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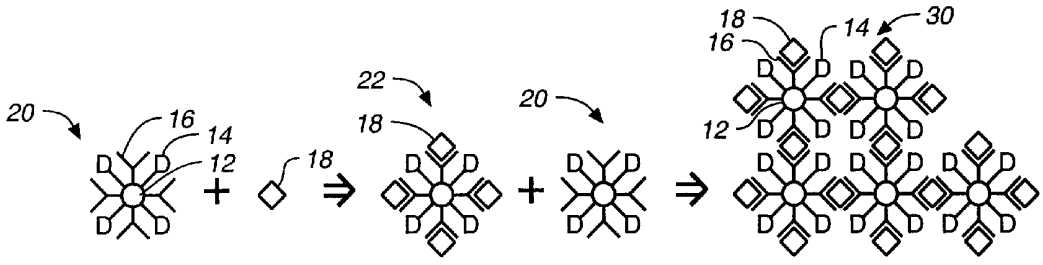
(54) Title: AGGREGATION-BASED ASSAYS



(57) Abstract: A method for measuring aggregation by the detection of individual particles (30) is disclosed. The method is adaptable for screening compounds, detection of low levels of a target (e.g. small molecules, large molecules, cells, or microbes), competitive binding assays and other assays. If the assay mixture contains a targeted compound (18), the particulate substrate (20) will aggregate to form a macroparticle (30). One of the components of the assay mixture is labeled with an optically detectable label (14). The macroparticle (30) has an associated macroparticle optical signal which would be identified by optical analysis of the assay mixture.

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Description

AGGREGATION-BASED ASSAYS

TECHNICAL FIELD

This invention relates to an assay to detect a target of interest through the formation of particles within a detectable size range.

BACKGROUND OF THE INVENTION

Detection of small amounts of compounds is required for current analytical and diagnostic assays. For example, small amounts of substances present in patient samples, such as blood or tissue, serve as an indication of disease states. Monitoring of substances in patient bodily fluid, such as pharmaceuticals, narcotics, toxins, hormones, antibodies, etc. is required for diagnosis and treatment of patient conditions. Numerous samples containing minute quantities of substances must be tested. The sample analysis must be rapid, sensitive, and specific for the substances tested.

Immunoassays are often used to detect small amounts of a target substance. Examples include radio immunoassay and enzyme immunoassay both of which have been used as sensitive assays for specific antigens. These assays allow detection of hormones, pharmaceuticals, and other small compounds present in bodily fluids at very low levels (e.g. picomoles/liter).

Immunoassays may either be heterogeneous (i.e. a separating phase is used to remove the bound detectable target from the assay mixture) or homogenous (i.e. the sample is directly detected in a native sample without a separation step). Because heterogeneous assay does not allow detection in a native sample, these assays present certain drawbacks. The washing or separating steps re-

quire additional material and experimentalist time and may alter the cell signal. In contrast, homogenous assays save time and resources by not requiring a separation step. Additionally, detection in a native sample reduces risk of error or contamination resulting from each manipulation step.

Aggregation assays provide one method for homogenous detection of compounds with the high sensitivity required to small quantities of compounds in solution. In aggregation assays, a solution of binding agents is mixed with a test sample. If the targeted compound is present, the compound will interact with the binding agent and produce a characteristic aggregation pattern. Such an aggregation pattern observed on a macro scale could be observed as a "clumping" of the aggregating particles. Two commonly practiced types of aggregation assays are latex-based tests and blood cell-based tests.

Latex agglutination

Latex agglutination assays generally detect the presence of an antigen by using a competitive binding assay system. An antigen and a latex bead having an associated antigen-analog or conjugate of the antigen compete to bind to the binding sites of the antigen specific antibody. If no antigen is present in the test mixture, the antibody binds to the antigen analog or conjugate of the antigen analog to form clumps that may be visually detected. If the antigen is present the antigen will compete with the attached compounds on the latex beads for the antibody binding sites. This prevents the reaction of the latex beads with the antibody, preventing aggregation of the latex beads. In this assay, the reaction mixture is generally visually observed to determine assay agglutination. Agglutination indicates the presence of the antigen of interest.

Various devices have been developed for the elimination of the subjective determination of the results of the latex-based assays. For example, U.S. Pat. No. 5,491,095 to Bepko et al. discloses an automatic reader for latex-based agglutination reactions. The reader directs light onto a flowing reaction mixture. A plurality of detectors positioned at a number of locations detect light that has passed through the sample. The variation in the amplitude detected by each detector indicates whether the sample is aggregated or uniform. The amplitude variation must exceed a threshold measurement to be detected.

An alternative assay described in U.S. Pat. No. 5,489,537 uses various dyes in a process that automates the detection of latex aggregation. In this assay, a test sample is incubated with a suspension of latex beads and a detectable colloidal dye. The latex particles are conjugated to a binding agent for binding a target antigen. If the targeted antigen is present, the latex beads aggregate and some of the aggregated latex particles entrap the colloidal dye. This reaction mixture is then brought into contact with a porous membrane. The pores of the membrane allow the non-aggregated latex particles to flow through but trap the aggregated latex particles containing the colloidal dye. The amount of colloidal dye remaining in the membrane is measured. The strength of this measurement is an indication of the amount of aggregation which in turn indicates the amount of the substance of interest.

Blood Based Aggregation

Hemagglutination assays use erythrocytes (such as sheep red blood cells) and anti-erythrocyte antibodies in an indicator system. The red cells, coated with the antibody are reacted with the sample. Antigens with

binding agents for multiple epitopes cause red cells to aggregate to form agglutinated masses. It is also possible to design an indirect assay in which the sample compound competes with the red cells for the binding to binding agents. In the absence of competitive binding, agglutination is observed.

U.S. Pat. No. 5,768,407 to Shen et al. describes a method for automating the detection of aggregation in a blood-based aggregation assay. In this method an illuminated image is produced and detected as an array of pixels, with each pixel having a value representative of the intensity of the illuminated image on the pixel. The values are subsequently processed, based upon a stored set of variables, to determine if aggregation has occurred and to classify the aggregation if it has occurred.

U.S. Pat. No. 5,583,003 to Hillyard et al. describes a direct agglutination measurement using blood cells. In this reference two reagents are provided, each of which binds to a non-overlapping epitope of a targeted antigen. In two separate assay mixtures, each of the reagents is separately mixed with a test compound that may contain the antigen. The two assay mixtures are then combined and the resulting agglutination indicates the presence of the antigen in the sample.

Although the present assays are capable of detecting low levels of a target antigen in a test sample, in general relatively large amounts of reagents are required to test for a target antigen. In addition, some of the present methods remain subjective and do not provide an automatic indication of the amount of the antigen of interest present in the test sample.

The present aggregation assays are generally designed to detect small compounds, such as narcotics or hormones. It would be advantageous to adapt present

aggregation assays to use currently available optical detectors to detect aggregation on a very small scale. Further utility would be added if the detection system is sufficiently flexible to be used for aggregation assays that could detect small compounds, large compounds and bacterial or other microbial cells.

It is an initial object of the invention to provide an agglutination assay for the detection of antigens and the screening of compounds that is able to detect small amounts of compounds in a homogenous assay. The assay should be adaptable both for diagnostic tests as well as for the screening of a library of compounds to determine the interaction of the compounds with a binding agent. It is a further object that the test be performed in microvolume reactions. It is a further object that the present assay be adaptable for use on presently available optical detection systems. It is a final object of the invention that the assay be able to detect both small and large compounds as well as bacterial cells in an aggregation assay.

SUMMARY OF THE INVENTION

The above objects are achieved through an aggregation assay which uses an optical system that can optically distinguish labeled macroparticles, preferably in a homogenous assay. This assay requires an optical system that may discriminate the macroparticles by detection of an associated optically detectable label.

In one embodiment of the assay, at least one microparticle and one binding agent are provided in an assay mixture. The binding agent may be present on the microparticle or could be a reagent free in solution. Either the microparticle or the binding agent or the antigen of interest is labeled with an optically detectable label. If the antigen of interest is present, the

antigen will compete for the binding sites on the binding agent. The assay mixture is subsequently optically interrogated. In one embodiment, this is effected by placing the assay mixture into a static chamber which is scanned in a limited depth of field. The optical scanner detects the optically detectable label and/or has a particle size gating function that detects the size of particles. The degree of formation of macroparticles or signal intensity in the presence of the test sample compared to a control with no antigen is an indication of the amount of antigen present in the sample.

In one embodiment, a microparticle having an associated binding agent, and an antigen labeled with an optically detectable agent are combined in an assay mixture with a test sample. The microparticle binding agent binds to an epitope of the target antigen (usually a large molecule) conjugate. Additional microparticles bind to other epitopes on the antigen. This linking of microparticles produces agglutination and formation of macroparticles. Macroparticles may then be detected by an optical system that detects the enhanced signal from the localized concentration of optically detectable label present on macroparticles. If the target antigen is present in the test sample, macroparticles will be formed similarly; however signal will vary depending on the concentration of the target antigen in the test sample. Additionally, the optical detection system may distinguish particles of the macroparticle size from microparticle.

Small antigens can be detected in a similar manner. Generally, small antigens are coated on optically detectable particles. A binding agent that binds the antigen is included in the assay mixture, either in solution or coated onto a particle in suspension. Agglutination will occur if a sufficient amount of free

antigen is present in the assay mixture. Several variations to this protocol are possible. Optical detection is important; at least one reagent of the assay should be optically detectable.

In one embodiment, multiple assays may be performed in a single assay mixture and analyzed in a single scan. The optical signal from each assay would provide a signal which the optical detection system could use to distinguish the assay results. The unique signal could be the emission wavelength of an associated dye, macroparticle size or shape or other feature detectable by the optical system. The ability to perform multiple assay in a single assay mixture could also be adapted for analysis of interaction of multiple epitopes of a large molecule to be analyzed in a single assay.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A is a representational illustration of the combination of a microparticle bead conjugated to binding agents and a detectable label. This combination causes aggregation resulting in the formation of a macroparticle when free antigen is added to the assay mixture.

Fig. 1B is a representational illustration of the combination of a microparticle bead having an associated antigen with a binding agent having two binding sites. The binding agent has an associated detectable label. This combination causes aggregation.

Fig. 1C is a representational illustration of the combination of Fig. 1B with the assay mixture that further includes analyte free in solution. In this combination, the analyte competes for the binding sites on the binding agent, inhibiting aggregation.

Fig. 1D is a representational illustration of rosetting.

Fig. 1E is a representational illustration of macroparticle formation in an assay using antibodies as a binding agent.

Fig. 1F is the illustration of Fig. 1E in which free antigen reduces the macroparticle signal.

Fig. 2A is a representational illustration of bacterial cell aggregation.

Fig. 2B is the representation of the assay of Fig. 2A in which aggregates did not form.

Fig. 3 illustrates a schematic of an optical system that could be used in conjunction with the present invention.

Fig. 4 illustrates a representation of a scan of an assay mixture containing microparticles and macroparticles and the corresponding detection signals.

Figs. 5A-5F are dot plots showing the size and intensity of detection events in a streptavidin-biotin binding system measured on the IMAGN 2000™.

Fig. 6 is a dot plot of the flow cytometry measuring the fluorescence intensity and light scattering in an assay mixture containing no Streptavidin Blue bead.

Figs. 7A-7D are dot plots of the flow cytometry analysis of the assay mixtures described in relation to Table 3.

