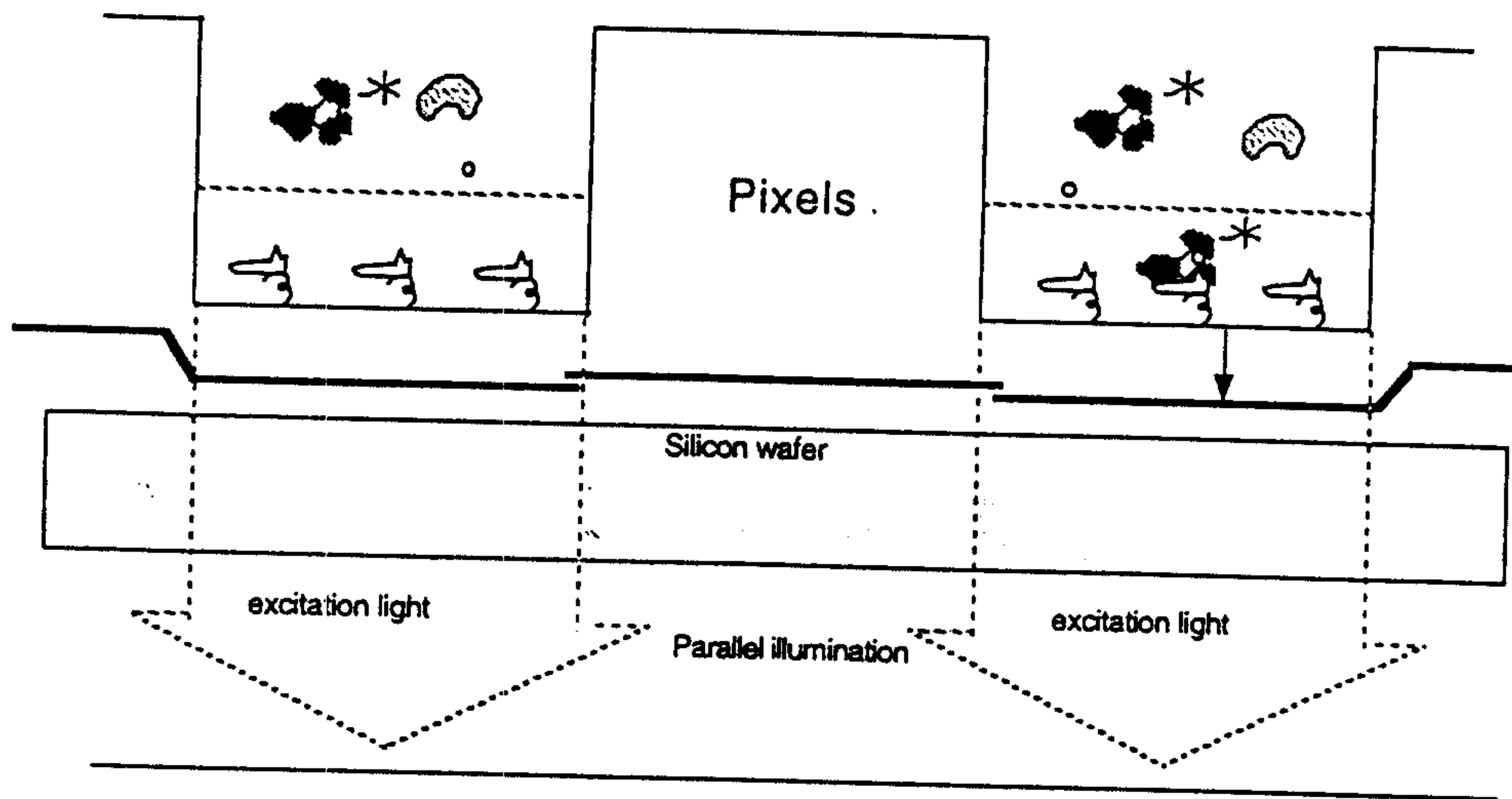


FIG. 16

Proximal CCD



Radioisotope or chemiluminescent labeling