Figs. 8A-8D are dot plots of the IMAGN 2000 analysis of the assay mixtures described in relation to Table 3.

Figs. 9A-9E are dot plots of the IMAGN 2000 analysis of the assay mixtures described in relation to Table 5.

Figs. 10A-10F are dot plots of the IMAGN 2000 analysis of the assay mixtures described in relation to Table 7. In the present assay the formation of individual macroparticles is detected. Each macroparticle is

composed of two or more smaller particles linked by a binding agent.

BEST MODE FOR CARRYING OUT THE INVENTION

Detection is effected by an optical detection system that preferably detects both the size of the macroparticle and the strength of a detectable label associated with the macroparticles.

For the present invention, a microparticle is defined as a single particle, cell, or compound.

A macroparticle is defined as an aggregation of a plurality of microparticles. A macroparticle produces an optical signal intensity greater than the signal produced by a microparticle. The size of the macroparticle will generally be between 0.01 μm and 50 μm.

The macroparticles are sufficiently dilute and sufficiently small that an optical detection system may detect the associated signal from a macroparticle as single, discrete detection events. In addition, the macroparticle must provide a sufficiently strong optical signal to distinguish the macroparticle from both microparticles and any optical background.

In one embodiment the macroparticle is detected by optical scanning systems which scan an assay mixture in a limited depth of field. The width of the macroparticle is preferably between 0.5 and 10 times the width of the beam spot. It is also preferred that the optical scanning system recognize a difference in signal profile between a microparticle optical signal event (if detectable by the system) and the macroparticle optical signal event.

In addition, the signal intensity from the detected macroparticle may be used to detect whether a labeled compound or competitive binding compound is present in the assay mixture. In addition multiple assays

may be performed in a single assay mixture. Each assay could include a different optically detectable label associated with each assay macroparticle. The optical detection system could be used to distinguish two or more optically detectable labels, each associated with a specific assay. Alternatively the macroparticle could be distinguished by size or other means.

In the present invention, the binding of a binding agent to a binding site produces the macroparticle. Binding sites may include a surface bound antigen, a surface bound receptor, a surface bound antibody, or any other class of compound which may serve as a site for a binding event. In addition, an enzyme and associated substrate could serve as the binding agent/binding site pair. Binding sites may be on the surface of a bead or cell collectively comprising a microparticle. Alternatively, receptor or antibody having multiple binding domains could act as binding sites.

Binding agents are any compound or complex which binds to a binding site to enable aggregation. The binding agent may be used as a reagent free in solution, or attached to the surface of a cell or bead. A binding agent may be multivalent, i.e. able to bind at least two sites, allowing linkages. Examples of binding agents include antibodies, enzymes, receptors, receptor domains, etc.

I. Assays

The assay of the present invention is illustrated in the representational diagrams of Fig. 1. In Fig. 1A a representational diagram illustrates a first method of detection of the presence of an antigen through the use of a bead and an associated binding agent. Bead 12 with a binding agent 16 covalently bound to the bead surface is provided. The binding agent is specific for

antigen 18. Also associated with the bead is a detectable labeling agent 14. This coated bead comprises microparticle 20. When the microparticle 20 is combined with antigen 18 in an assay mixture, the antigen 18 binds to the binding sites. The antigen has multiple copies of one epitope that binds to the binding sites. The antigen may be bound to multiple binding sites, with each binding site binding one of the antigen's epitopes. Multiple microparticles aggregate as the binding agents on each microparticle bind to one of the repeated epitopes on the antigen, linking the microparticles together. The aggregation produces a macroparticle 30.

The macroparticle may be detected by scanning the assay mixture to detect a localized concentration of signal from the detectable label associated with the presence of a macroparticle 30. In addition, the optical system could sort the particles by size to distinguish particles of the size of a macroparticle from the smaller sized microparticles. In this assay a macroparticle will form only in the presence of antigen. Thus the detection of macroparticles, the intensity of the signal from the associated optical labels detected from macroparticles, and the size (indicated by signal profile) of macroparticles are all indications of the presence of the antigen in the assay mixture. These combined measured signals (collectively the macroparticle signal) may increase as the amount of antigen in the assay mixture increases, depending on assay design. The macroparticle signal measured from assay mixtures containing various known concentrations of the antigen may be compared to the signal measured from an assay mixture containing an unknown concentration of the antigen. This comparison determines the unknown antigen concentration.

In this embodiment of the assay, the antigen must have two binding sites for aggregation to occur.

This assay is suitable for detection of large molecules and cells. Two binding reactions are required for the aggregation of microparticles, giving the assay high specificity. A single, non-specific binding event would be insufficient to create aggregated macroparticles. In this respect, this embodiment of the assay is similar to the sandwich type immunoassays.

Fig. 1B is a representational illustration of an alternative assay. In this assay, a bead 12 is coated (e.g. by covalent bonding) with an antigen 18. The bead and associated antigen comprise a microparticle 20. Microparticle 20 is combined in an assay mixture with a soluble binding agent 16 conjugated to a detectable label 14. The binding agent 16 binds to antigen 18 attached to the surface of the bead. The binding agents 16 have multiple attachment sites, allowing the binding agent to bind to the antigen associated with multiple microparticles. This results in the formation of macroparticle 30 comprised of multiple antigen coated beads linked by the binding agents. The presence of a macroparticle will correspond to a localized concentration of the detectable label associated with the binding agent. The macroparticle may be detected by detection of a localized concentration of the detectable label. If the free antigen is present in the sample, then it will bind to binding agent and fewer macroparticle aggregates will be formed.

The assay of Fig. 1B may be used for the detection of an antigen in an assay mixture. This is illustrated in Fig. 1C. In this assay, microparticle 20 comprised of a bead 12 with an associated surface bound antigen 18 is combined with the binding agent 16 having an associated detectable label 14. In addition, the assay mixture also contains antigen 18 free in solution. In this assay, some of the antigen that is free in solution will bind to binding agent 16. The binding agents

that are bound to the antigen that is free in solution will not be available to bind to particle associated antigen. When less binding agent is available to bind particles, aggregation of the particles will be inhibited. When aggregation is inhibited, there will be reduced detection of localized concentration of the detectable binding agents. The detectable label associated with the binding agent that is free in solution will be detected as part of the optical background. In the assay embodiment depicted in Figs. 1B and 1C, when the target antigen is not present as a soluble compound, the formation of macroparticles, as indicated by the macroparticle signal, will be greatest. The macroparticle signal will decrease as additional soluble antigen is added to the assay mixture. In this way, the amount of an antigen in a test sample may be determined by detecting the inhibition of the macroparticle formation and attendant macroparticle signal.

In the assay depicted in Fig. 1A, aggregation occurs when the antigen was bound by two binding agents. In contrast, in the assay depicted in Figs. 1B and 1C aggregation occurs when the antigen associated to each bead is bound by the binding agent at a single binding site.

In another embodiment, the presence of a binding agent may be detected through "rosetting". In Fig. 1D, fluorescent beads 32 have a surface bound antigen 34. When an excess concentration of the beads are combined with a binding agent 36, the binding sites 38 bind the surface antigen 34. The resulting "rosette" macroparticle 39 may again be detected by size gating and detection of the increased optical signal. In this assay the limited number of binding sites on the binding agent limits the total size of the final aggregated macroparticle. In example 1D a tetrameric binding agent 36 has

four binding sites 38 and may bind four beads 32. This assay may be adapted for screening liquid samples to determine if a soluble target compound is present in the sample. If the antigen is present in solution, it would bind to the binding agent. Fewer beads would bind to the binding agent, inhibiting the rosette formation. Subsequent analysis of the assay mixture would detect fewer rosettes with reduced signal intensity.

The assay could also be modified for use as a competitive binding assay. In such an assay, a screened compound from a compound library would be combined with the assay mixture. If the compound has an affinity for the binding sites, the macroparticle or rosette formation would be inhibited if the screened compound binds to the binding agent. If the screened compound did not have an affinity for the binding agent, no inhibition of rosette formation would occur.

The present invention may use an antibody as a binding agent. Antibodies are commercially available, may have highly specific binding, high binding constants and may be conjugated to dyes and surface associated functional groups.

In Fig. 1E, a fluorescent bead 40 has a surface associated antigen 42. When bead 40 is combined with antibody 44, antibody binding domain 46 may bind to antigen 42. If antibody 44 has binding regions that may bind to two separate antigens, one antibody may serve as a link between beads, causing formation of macroparticles. Macroparticle 48 may then be detected, again by size gating and optical label intensity detection. Fig. 1F depicts the same assay with free analyte 49 present in the assay mixture. In this assay macroparticle 48 signal intensity and size would be diminished because fewer beads would be incorporated into the macroparticles.

Instead, the antibodies would bind to the soluble analyte.

The assays of Figs. 1A-1F are useful in screening samples to determine the amount of antigen present in the sample. The antigen to be detected may be a narcotic, a pharmaceutical agent, a hormone, a protein or glycoprotein or cells (e.g. microbes).

In addition to detection of specific compounds, this assay may be used to screen a compound library to determine if the component compounds in the library bind to the binding agent used in the assay. This screening may be used to determine if the compounds have an affinity for the binding agents. If the amount of the compound from the library is known, detection of macroparticle formation and associated optical signal may serve as an indication of the strength of the affinity of the screened compound for the binding agent. Any of the assays illustrated may be used either to determine the amount of an antigen in an assay mixture or to screen a library of compounds to determine binding activity. U.S. Pat. No. 5,876,946, hereby expressly incorporated by reference herein describes techniques useful for screening chemical libraries.

In the assays of Figs. 1A-1F, a number of different beads or other microparticle supports may be used. Latex beads between the size of 0.01 μm and 10 μm are suitable. The preferred size is between 0.05 to 2 μm, and most preferably is 0.2 μm in size. Both functionalized beads (beads with functional groups such as amino, hydroxyl, carboxyl, or halo groups bound to the surface of the bead) and non-functionalized beads are adaptable to the present assay as long as appropriate methods for conjugating fluorescent dye, analyte and/or binding agents to beads are developed. Fluorescent beads are currently available from various commercial sources

such as Spherotech (Libertyville, Ill.), Bangs Laboratories (Fishers, IN); Molecular Probes (Eugene, OR). Suitable beads include latex polystyrene beads, either uncoated or coated with avidin having 10^6 binding sites per bead. In addition to latex beads, suitable beads may be made of cellulose, silica, glass, grafted co-polymers, polyacrylamide, dimethyacrylamide, or other suitable materials. Beads of various sizes labeled with a fluorescent or other optically detectable dye are available from Molecular Probes (Eugene, Oregon) and Spherotech (Libertyville, IL). These beads are available either with or without associated functional groups. Additionally these beads are available coated with biotin, streptavidin, NeutraLite avidin and Protein A.

In one embodiment of the assay the bead was coated with a receptor or antibody that functions as the binding agent. Alternatively, the receptor or antibody may be free in the assay mixture and function as binding agents that attach to binding conjugates (binding sites) present on the surface of a bead. Numerous binding agents are adaptable to these assays. Such binding agents include nucleic acids (e.g. for hybridization assays), antibodies, DNA binding proteins, receptor proteins or receptor binding domains, avidin/biotin and other binding proteins or binding agents. These binding agents preferably have functional groups that allow the covalent linkage of the binding agent to the surface of a solid substrate. Alternatively the binding agent may be expressed on the surface of a cell.

An optically detectable labeling agent is also required for the present assay. The macroparticle signal is detected as a localized concentration of the optically detectable label. The optically detectable label may be present on the bead (as in Fig. 1A, 1D), on a binding agent in solution (as in Figs. 1B-1C), or on the antigen.

The optically detectable label, such as a dye, may be attached to a bead. The dye may be tagged onto the bead during manufacturing or bound to the bead by a surface reaction. For example, beads functionalized with an amino or carboxyl group can be conjugated to variety of dyes such as cyanine dyes (e.g. Cy5, and Cy5.5), APC; (Becton Dickinson BioSciences, San Jose, CA), fluorescein, Texas red, rhodamine, FITC, PE, and numerous other dyes. Conjugation to a functional group, such as amino or carboxyl group, may be used to attach a dye to the binding agent (such as an antibody) or to the antigen. If the optically detectable label is a fluorescent dye, it may be preferred for the dye to have an excitation and emission wavelength above 590. Various substances present in biological samples may produce autofluorescence when excited by wavelengths below 590 nm. The use of a dye that has an excitation and emission wavelength above 590 nm, such as Cy5, Cy5.5, APC, Nile Blue or FD&C Blue #1, is preferred for the assay of blood samples. Components of blood (e.g. hemoglobin) autofluoresce when excited by light below 590 nm. Methods for limiting autofluorescence are further described in U.S. Pat. No. 5,585,246, hereby expressly incorporated by reference herein. Alternatively, the optically detectable compound may be chemiluminescent.

Bacteria or other microbes may be detected using the aggregation assay of the present invention. A bacterial cell assay would be similar to the assays in Figs. 1A-1F. The average size of bacteria is 0.2 μ, roughly the same size as the preferred particulate substrate. A typical microbial cell has numerous binding sites on the cell surface. An assay could be developed in which the binding agent binds to binding sites on the microbial cell surface. As in the previous, the binding agent or other reaction reagent is labeled with an opti-

cally detectable label. This agent could be combined in an assay mixture with a liquid sample that may contain microbes. If microbes are present, the bacteria would bind to the binding agent and aggregate, forming detectable macroparticles. If no bacteria are present, the unbound binding agent would remain as a background optical signal. In an alternative embodiment of the assay, the binding agent may be attached to a fluorescent bead. This bead may be from 0.05 μ to 10 μ in diameter, more preferably 2 μ. As in the previous references, the aggregated macroparticles could be gated by particle size and signal intensity.

In reference to Fig. 2A, bacteria 13 are shown having surface binding sites 15. When combined with a binding agent 17, the binding agent 17 will bind to the binding site 15 on the bacteria 13. Each binding agent 17 has multiple binding domains which may bind to multiple binding sites 15. If a number of cells are joined, an aggregated macroparticle 25 forms. The macroparticle is detected by size and/or signal intensity from the localized concentration of the optically detectable label 19 associated with the binding agent.

In contrast, Fig. 2B illustrates the assay in which no or very few bacteria are present. In this assay mixture, the bacteria 13 having binding sites 15 bind to binding agent 17 at binding site 15. Because few bacterial cells are present, additional binding agent domains 21 will attach to the binding sites 15 on bacteria 13. As a result, multiple microbial cells do not aggregate, and no microparticle forms. If no macroparticle signal is detected, microbial cells are not present.

The binding agent may, for example, be an antibody with a conjugated fluorescent label. The technology is adaptable for detecting large classes of microbes in a sample. For example, an antibody that binds to

peptidoglycan could be used as a universal bacterial binding agent. Peptidoglycan is a structural protein found in nearly all true bacteria. This antibody is further described in U.S. Pat. No. 4,596,769, hereby expressly incorporated by reference herein. The use of this antibody allows broad spectrum bacterial detection. A more specific assay could also be developed using a binding agent specific to only certain species of bacteria.

The binding agent may be used as a reagent in solution in an assay mixture or may be conjugated to fluorescent bead. As in other applications, beads 0.05 μ to 10 μ may be used, with 0.2 μ beads preferred. The use of beads provides a larger macroparticle, which may be preferable in some applications or with certain optical detection systems.

The same technique may be applied for the detection of viruses, viroids, and virus-like organisms. Viruses are generally much smaller than bacteria (e.g. Tobacco mosaic virus size: 3000 Å long x 180 Å diameter; mass = 40,000 Kd). Detection of these organisms would generally require a bead or other particulate substrate to act as a solid support for the binding agent to which the viruses bind.

In the prior art, agglutination assays required relatively high amounts of reagents, assay volumes, and particle counts. These assays were typically analyzed by turbidity or light scattering measurement or by direct visual examination. This generally requires analysis of at least milliliter quantity of assay mixture containing high concentrations of beads or cells.

In contrast, the present invention, through sensitive optical detection of individual macroparticles, enables sensitive assay of aggregation using microliter quantity assay volumes and using bead concentrations

which are at least 1,000 times more dilute than concentrations used in standard turbidity agglutination.

The sensitivity of the present method may be adjusted to modify the assay for a selected application. By limiting the concentration of binding agent and/or ligand (antigen), (e.g. concentration free in solution, concentration on beads or cells, etc.) sensitive assays could be developed. Optical detection using fluorescence and chemiluminescence increases sensitivity several fold over the usual turbidity measurement of agglutination.

Conversely, increasing ligand intensity on a fluorescent bead will decrease the sensitivity of an aggregation assay. Optimization of concentrations of binding and ligand reagents are important to modify the assay sensitivity. Such modification allows a "tuneable" sensitivity whereby the sensitivity of the assay may be modified to fit a selected application.

II. Description of An Optical Detection System

As noted in the description of the assay, the optical detection system must be able to detect and enumerate macroparticles by the detection of macroparticle size and measurement of an optical label associated with the macroparticle. One type of suitable optical detection system confines the detection to a limited depth of field within an analytical container. The macroparticles will settle within the limited depth of field and be scanned by a focused light source. The optical detection system has a light source that is selected to provide light of a wavelength matched to the excitation or activation wavelength required for the optically detectable label. In addition, the system has a detector that collects light of the wavelength produced by the optically detectable label. This system also measures signal profile, which indicates macroparticle size. Various opti-

cal detection systems are compatible with these criteria. One such system is the IMAGN 2000 sold by Biometric Imaging (Mountain View, CA).

In Fig. 3, an optical system for limiting the depth of field is illustrated. In this system a laser 10 directs laser light 80 onto a beam splitter 14. The beam splitter is configured to direct light of the wavelength of laser light 80 onto objective lens 19. Lens 19 focuses laser light 80 into a Gaussian waist 33. The Gaussian waist 33 intersects the container 40 at the beam waist having a width w. The area is thus provided with the highest energy illumination.

The assay mixture is contained within container 40. The wavelength of laser light 80 is selected to excite fluorescence from the optically detectable label used in the assay. Excitation by laser light 80 produces emission light 83 from target fluorescent compounds in the assay mixture. This emitted light is gathered by lens 19, which functions as a wide angle light collector. The emission light falling on lens 19 from the illuminated area is collimated by lens 19. This collimated emission light 83 passes through beam splitter 14, which is selected to allow light of the wavelength of emission light 83 to be transmitted. The collimated light is directed by steering mirror 22 onto focus lens 23. Lens 23 focuses the light through the aperture in spatial filter 24 onto detector optics 31. Detector optics 31 may be configured to split the focused emission beam and have distinct spectral ranges detected by separate detectors.

The use of multiple detectors creates separate detection channels to detect light above and below a cutoff wavelength. This would allow detection of two or more dyes at the same time if each dye has a specific emission profile related to the selected wavelength.

By matching objective lens 19 to spatial filter 24, the depth of field is confined to depth d. The positioning and optical properties of objective lens 19 will determine the size and location of the Gaussian waist of the excitation beam 80. Outside of the waist location, the energy of the wavefront of the excitation light rapidly falls off. In addition, the position of lens 19 will determine the area from which light is collected. Excitation light which originates beyond the depth of field will be less likely to be collimated by lens 19. The size of the aperture of spatial filter 24 is matched to objective lens 19 to function as a stop for light originating outside of the selected depth, limiting the depth of field of detection to depth d.

The focused excitation beam is scanned by the optical detector in a raster line scan of a depth within the assay container. An optical oscillator scans the excitation beam in a first direction while keeping the waist in register within the analytical container. The container is incrementally moved to provide for movement of the beam in relation to the container in a second direction. This allows optical interrogation of a volume defined by the depth of field and the two dimensional scan area. Additional description of the elements of this system are found in U.S. Pat. No. 5,547,849 hereby expressly incorporated by reference herein.

The detector measures the emission light intensity, with signal capture recording measurements from overlapping beam spot widths. Each captured measurement is pressed and stored as a pixel within a plot. Intensity measurement over a baseline intensity (average light intensity measurement) are recognized as potential detection events. The pixels surrounding a detection event are stored. The pattern of pixels in a neighborhood surrounding a detection event are indicative of the size

of the particle detected. The intensity of the detection event indicates the amount of detectable label present at a scan location. This detection method is further described in U.S. Pat. No. 5,556,764 hereby expressly incorporated by reference herein.

The optical detection system shown in Fig. 3 focuses light into the bottom of a container, such as a microplate well. The use of this system orientation provides an advantageous analytical platform for screening a number of compounds for binding inhibition, binding to a substrate, or other screening assays. One such system for scanning analysis of microplates is fMAT™ available from Perkin Elmer, Foster City, CA. If the optical system analyzes a container deeper than the depth of field of detection, some macroparticle settling time would be required to allow the macroparticles to settle into the detection depth of field. A five to ten minute settling time should be sufficient to allow between 75 and 90% of the macroparticles to settle into the optical depth of field in a microplate well.

Macroparticles could also be detected by a flow cytometry system such as FACScan (Becton Dickinson, San Jose, CA). This system passes a stream of the assay mixture past a focused light source. Particles in the stream scatter light which is detected by sensors. Emitted light may also be detected by sensors. The scattering measurement indicates particle size while fluorescent intensity indicates amount of optically detectable dye that is associated with the macroparticle.

Fig. 4 provides an illustration of the detection of microparticles and macroparticles in an assay mixture. In assay mixture 90, some fluorescent reagents 91 are in solution, not bound to any associated target. These unbound reagents produce a background optical signal 112. Also in the assay mixtures are a number of

microparticles 93 that will produce a microparticle signal 114 which may be detectable over the background signal 112. The microparticle signal is a localized concentration of the detectable label either bound to the bead or associated with the bead. The macroparticle 95 gives a macroparticle signal intensity 114 that is detectably greater than the microparticle signal intensity. This results from the macroparticle having a greater localized concentration of the detectable signal than the microparticle. In addition to signal intensity, the signal profile of the macroparticle detection signal 116 is detectably different than the signal profile of the microparticle detection signal 114. The signal intensity and two dimensional profile may be used alone or in combination to detect microparticles and macroparticles. Detection is most robust if the size of the macroparticle is at least 50% of the beam spot size (size of focused beam waist).

III. Examples

The preceding generalized description is further illustrated with the following examples.

Example A. Detection of digoxin using an aggregation assay

Digoxin is a small molecule pharmaceutical compound with a molecular weight of 781 used to treat heart conditions. Monitoring digoxin levels in a patient could be important in determining efficacy of cardiac treatment. Additionally, competitive binding assays containing soluble digoxin could serve as an initial indication of compound binding to cardiac receptors. This information would be useful in evaluation of a prospective cardiac therapeutic compound.