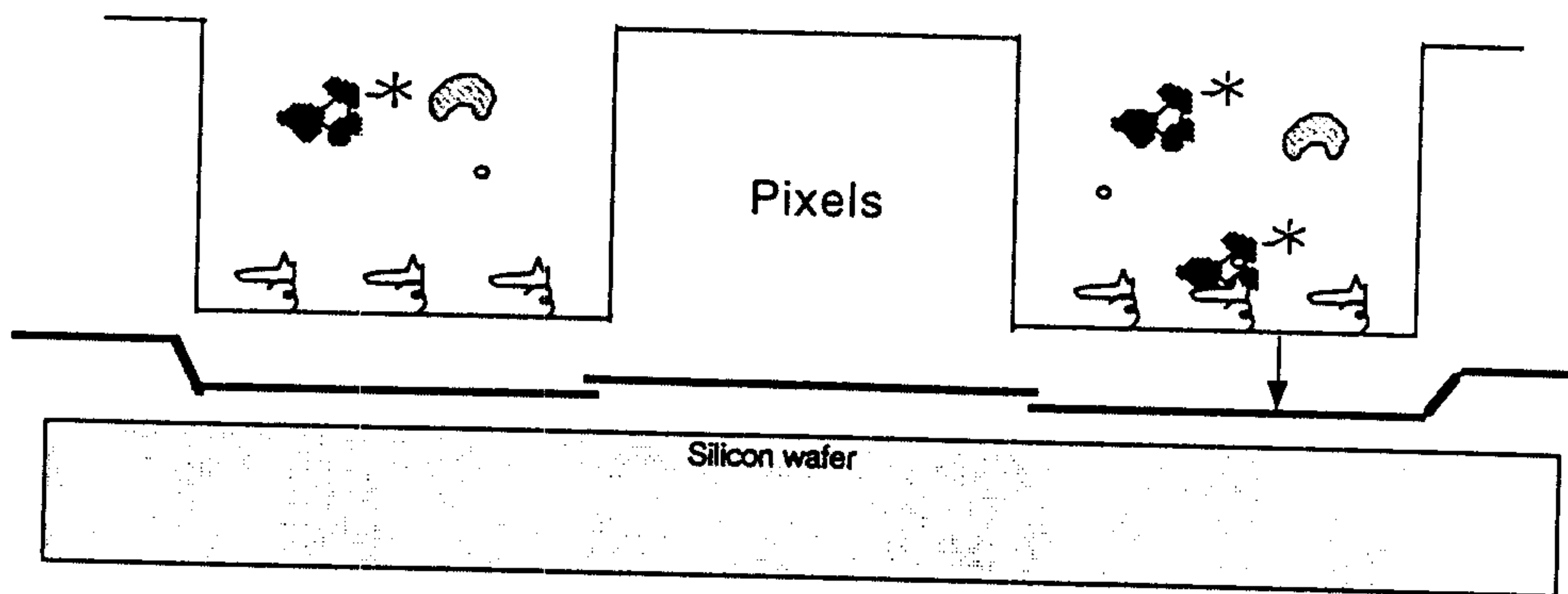
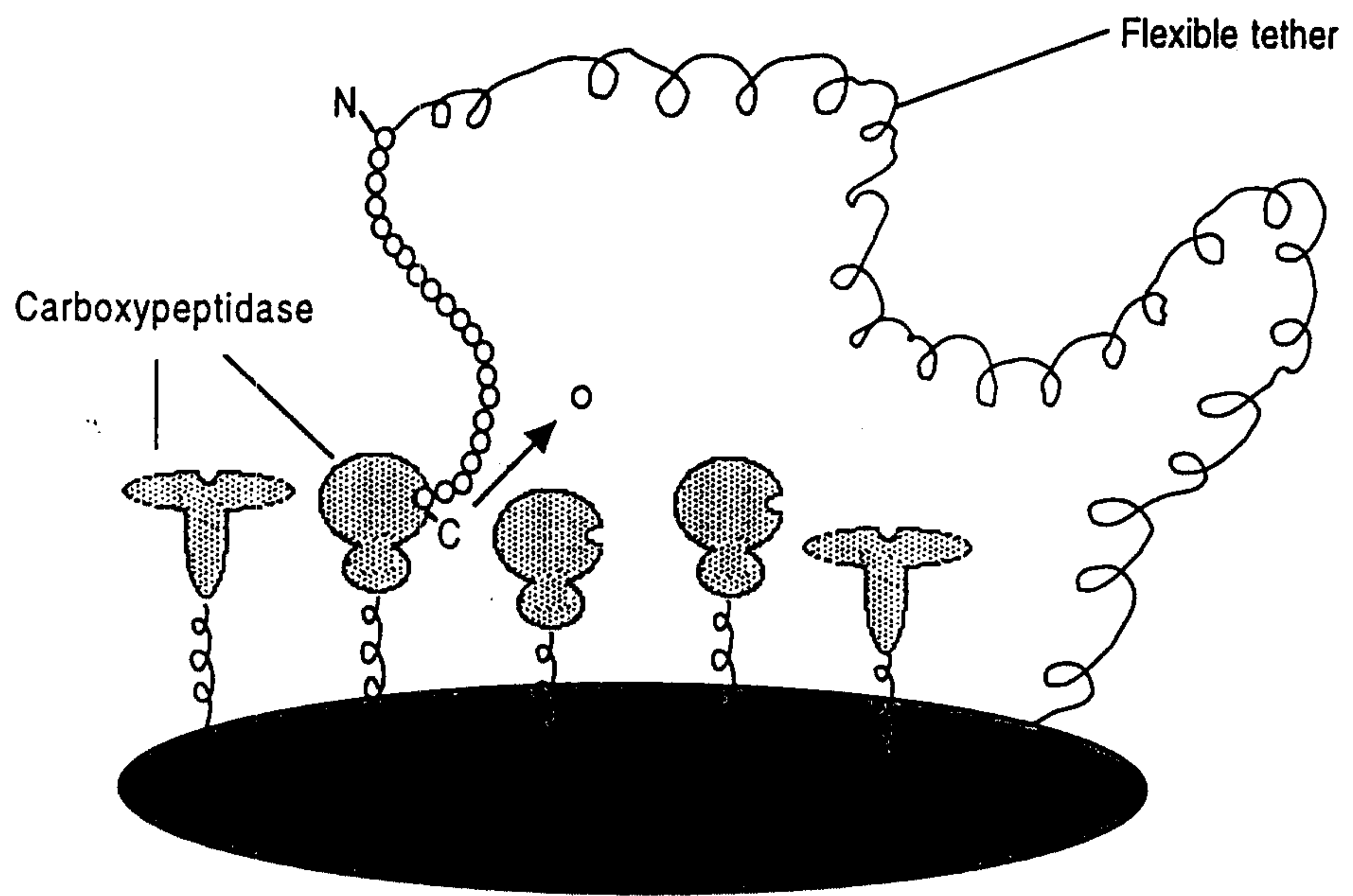