Aggregation assay of hapten or small molecule

1. Conjugation of digoxigenin to Bovine Serum Albumin:

Digoxin can be conjugated to BSA by following the periodate and other procedures (Butler, V.P. and Chen, J.P. Digoxin specific antibodies. PNAS 57:71-78 (1967); Tijssen, P. in PRACTICE AND THEORY OF ENZYME IMMUNOASSAY, pp. 279-296, 1985). Step-by-step procedure for preparing fluorescent Blue bead (Bangs Lab, Fishers, IN) digoxigenin conjugate is as follows:

First, digoxigenin (hapten) was conjugated to bovine serum albumin (BSA). Digoxigenin which has similar cross-reactivity to digoxin is used in place of digoxin. Digoxigenin can be functionalized with a carboxyl group (Brinkley, M. A brief survey of methods for preparing protein conjugates with dyes, haptens and cross-linking reagents. Bioconjugate Chemistry 3:1-13, (1992)). Once the digoxigenin hapten has been prepared containing the carboxyl group, it can now be conjugated to BSA as follows.

10 mg of digoxigenin hapten was placed in a 5 ml glass vial. 1.2 M excess each of N-hydroxysuccinimide (NHS) (3.2 mg) and 1-ethyl-3-(-dimethylamino-propyl)carbidiimide HCl (EDAC) (5.3 mg) were added to the glass vial containing the digoxigenin-COOH. The mixture was dried for 5 hours in vacuum in a desiccator. The vial was removed from the vacuum and opened under nitrogen. The vial was immediately capped with a rubber septum. 1 ml of HPLC grade dimethylformamide (DMF) was added to the vial through the septum using a syringe. The mixture was stirred overnight at 4°C, forming the activated digoxigenin hapten.

Activated digoxigenin hapten was added to BSA to prepare hapten BSA conjugate. BSA was dissolved in 50 mM Tris buffer, pH 8.0 at 10 mg/ml. Activated digoxigenin hapten was placed in a 1-ml glass syringe

suitable for using with a syringe pump. The syringe pump device was set up at room temperature and set at 1 ml/hour addition. For 1 mole of BSA, 85 mole of activated digoxigenin hapten was added to produce the conjugate as follows: One ml BSA solution was placed on a magnetic stirrer to stir the solution. Activated digoxigenin hapten was added to the BSA solution at the 1 ml/hour rate specified above. This mixture was continuously stirred while the activated digoxigenin hapten was added. Once all of the activated digoxigenin was added, the mixture was stirred for an additional hour.

Following the combination of BSA to the activated digoxigenin hapten, the conjugate mixture was dialyzed over running tap water for 1 week, followed by purification on Sephadex G50 column in 25 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 6.1. The protein in the conjugate was determined by Pierce BCA protein assay (P/N 23225, Pierce, Rockford, IL). The amount of digoxigenin conjugated to BSA was determined by differential absorption spectrophotometry.

2. Conjugation of BSA-digoxigenin to Fluorescent Blue Bead

Fluorescent beads were used in this experiment. These beads are available with various types of functional group on them such as amino (NH_2) group (P/N 2600, size 0.06 µ), carboxyl (-COOH) group (P/N 4169, size 0.19 µ) from Bangs Lab (Fishers, IN). Similar beads are available from other commercial suppliers [e.g. Sky Blue bead from Spherotech, Libertyville, IL].

The instant assay used -COOH Blue bead (0.19 µ) and covalently attached to the -NH_2 group on BSA of BSA-digoxigenin conjugate. Adsorption of the BSA digoxigenin conjugate protein precedes the covalent coupling. Therefore, a protein binding isotherm must be first estab-

lished to determine the appropriate concentration of BSA-digoxigenin to be conjugated to the -COOH Blue bead. A 100 µg protein (BSA-digoxigenin)/mg bead is preferred.

Set up the binding reaction by pipetting the following in a tube:

50 µL of 500 mM MES stock buffer (MES=(2-(N-morpholino)ethan sulfonic acid, pH 6.1): 25 mM final concentration.

100 µL of 1% solid stock bead particles: (0.1% solids final volume)

1 mg BSA-Digoxigenin based on 280 µm protein determination (≃ 15.4 µM)

Water to make 1 ml final volume

These compounds are mixed in the tube for 30 minutes on a mixing wheel at room temperature.

Calculate the EDAC amount and use it in 2.5-fold molar excess over the BSA carboxyl group (0.011 mg in this example).

The EDAC solution is prepared just before use and the calculated amount is mixed rapidly into the reaction by mixing repeatedly with the pipettor.

Following addition of EDAC, the tube is mixed at room temperature on a mixing wheel for 1 hour. After the mixing, the unbound protein is removed by 5 times centrifuging the tube at 20,000 g for 20 min and decanting the supernatant. The pellets are resuspended to 0.1% solids in the 25 mM MES buffer by probe ultrasonication.

Finally the BCA protein assay is run to determine the protein conjugated to the bead. The reading of the supernatant is made after centrifuging the protein assay tubes to remove particles (Ref: Micro reagent Optimization Manual; Seradyne, Indianapolis, IN).

3. Preparation of Digoxigen Standard

A digoxin stock solution is prepared at 1 mg/ml in DMF. Dilute in 50 mM -[2-hydroxyethylpiperazine-N'-[2-ethanesulfonic acid] or HEPES, pH 7.2 to give final concentration in ng/ml: 0, 1, 2, 3, 5, 10.

4. Preparation of antibody reagent

Digoxigenin specific antibody can be produced by using methods known to those of skill in the art. Many antibodies are commercially available. Prepare appropriate antibody dilution in borate buffer.

5. Assay protocol

Both bead and antibody reagents are optimized to give maximum signal in assay containing no test sample. Optimization protocols are similar to those used in example E. The zero sample will give maximum relative cell intensity (RCI). The RCI will decrease proportionately with increase of sample in the reaction mixture. A representative protocol is given below:

7 tubes containing the assay mixture are assembled: six for the digoxin standards at 0, 1, 2, 3, 5, and 10 µg/ml respectively, and one tube for the unknown sample. 20 µL of standard or sample is added to each tube. 100 µL of Bead reagent is added to each tube. Bead reagent and antibody reagent are added at an optimized level as determined by the protocol mentioned in Example E. The final volume in each tube was made to 1 ml with borate buffer. The tubes are vortexed and subsequently incubated for 1 h. 50 µL of the solution from each tube is then added into a VC120 capillary sold by Biometric Imaging (Mountain View, CA). The sample is then analyzed on an IMAGN 2000™. The results may be used to plot RCI vs. digoxin concentration for the 6 (six) digoxin standards. The concentration in the unknown test

sample can then be determined from the graph by correlating the intensity from the test sample to a position on the graph.

Example B. Detection of Alpha-fetoprotein in an aggregation assay

Alpha-fetoprotein is a large molecule (MW=70,000) glycoprotein used as a marker for prenatal detection of fetal anomalies and certain types of cancer in non-pregnant patients. The carbohydrate portion of the molecule has a vicinal -OH group which can be converted to aldehyde group by periodate oxidation. This aldehyde group will react with an amino group to form Schiff's base. Therefore, for this application, bead with amino functional group is used. The alpha-fetoprotein is conjugated to an amino functionalized fluorescent bead via the aldehyde group in methods similar to those used in example A.

It is not necessary to use only particles having carboxyl or amino groups. The alpha-fetoprotein could be adsorbed directly on to the particle surface. However, the scheme described above is preferred to adsorption because covalent bonding produces a more robust conjugate compared to adsorption. Protocol and optimization remain the same as given in example E.

Depending on the use, either a quantitative or threshold determining assay can be developed. Alfa-fetoprotein (AFP) levels greater than 1000 µg/L may be indicative of some types of cancer (except during pregnancy). For a threshold determining assay, one standardized level of AFP would be run with samples and the RCI of the standard or test sample compared. For quantitative assays, a standard curve can be prepared as discussed for digoxin. Procedures for reagent preparation and optimization remain the same as discussed for

streptavidin in example E. Many variations of this technology are possible as shown in Figs. 1A-1F.

Example C. Avidin/Biotin Macroparticle Formation

In a third example, biotin is used as a binding agent to cause aggregation of avidin coated particles. Biotin is a 244 dalton molecule found in blood and tissue. It has a very high affinity for avidin/streptavidin. Avidin is a tetrameric glycoprotein with a molecular weight of approximately 66,000. It is primarily derived from egg whites. Like avidin, streptavidin is a tetrameric protein with similar molecular weight and binding characteristics. Streptavidin is isolated from *Streptomyces avidini*. Streptavidin has advantages in certain applications in comparison to avidin because avidin has differing protein glycosylation. Avidin may be subject to non-specific binding in certain application because of its associated carbohydrates.

In the present example, avidin-biotin binding is used in the agglutination of beads. This agglutination is subsequently detected on an optical detection system in a limited depth of field. In this assay, a conjugate of streptavidin-Cy5 is utilized. Biotin coated beads (biotin-bead 6 μ in size) are used. These beads are non-fluorescent alone, but become fluorescent after streptavidin-Cy5 binds to biotin, which is covalently attached to the bead. As noted earlier, such an assay alternatively could include fluorescent labeled bead instead of a fluorescent reagent.

The optical system used to analyze the assay mixture was the IMAGN 2000™ (Biometric Imaging, Mountain View, CA). This system has an optical configuration similar to that shown in Fig. 3. A laser produces a beam which is focused by a lens into a container. The focused light excites fluorescence which returns in a retrobeam

to a detector. The focusing lens also acts as a light collector. A spatial filter, acting in combination with the light collector, limits the depth of field from which light is collected. In general, this limited depth of field is from 25 to 250 microns. The IMAGN 2000 system scans from the top down into a container, an opposite optical configuration of that shown in Fig. 3. This system is generally used to scan rectangular capillaries having a limited fluid depth. Thus the capillary depth may structurally limit the scanned depth and act in conjunction with the optical system to limit the depth of field. As noted in relation to Fig. 4, such a system is able to detect discrete events which are measured by detection of intensity of the retrobeam over a selected threshold. The scanner analyzes a specific volume of fluid defined by the scan width and length and the optical depth of field. The number of detection events may be tabulated as detection events per unit volume (e.g. cells/μL, beads/μL or macroparticles/μL). The optical system detects the relative cell intensity (RCI) which is a measurement of the average intensity of detection events. The peaks are detected from macroparticles, cells, or beads. In addition the system measures background limiting intensity (BLI), which is a measurement of the average intensity of the background wavelength. By scanning in a limited depth of field, an assay mixture may be analyzed without removing unbound optically labeled reagents. The relatively high signal from targets with higher localized concentration of associated optical dye allows detection in the presence of unbound dye. This enables homogenous assays, i.e. assays that do not require a separation step. In addition to RCI, the system also detects emission signal profile, allowing size gating. This optical analytical system may be used to analyze detection assay mixtures for detecting small

molecules, large molecules, insoluble antigens, and a variety of cells. It is anticipated that any optical system which provides detection in a limited depth of field and is capable of measuring optical signals may be adaptable to the present assays.