FIG. 17





**FIG. 18**

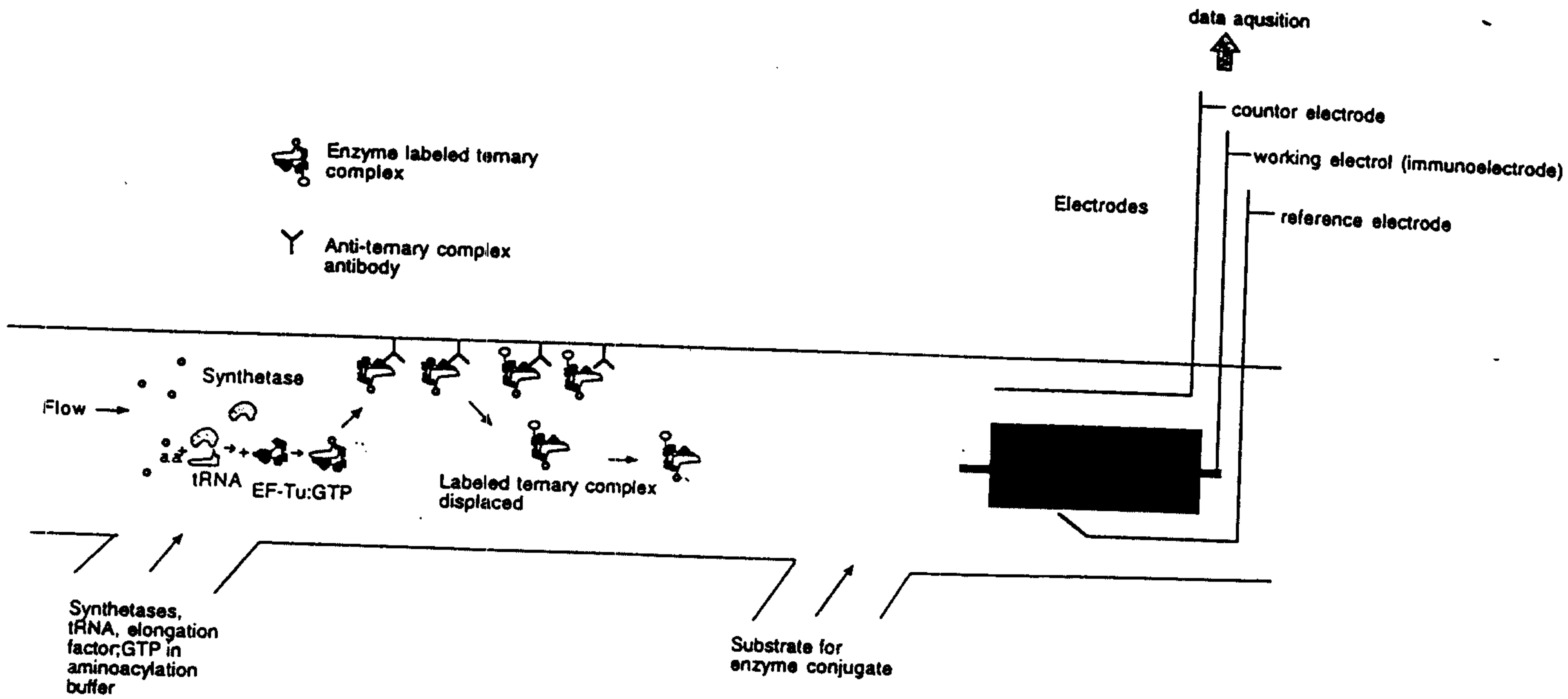


FIG. 19A