In this example, amino beads (Becton Dickinson Biosciences, San Jose, CA) were used. Biotin was then conjugated to the amino group on the bead (Stock 200,000 beads/µl). The biotin coated beads were diluted with borate buffer (100 mM borate buffer contains 0.1% bovine serum albumin, 0.05% Tween 20, 100 mM EDTA and 0.05% azide) to give a final bead number per unit volume (200-1000 beads per µl). Concentration of avidin/streptavidin that will give a useful dynamic range in the assay was determined experimentally as illustrated in the next example. All dilutions were made in borate buffer unless otherwise specified.

A number of assay mixtures were assembled, each containing a standard amount of the suspended biotin beads, streptavidin-Cy5 (FluoroLink™ Cy™5 Amersham Pharmacia Biotech, conjugated to streptavidin) in borate buffer to a total volume of 1 ml. A fixed aliquot of biotin beads were added initially to a 1 ml eppendorf tube. A varying amount of avidin was added to each tube, that was subsequently mixed by vortexing. Following vortexing the tubes were incubated. Following incubation, a fixed amount of streptavidin conjugate was added to each tube, vortexed and incubated. Borate buffer was added to each tube to achieve a final volume of 1 ml. The tube was vortexed to mix the contents of the tube. 50 µL of the mixture was loaded into each of two VC120 capillaries (Biometric Imaging, Mountain View, CA) and placed on a VC2 cartridge (Biometric Imaging, Mountain View, CA). The cartridge was loaded on the IMAGN™ 2000 instrument and analyzed using CD34 assay software (Bio-

metric Imaging, Mountain View, CA). Initial experiments indicated that signal intensity of produced macroparticles was very intense. To compensate the instrument photomultiplier tube (PMT), gain was reduced by 50%. Other gating parameters were unchanged from those specified for the CD34 assay.

Table 1 - Experiment Protocol

Cartridge Set	1	2	3	4	5	6
µL Avidin (5 mg/ml), µL	0	5	10	20	50	100
Borate buffer, pH 8.6, µL	100	95	90	80	50	0
µL Steptaviden -Cy5 conjugate, **	10	10	10	10	10	10

Mix contents by vortexing

** Steptaviden-Cy5 stock 1 mg/ml diluted to 1000x. 10 ng SA-Cy5 in 10 µL volume

Biotin Bead (20x dil.), µL	20	20	20	20	20	20

Mix contents by vortexing
Incubate at RT, 30 min, shaking, medium speed on American rotator.

Borate buffer, pH 8.6, µL	770	770	770	770	770	770
FD&C Blue #1, (1 mg/ml stock; 200x diluted) µL	100	100	100	100	100	100

Mix contents by vortexing
Load VC cartridge with VC120 capillary and read

Table 1 lists the assay mixture components for each of six cartridges analyzed. As noted, each cartridge contains two capillary tubes containing 50 µL of assay mixture. The streptavidin Cy5 conjugate is a 1 mg/ml streptavidin-Cy5 stock solution diluted 1000X in borate buffer.

Table 2 - Avidin-Biotin Macroparticle Formation Assay

STELLer CD34 Assay, Version CD34-3.07B
Gain0=84; Gain1=144 (50% less gain)

	Avidin µL added	Avidin µg/ml*	BLI A	RCI A
Cartridge 1	0	0	265	6879
Cartridge 2	5	25	272	668
Cartridge 3	10	50	272	486
Cartridge 4	20	100	283	312
Cartridge 5	50	250	288	259
Cartridge 6	100	500	289	179

* µg in 1 ml reaction mixture

The results of the analysis of the assay mixtures are listed in Table 2. In general, with increase in avidin concentration, a decrease in RCI is observed. The RCI provides a good correlation with the concentration of avidin at the current instrumentation parameters.

Dot plots at each concentration of avidin are shown in Figs. 5A-5F. Fig. 5A is a dot plot showing measurements of detection event intensity (MaMo) and size (FitArea O), measuring the assay mixture containing 0 µL avidin. In this reaction, streptavidin-Cy5 binds to the biotin bead without competition from free avidin in solution, resulting in macroparticle aggregates ranging in size up to index 4. This aggregate produces a very intense optical signal, indicated in the dot plot by the number of intensity events measured in the extreme right of the intensity scale (X-axis). Fig. 5B illustrates the measurements made of the assay mixture containing 5 µL (25 µg) of avidin. This graph illustrates that the detection events are significantly less intense than seen in Fig. 5A. This trend continues in Figs. 5C, 5D, 5E, and 5F. The reduction in signal intensity correlates to the amount of free avidin in solution. Addition of increasing amounts of avidin increases competition between labeled streptavidin and free avidin for binding to biotin on the biotin coated bead. When avidin binds to the bead, less labeled streptavidin may bind, reducing the detected signal. From these dot plots, it is apparent that this assay allows very sensitive detection of analyte in solution. The macroparticle size acts as a

second indicator of competition. Each of avidin and streptavidin has tetrameric binding sites and each promotes macroparticle formation. Since the concentration of streptavidin-Cy5 conjugate remains constant, the increasing concentration of avidin in the assay mixture participates in aggregation reaction and maintenance of the aggregate size. However, the aggregate moves to the left on the intensity scale with increasing avidin due to reduced label in the aggregate. This movement can be measured in RCI and is related to avidin concentration in the reaction mixture. This is observed in Figs. 5A-5F.

Example D. Avidin-biotin macroparticle formation with detection using flow cytometry

In the next example, avidin-biotin binding and the formation of macroparticles are analyzed using IMAGN 2000 system and compared to analysis using a flow cytometer. In the assay mixture assembly, two types of beads are used. One bead is coated with biotin. The second bead is conjugated to streptavidin and is optically detectable through an associated blue label impregnated into the bead.

Avidin-Biotin System: Flow vs. Imagn

Table 3 - Experimental Protocol

Tube No.	Biotin bead	SA Blue bead dil.	SA Blue bead	SA-APC	Buffer
1	25	-	-	-	475
2	25	1000X	68	-	475
3	25	100X	68	-	475
4	25	10X	68	-	475
5	25	-	-	20	475

* Tubes #2, 3, 4 have SA blue bead 360, 3600 & 36,000 beads per biotin bead, respectively.
* Biotin bead diluted 10X (Stock: 200,000 beads/µl).

Note:

a. All additions are µL.
b. Biotin bead are 6 µM; 200000 bead/µL.
c. SA Blue bead (Bangs) are 0.19 µM; 2.628+e9 beads/µL.
d. SA = Streptavidin

The assembly of the reaction mixtures is described in conjunction with Table 3. In this method the biotin beads as used in Example C were diluted 10X with borate buffer. 25 µL of diluted beads was added to each tube, as stated in Table 3. Streptavidin Blue bead was added as indicated in Table 3. The final volume in each tube was brought to 500 µL with borate buffer. The tubes were vortexed and incubated at room temperature for 15 minutes. The beads were subsequently washed two times by adding 2 ml borate buffer for each wash, and centrifuging the contents at 5000 RPM in a Sorvall RT6000 centrifuge for 5 minutes. Liquid in the tubes was decanted to remove unbound streptavidin Blue bead. The remaining beads at the bottom of the tubes were suspended in the 0.5 ml borate buffer and analyzed both on FACSCalibur™ (Becton Dickinson, San Jose, CA) and IMAGN 2000™ analysis system. The first reaction mixture does not contain any streptavidin Blue bead. The light scattering and fluorescent intensity measurements made on flow cytometry instrumentation are shown in Fig. 6. The detected Biotin beads are in group R1 with very low non-specific aggregates in group R2. These particles are of relatively small size and relatively low fluorescent intensity, and this set serves as control for tubes 2, 3 and 4 (Table 3).

The second assay mixture has 360 streptavidin Blue bead per biotin bead. In flow cytometry analysis of this assay mixture, macroparticles containing two, three and four biotin beads are seen as indicated by the scattering plot 7A and are indicated by squares R2, R3 and R4 respectively. The fluorescent intensity shows a similar grouping. In addition to the relatively low fluorescent intensity characteristic of group I (RI) positioned at 200 on the X axis of the fluorescent intensity graph, additional groups are seen at 400, 600, and near 800. These three additional intensity groups correspond to scattering groups R2, R3 and R4. The analysis of the second reaction mixture was also performed on the IMAGN

2000 the results of which are shown in Fig. 8A. The combined signal intensity and particle size are measured by this system. These results in terms of size index correspond well to the results determined by flow cytometry. The size index (signal fit area) ranges from 0.5 to 4 in IMAGN dot plot (Fig. 8A) that indicates scattering groups R2, R3 and R4 in flow scattergram (Fig. 7A).

The third assay mixture has 3600 streptavidin Blue beads per biotin bead. Again the results are shown for flow cytometry in Fig. 7B and for IMAGN 2000 in Fig. 8B. The flow cytometry results are similar to those for Fig. 7A. Macroparticles of four different sizes were detected. For the IMAGN 2000 detection, Fig. 8B corresponds to the flow cytometry results with the macroparticles detected having a signal fit area between 0.5 and 4.

The fourth assay mixture has 36,000 streptavidin Blue beads per biotin bead. At this high streptavidin Blue bead concentration, the flow cytometry plot shown in Fig. 7C indicates that groups R3 and R4 (detected larger macroparticle aggregates) have significantly diminished. This is likely due to the increased competition of the streptavidin beads for biotin bead binding. Fig. 8C illustrates detection on the IMAGN 2000 system. The results correlate well with flow cytometry, with particles detected ranging in size index (fit area) from 0.5 to 3 with less number of bigger particles present. Finally assay mixture 5 contains only streptavidin-APC, 6 μ beads. Analysis of this assay mixture by flow cytometry produces an intense measurement in the R1 group (indicating 6 μ size as Biotin bead in Fig. 6) as shown in Fig. 7D. This very intense reading was similarly measured in the IMAGN 2000 as shown in Fig. 8D.

The flow cytometry results correlate well with the IMAGN 2000 results. In both systems, the analysis shows both aggregation, and rosetting are occurring depending on the amount of streptavidin blue bead used in a

sample. In assay mixtures containing very high concentrations of streptavidin Blue bead, additional rosetting and less aggregation are seen in both flow cytometry measurement and IMAGN 2000 plots; as seen in comparison of assay mixture 2 (high aggregation as illustrated by large macroparticle formation) and assay mixture 4 (relatively low macroparticle size and decreasing intensity indicating rosetting).

Example E. Antibody/antigen system for macroparticle formation

In the next example, an antibody is used as the binding agent for the aggregation of particles.

Table 4. Aggregation Using Antibody/Antigen System Experiment Protocol for Streptavidin (SA) Blue bead and streptavidin antibody

Item description	Mixture 1	Mixture 2	Mixture 3	Mixture 4	Mixture 5
Rabbit anti-SA antibody	100	100	100	100	100
SA Blue bead	100	50	20	10	0
Borate buffer	0	50	80	90	100

Incubate overnight shaking at room temperature on American rotator.

Background dye (200Xdil)	100	100	100	100	100
Borate buffer	700	700	700	700	700

Mix and load VC120 capillary. Run STELLer CD34 assay.

SA = Streptavidin

Antibody was diluted 32X in borate buffer per manufacturer's recommendation.

SA Blue bead was diluted 1000X in borate buffer. Aliquots were added from this dilution as indicated above. The SA Blue bead is supplied as 1%. After 1000X dilution, its conc. will be 10 ng/μL.

Background dye is 1 mg/ml stock of FD&C#1 in water. All dilutions made from this stock.

Assay mixtures were assembled each containing a varying amount of the antigen coated beads. Table 4 describes the reagents combined to form each of the five assay mixtures. The streptavidin antigen and rabbit anti-streptavidin (anti-SA) antibody were obtained from Sigma (St. Louis, MO). The antibody was diluted 32X in borate buffer as per manufacturer's recommendation. Streptavidin Blue beads were diluted 1000X in borate buffer. The final concentration of the streptavidin Blue beads after 1000X dilution will be 10 ng/μL. The background dye supplied is 1 mg/ml stock of FD&C Blue #1 in water. All dilutions of background dye are made from this stock. The five assay mixtures assembled contain a fixed amount of the antibody reagent (100 μL) added into each assay mixture. Varying amounts of streptavidin Blue bead are added to each assay mixture. Borate buffer was added to bring the mixture volumes to a common amount. Following assembly, 50 μL of the assay mixture was added to VC120 capillaries and analyzed using the STELLer CD34 software (gain 0 = 67, gain 1 =124; normal noise and size gate) run on the IMAGN 2000™.

Aggregation Using Antibody/Antigen System

Table 5
Effect of varying concentration of streptavidin (SA) Blue particle on streptavidin antibody

Assay Mixture	SA bead, μL added	Beads/μL	BLI	RCI	IMAGN error messages
5	0	2.5	401	860	value outside range
4	10	18.5	384	988	-
3	20	73.5	394	1991	-
2	50	293	427	3819	-
1	100	274.7	427	3118	too many events

Table 5 and Figs. 9A-9E set up the results of this analysis. An assay mixture 1 using 100 microliters of streptavidin blue beads resulted in graph 9A in which the detection events were too numerous for the optical analytical system to distinguish as indicated by error message. In the second, third and fourth reaction mixtures, the relative cell intensity and background light

index are within the detectable ranges. Figs. 9B, 9C and 9D illustrate the dot plots of the cellular detection events. In assay mixture 5 (Fig. 9E), no streptavidin Blue beads were present and very few detection events were measured. With respect to Table 5, increasing concentration of streptavidin Blue bead and the reaction mixture corresponds with increasing amounts of Blue bead which are being bound to the anti-streptavidin antibody. The numbers of beads/μL and relative cell intensity are decreasing with decreasing addition of SA-Blue bead to the reaction mixture as shown in plots 9A-9E. The background light intensity remains similar at all concentrations as expected. With no beads present in the reaction mixture, fluorescence from beads will not be generated and the instrument will detect no beads. With increasing concentrations of beads, the aggregation into macroparticles will occur and the instrument will detect aggregated macroparticles as events. These events will be enumerated and the intensity of the events measured. The minimum size index threshold for this experiment was set to 1.25. This setting gates out non-aggregated beads, targeting detection of only macroparticles.

The gating out of non-aggregated beads is shown in dot plots 9A-9E generated by the IMAGN 2000. In assay mixture 5 containing no streptavidin Blue beads, only the background noise is present as events below the selected size index (Fig. 9E). At 10 μL bead mixture used in assay mixture 4, the agglutination to form macroparticles is seen although most of the events have detection at relatively low intensity and the size of detection events is concentrated near the 1.25 gating threshold, with a few macroparticles ranging from 1.5 to 4, as shown in Fig. 9D. Fig. 9C illustrates the dot plot of the analysis of assay mixture 3 containing 20 μL streptavidin Blue bead. This plot shows many additional detection events above the 1.25 gating threshold with many additional detection events ranging from 1.5 to 4. This trend continues in the second and first assay mixtures, the detec-

tion results of which are illustrated in Figs. 9B, 9A respectively. It appears that in this assay macroparticles aggregate to a maximum size index of 4. With additional streptavidin Blue bead concentration, additional aggregated macroparticles are produced, with additional beads also correlating to higher macroparticle intensity. Excluding intensity measurement, the maximum number of large size macroparticles are formed when the bead concentration is between 20 and 50 µL in the assay mixture. In this initial assay, the optimized amount of streptavidin Blue bead concentration in a streptavidin detecting assay is determined. When excess Blue beads are used, the instrument is unable to detect discrete events resulting in error signal "too many events detected". This results from an excess of non-aggregating beads providing an optical signal in excess of the ability of the optical system to detect and process.

Table 6 - Experiment Protocol

Cartridge Set	1	2	3	4	5	6
*SA --> ng	0	125	250	500	1250	2500
Free SA (25 µg/ml)	0	5	10	20	50	100
Borate buffer	100	95	90	80	50	0
SA-Blue bead (1000x dil)	25	25	25	25	25	25
Rabbit anti-SA antibody	100	100	100	100	100	100

*ng added in 1 ml reaction mixture.

Incubate over the week end at 4C and then 1h shaking at RT

Borate buffer*	675	675	675	675	675	675
Background dye (200x dil)	100	100	100	100	100	100

Mix and load VC120 capillary. Run STELLer CD34 assay.

*After adding borate buffer, 0.4 ml was taken out and 40 µL of 200x diluted dye was added.

SA stock of 25 µg/ml used in this experiment

Background dye is 1 mg/ml stock of FD&C#1 in water. All dilutions made from this stock.
All additions are in µL.

The experiments described in conjunction with Table 4 and Table 5 provided a concentration of streptavidin Blue bead which would provide a sensitive assay for streptavidin detection via aggregation. Table 6 outlines the experimental protocol in assembling the assay mixture. As was determined in the previous set of experiments, 25 µL streptavidin Blue bead was prepared in borate buffer and used in the assay. A rabbit anti-streptavidin antibody was used with 100 µL added per reaction mixture. Amounts of free streptavidin varying from 0 µL of free streptavidin to 100 µL of free streptavidin was added to each assay mixture. Other remaining components of the reaction mixtures were identical to those in the previous assay mixture (Table 4). The noise gating in the STELLer CD34 software was set to 10 to avoid "too many events detected" error message on the IMAGN 2000 instruments.

In this group of assay mixtures, both free streptavidin and streptavidin Blue bead will bind to the anti-streptavidin antibody. Either binding agent will effect formation of macroparticles. If most of the antibody binding sites are occupied by free streptavidin, the relative cell intensity signal detected by the optical detection method will be weak. In contrast, if the majority of the sites are bound by streptavidin Blue beads, the macroparticles detected will have relatively high signal intensity.

Table 7

Effect of free streptavidin on aggregation: Antibody-Antigen system

SA, µL	BLI A	RCI A
0	293	3202
5	288	1103
10	274	879
20	319	802
50	273	828
100	283	516

Table 7 shows the data from an IMAGN 2000 analysis of the six assay mixtures. These results are also shown in Figs. 10A-10F. Data in Table 8 demonstrate that there is a linear relationship between the free streptavidin (between 20 µL to 100 µL streptavidin) and RCI in the assay mixture. The RCI decreases with increasing free streptavidin concentration. Fig. 10A shows that when no free streptavidin is included in the assay mixture, only streptavidin Blue beads bind to the anti-streptavidin antibody resulting in very high RCI of 3202. The assay mixtures containing 5 µL, 10 µL and 20 µL free streptavidin in the assay mixture, when analyzed, show macroparticle formation with increasing macroparticle size index and corresponding decrease in relative cell intensity. This is shown in Figs. 10B, 10C and 10D. In assay mixtures with 50 µL and 100 µL, free streptavidin in the assay mixture, the relative cell intensity has decreased significantly. This is shown in Figs. 10E, 10F. Because of the dim signal, some events may not be detected by the instrument. It is thus difficult to assess the size of the aggregated macroparticles in the presence of high concentration of free streptavidin. However, within the range in assay mixtures 1, 2, 3 and 4 sensitive detection of the free antigen through measurement of both size of macroparticle and intensity of macroparticle signal is achieved.

As the foregoing examples illustrate, the present assay provides a highly sensitive assay for the detection of haptens or large molecules. The assay can be used for detection of a target compound in solution, the assay of binding to a compound, or a competitive binding assay for screening. A number of different optical systems may be used to analyze the assay mixtures and detect macroparticle optical signals.

Claims

1. A method for the detection of individual aggregated macroparticles, the method comprised of the steps:

combining in an assay mixture a particulate substrate or cell, said particulate substrate or cell associated with an antigen binding site or binding agent, with an associated assay solution wherein either the binding agent, or ligand, or the particulate substrate or cell has an associated optically detectable label, wherein under binding conditions the particulate substrate or cell may aggregate to form a macroparticle;

directing a beam to optically interrogate the assay mixture, the interaction of the beam and the macroparticle producing a macroparticle optical signal event wherein the assay solution is scanned such that individual macroparticles are detected; and

detecting optical signal events wherein said macroparticle optical signal event is distinguishable from optically detectable label not associated with a macroparticle, thereby measuring the agglutination in the assay mixture.

2. The method of claim 1, wherein the step of directing a beam to optically interrogate the assay mixture is effected by,

adding the assay mixture to an analytical container;

scanning a focused beam spot in two dimensions through the assay mixture in said container;

measuring the light intensity emitted from a limited depth of field within the scanned depth; and

determining if localized light intensity measurement are above a background intensity.

3. The method of claim 2, wherein directing the beam spot includes directing a focused beam spot having a beam spot size selected so that the non-aggregated cells or particulate substrates have a width which is 0.5 to 10 times the width of the beam spot.

4. The method of claim 1, wherein the step of detecting optical signal events includes at least one step selected from the steps of detecting optical signal intensity and detecting optical signal profile.

5. The method of claim 1, wherein the step of contacting a particulate substrate or cell with a binding agent is effected by contacting a bead coated with surface receptors with a binding agent.

6. A system to measure aggregation, the system comprising:

an assay container;

an assay mixture contained within the assay container, the assay mixture including,

a) at least one microparticle,

b) at least one binding agent,

c) at least one optically detectable label,

d) at least one binding site wherein the microparticle is conjugated to one of the group consisting of the binding agent and the binding site such that under binding conditions microparticles aggregate to form macroparticles which settle to a specific depth of the container, said macroparticles at a concentration within the container such that the macroparticles are in a non-overlapping, detectably spaced orientation within the analytical container; and

an optical detection system configured to optically interrogate the container in a limited depth of field, the depth of field containing the macroparticles, said optical system distinguishing macroparticles from microparticles in a homogenous assay mixture.

7. The assay system of claim 6, wherein the container is a capillary.

8. The assay system of claim 6, wherein the microparticle is a bead.

9. The assay system of claim 8, wherein the bead is labeled with the optical label.

10. The assay system of claim 6, wherein the optically detectable label is selected from the group comprising a fluorescent dye and a chemiluminescent agent.