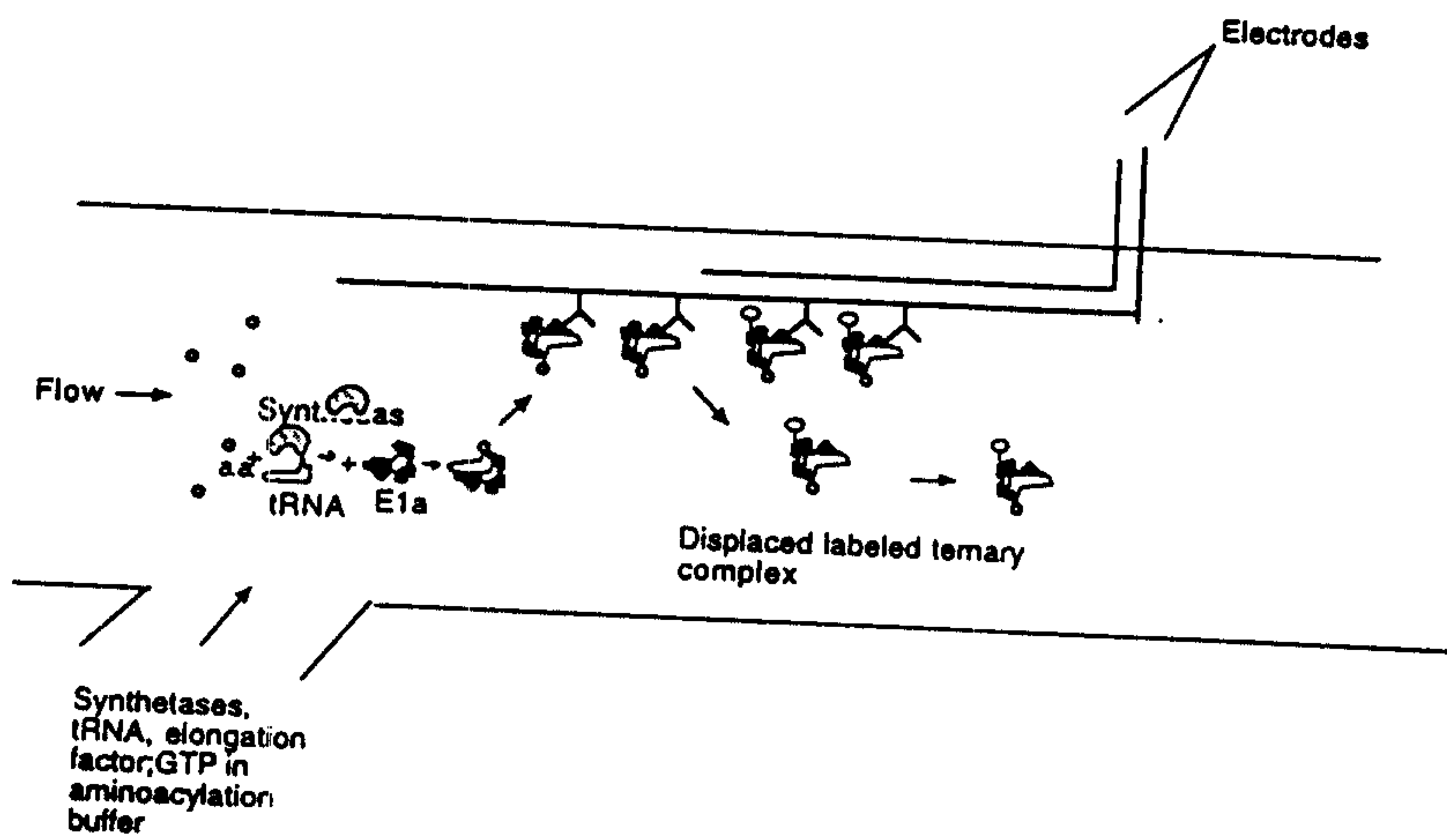
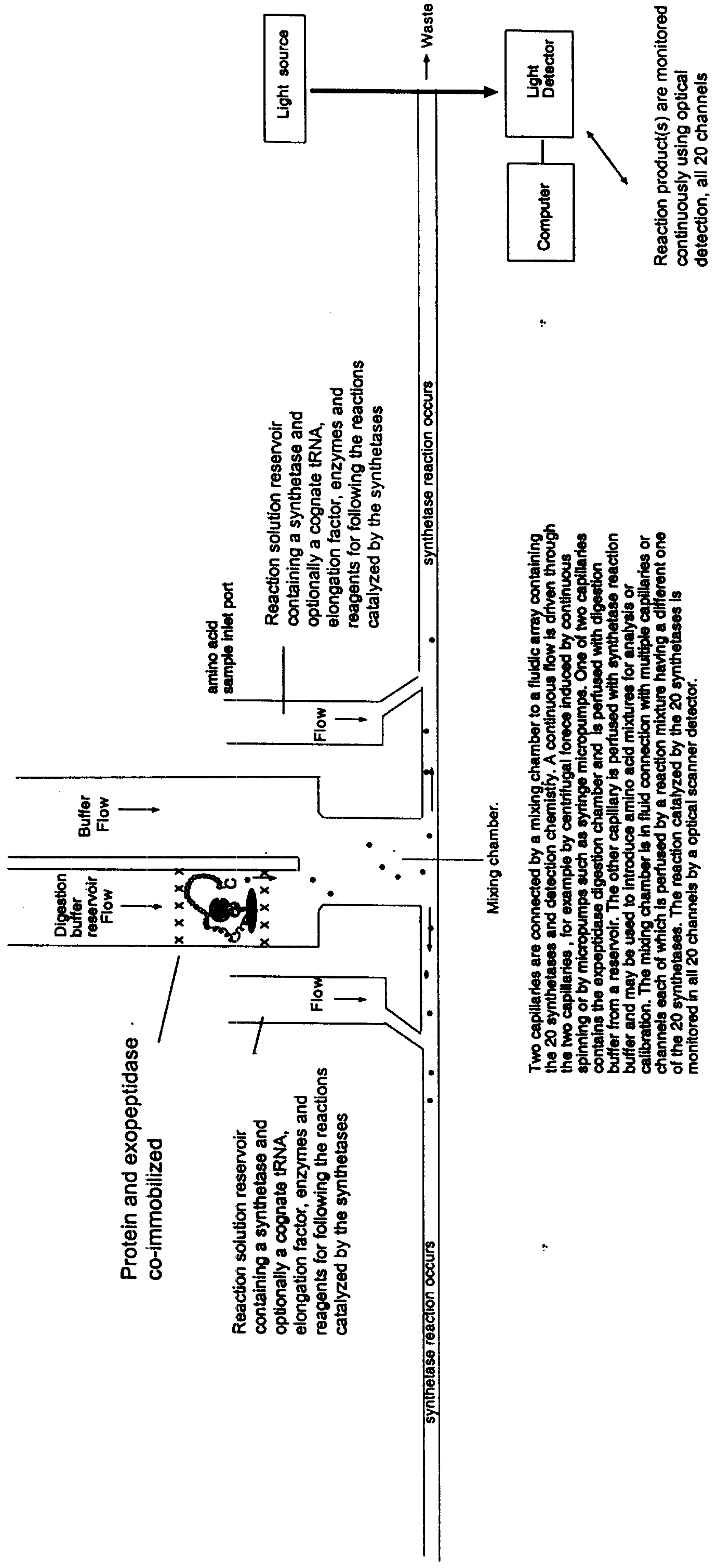