11. The assay system of claim 6, wherein the optical system is a laser scanning system.

12. The assay system of claim 6, wherein the binding site is present on an antigen.

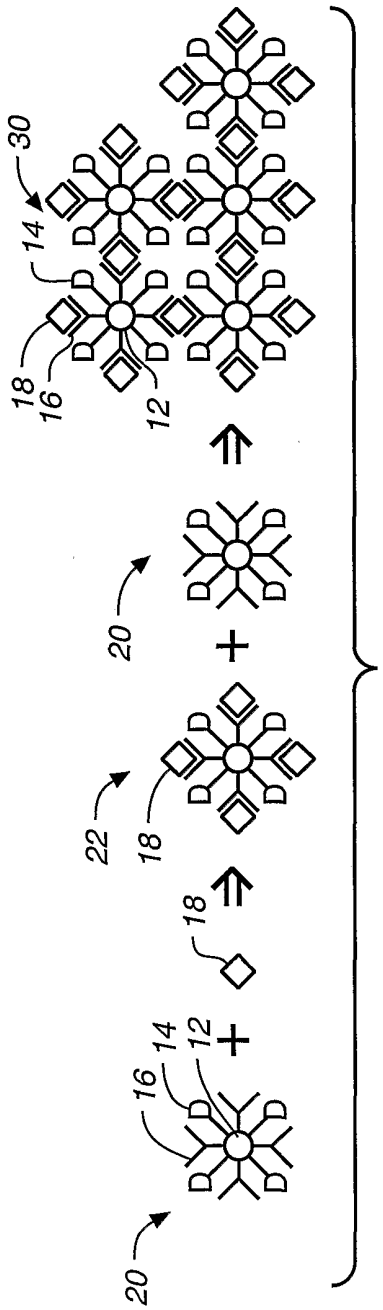


FIG._1A

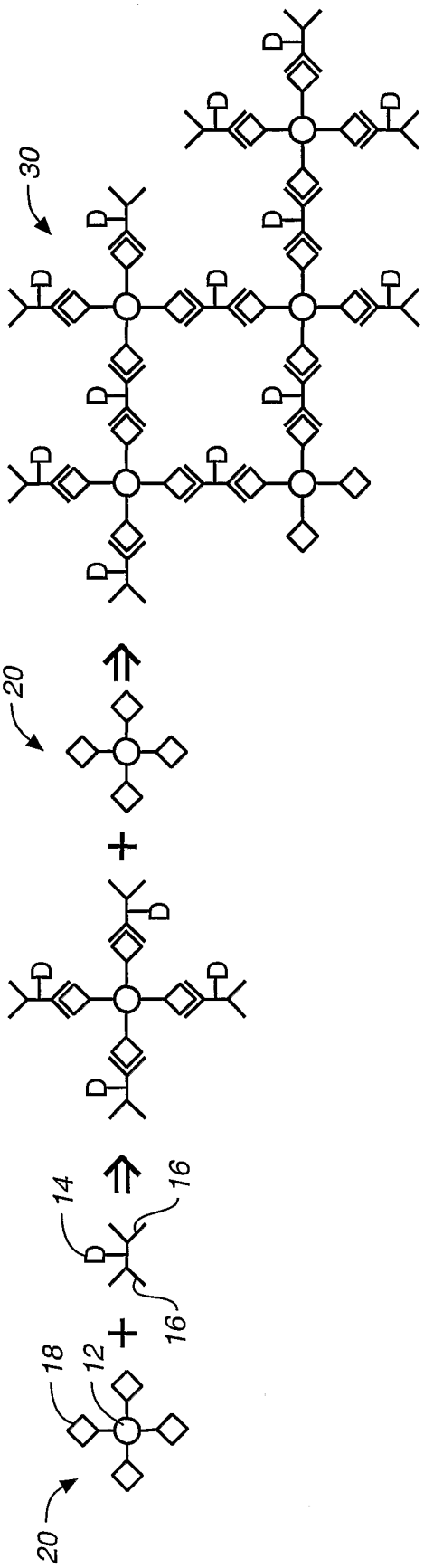


FIG._1B

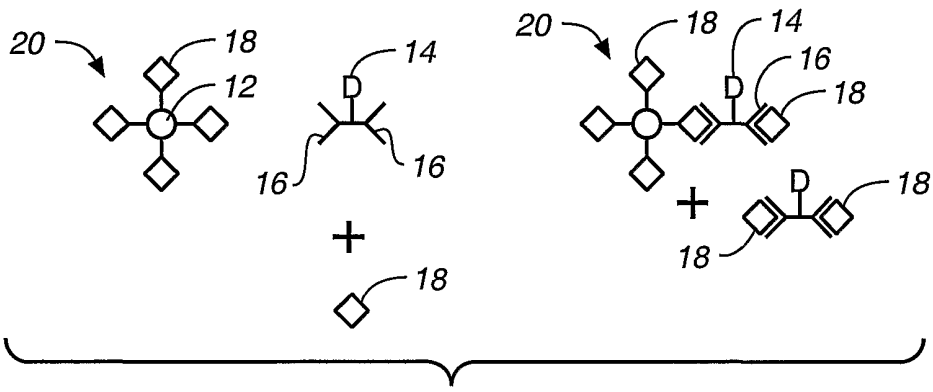


FIG._1C

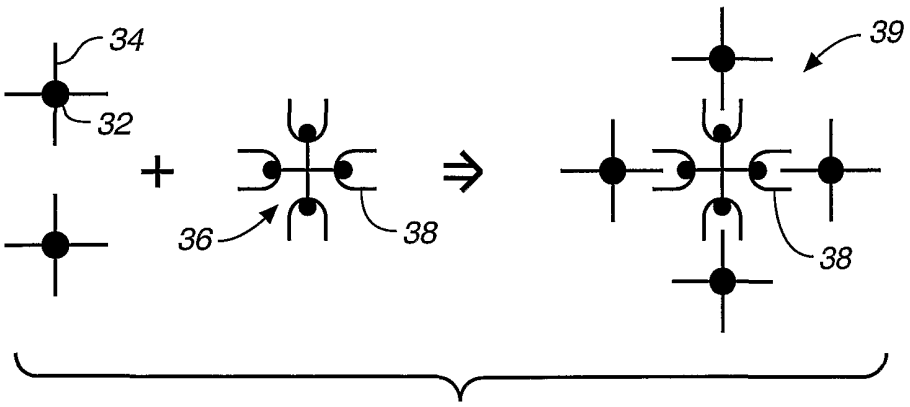


FIG._1D

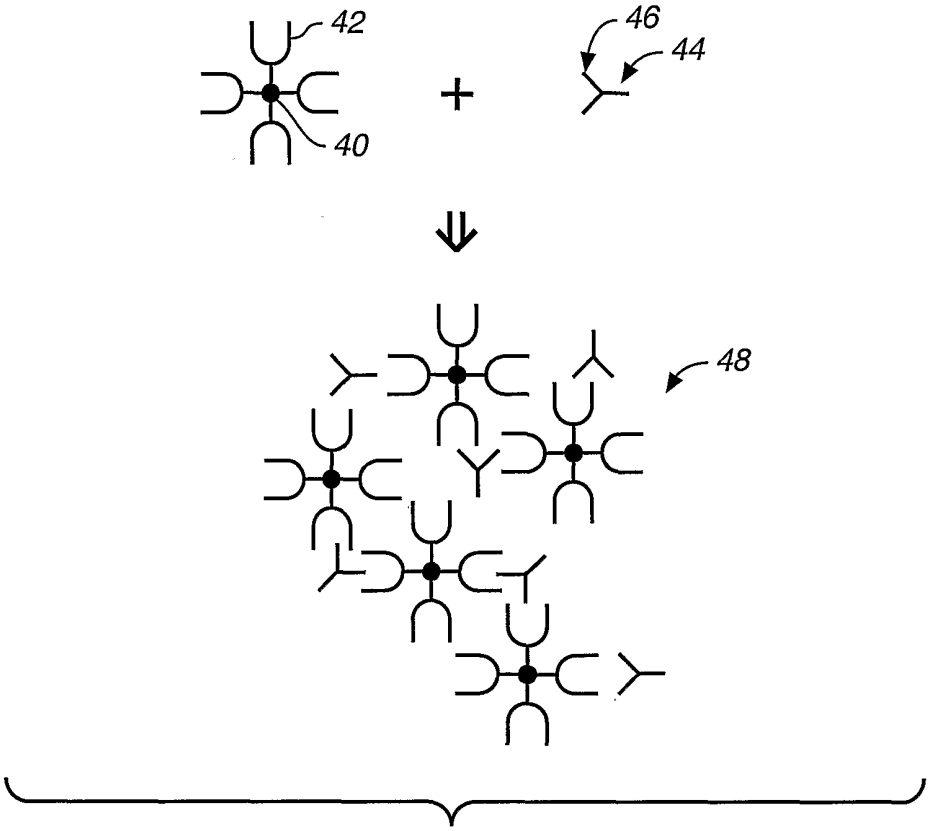


FIG._1E