FIG. 19B



Two capillaries are connected by a mixing chamber to a fluidic array containing the 20 synthetases and detection chemistries. A continuous flow is driven through the two capillaries, for example by centrifugal force induced by continuous spinning or by micropumps such as syringe micropumps. One of two capillaries contains the exopeptidase digestion chamber and is perfused with digestion buffer from a reservoir. The other capillary is perfused with synthetase reaction buffer and may be used to introduce amino acid mixtures for analysis or calibration. The mixing chamber is in fluid connection with multiple capillaries or channels each of which is perfused by a reaction mixture having a different one of the 20 synthetases. The reaction catalyzed by the 20 synthetases is monitored in all 20 channels by an optical scanner detector.

Fig. 20

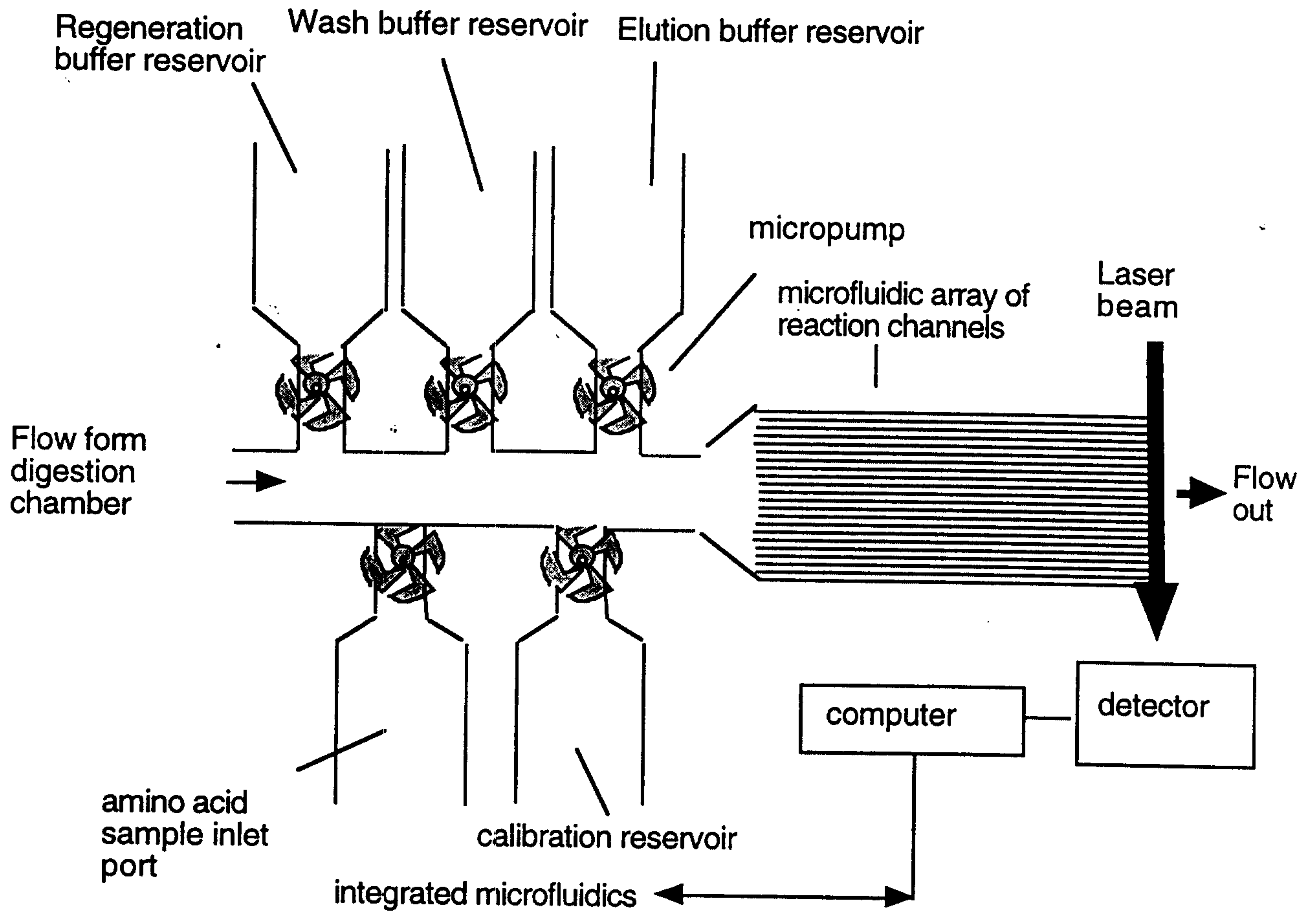


Fig. 21 A

integrated  
microfluidics

# Fig. 21 B

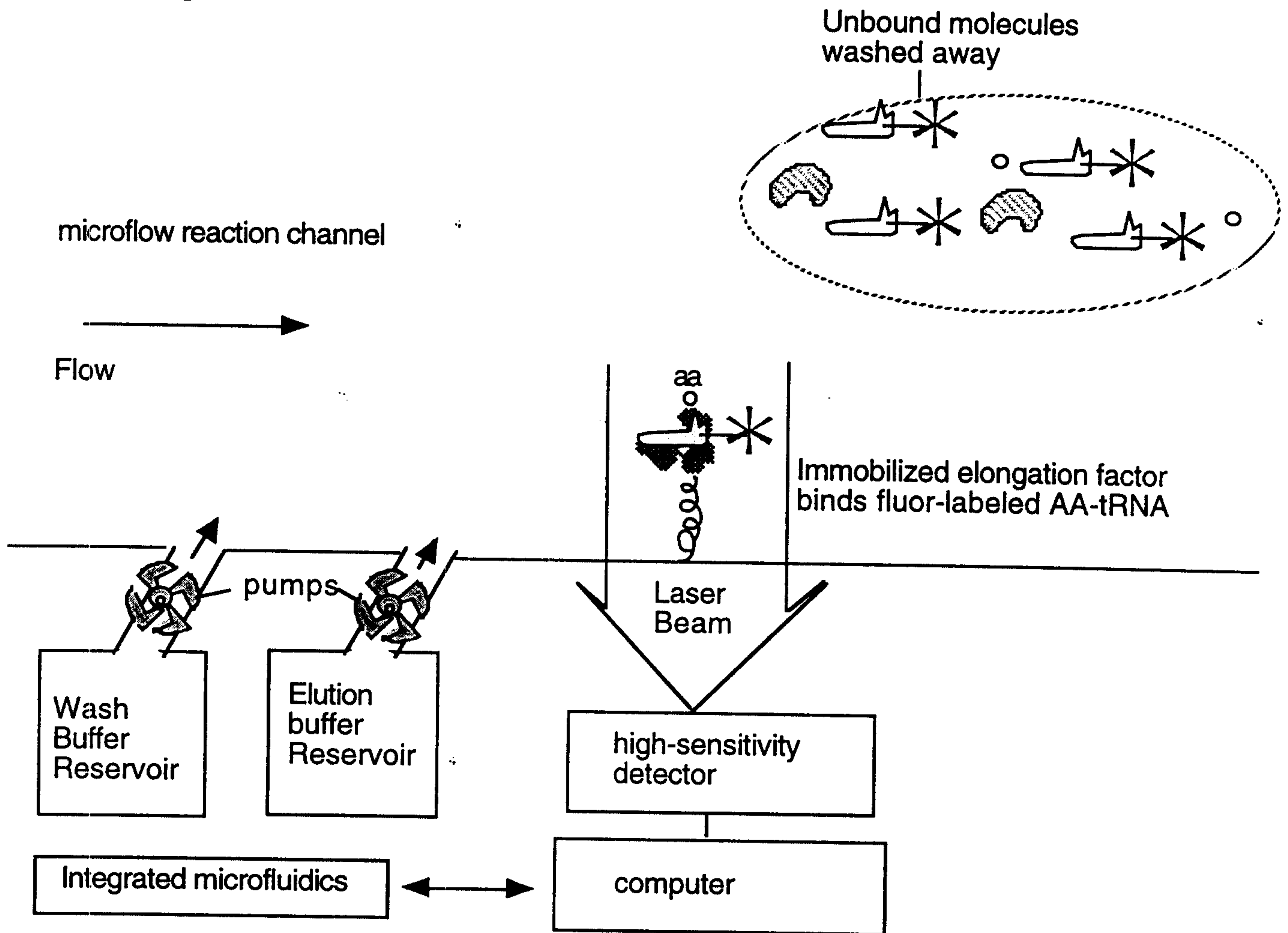
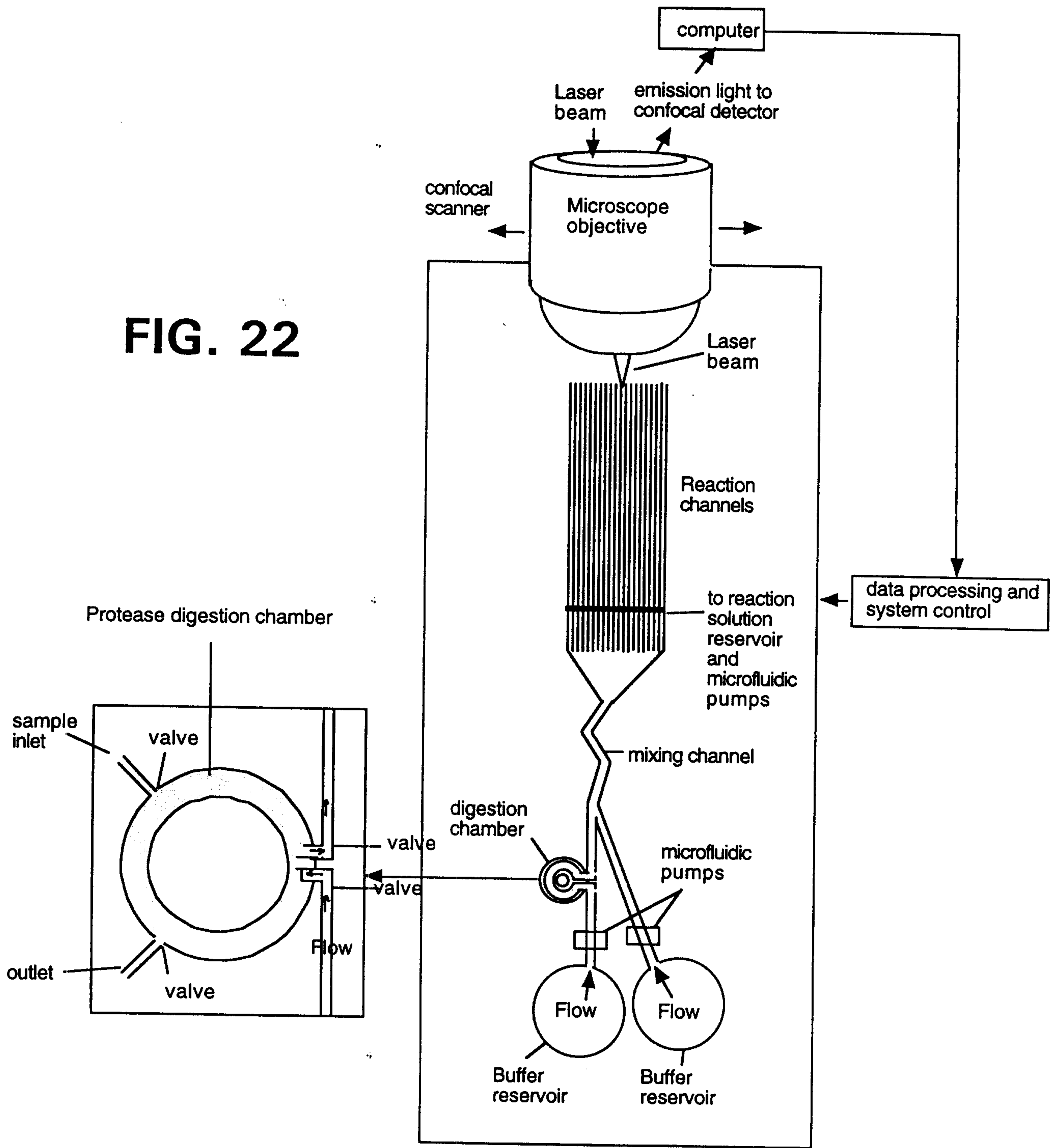
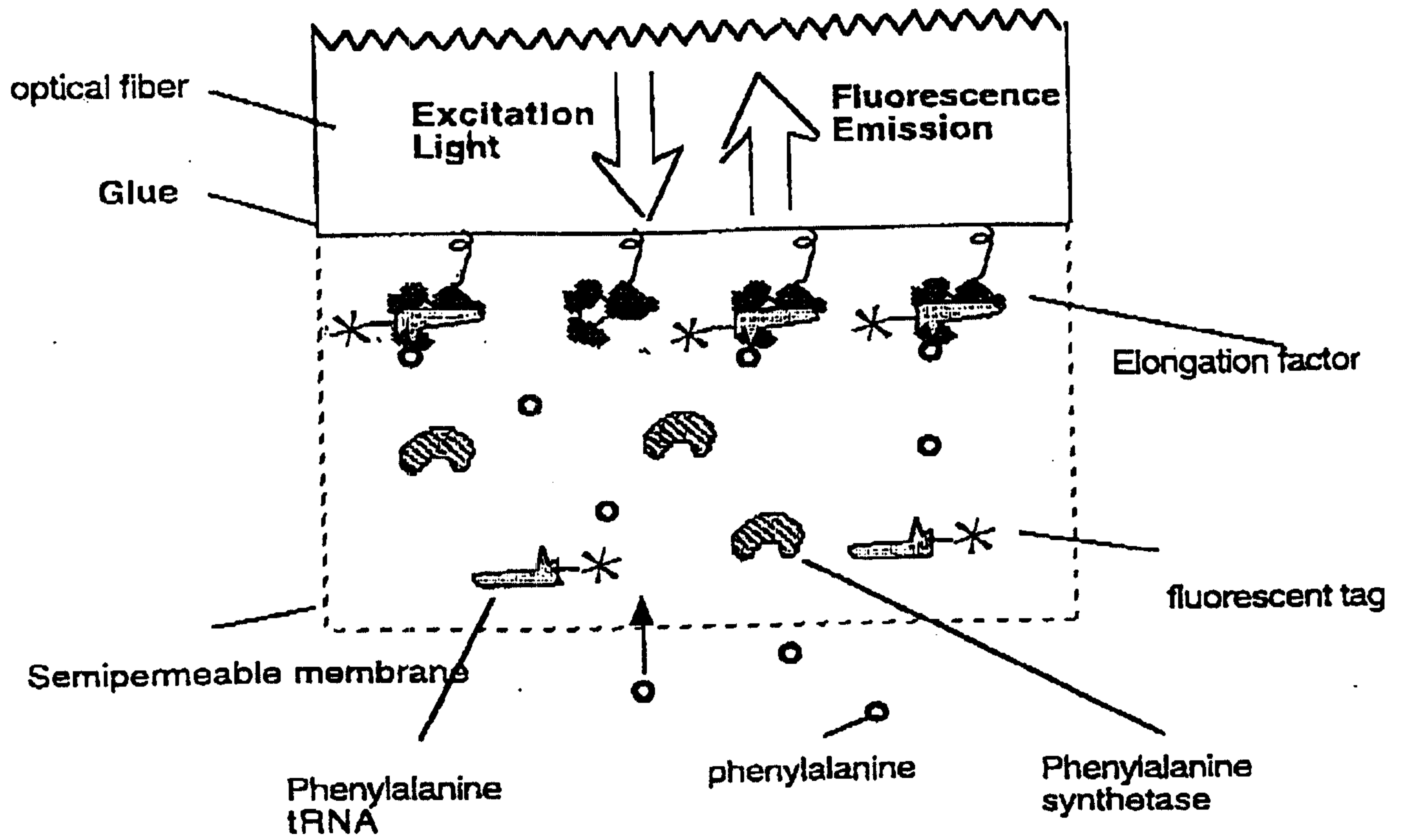


FIG. 22





**FIG. 23**