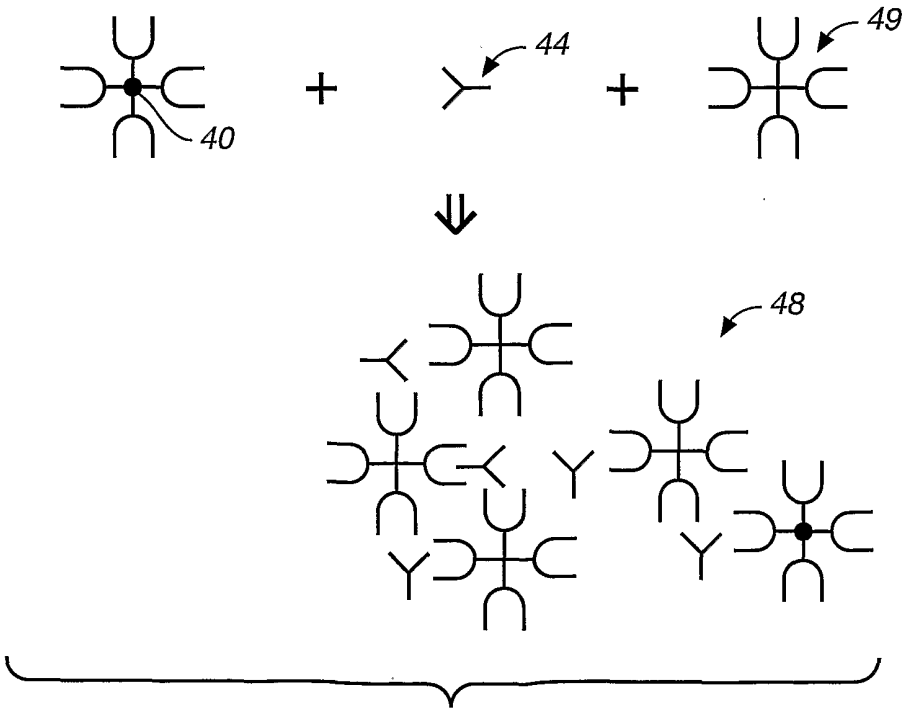


FIG._1F

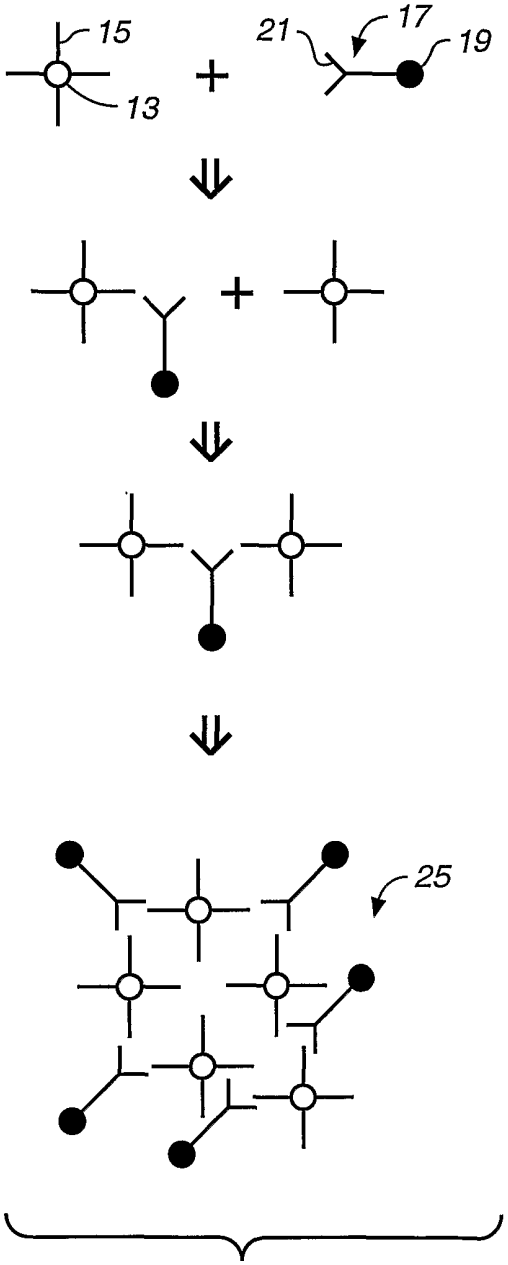


FIG._2A

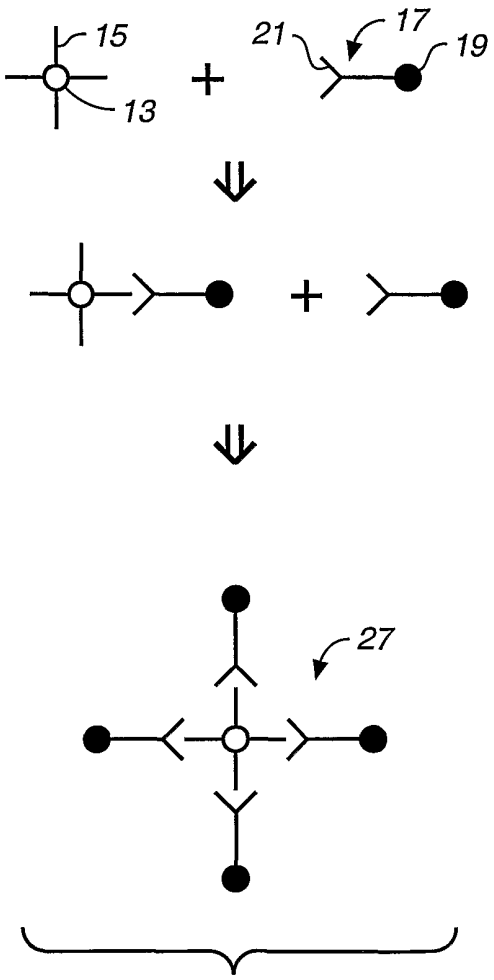


FIG._2B

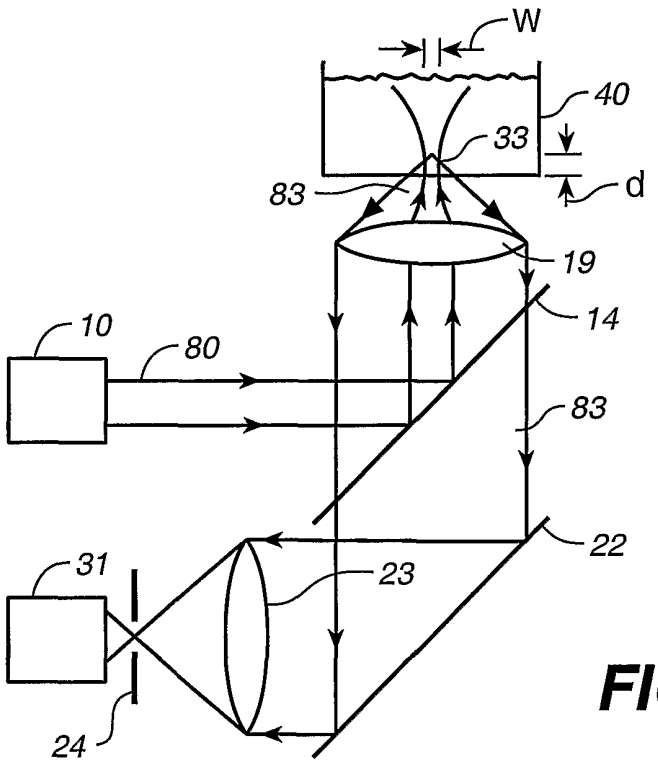


FIG._3

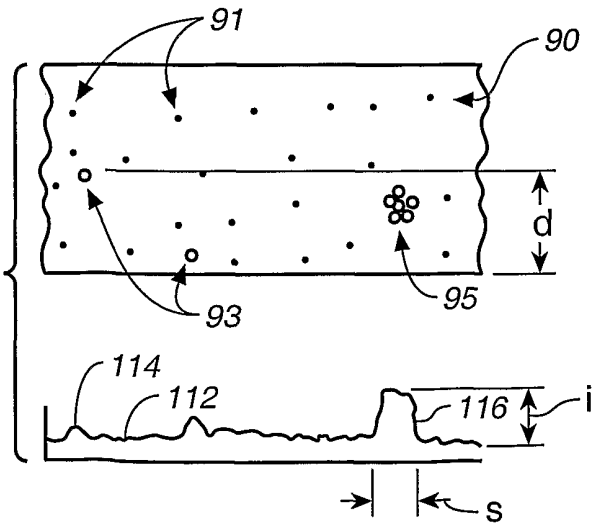


FIG._4

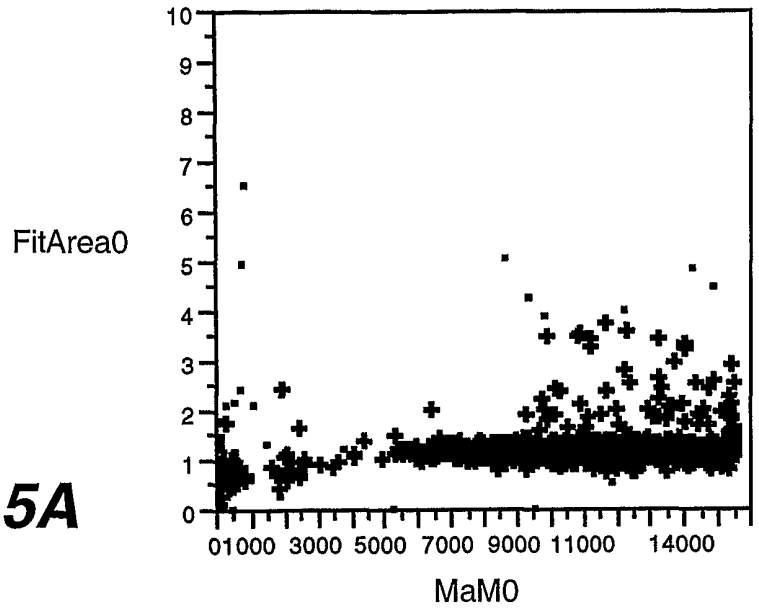


FIG._5A

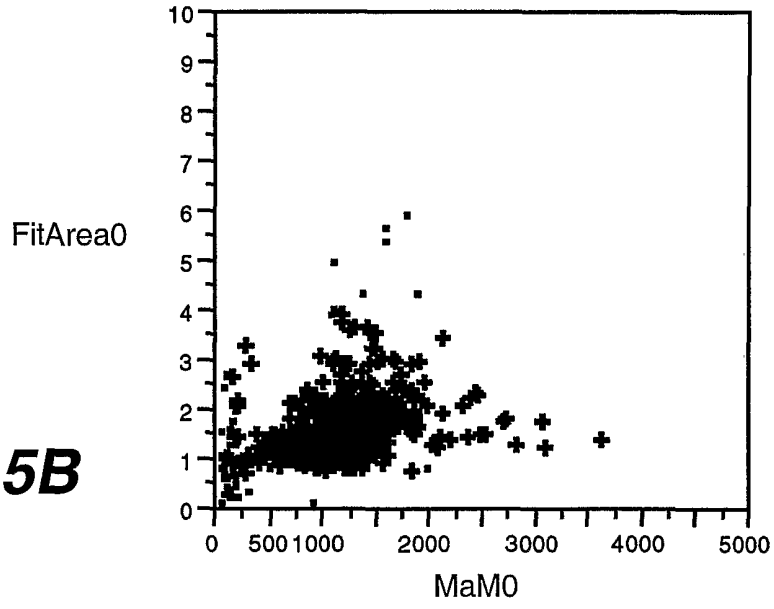


FIG._5B

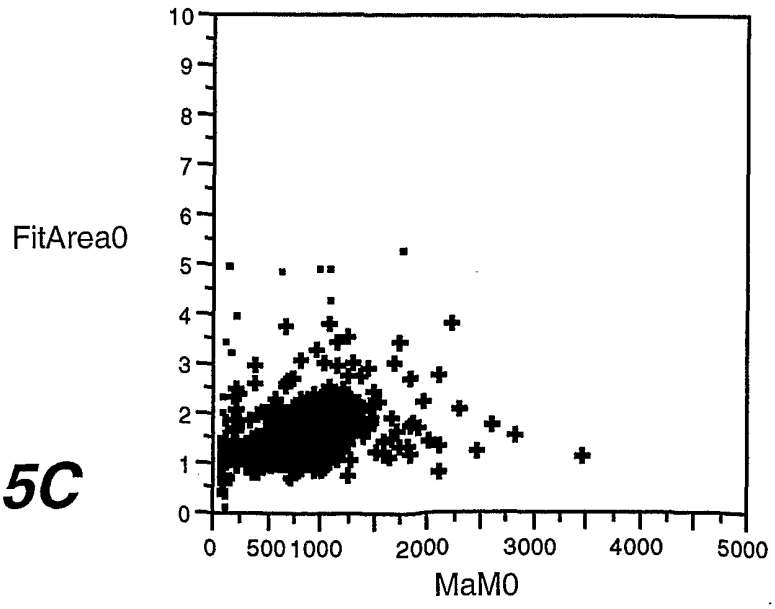


FIG._5C

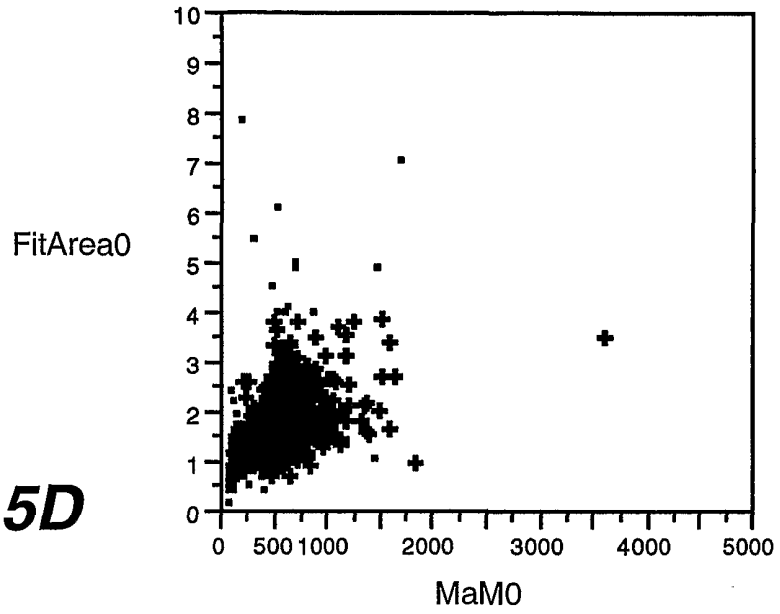


FIG._5D

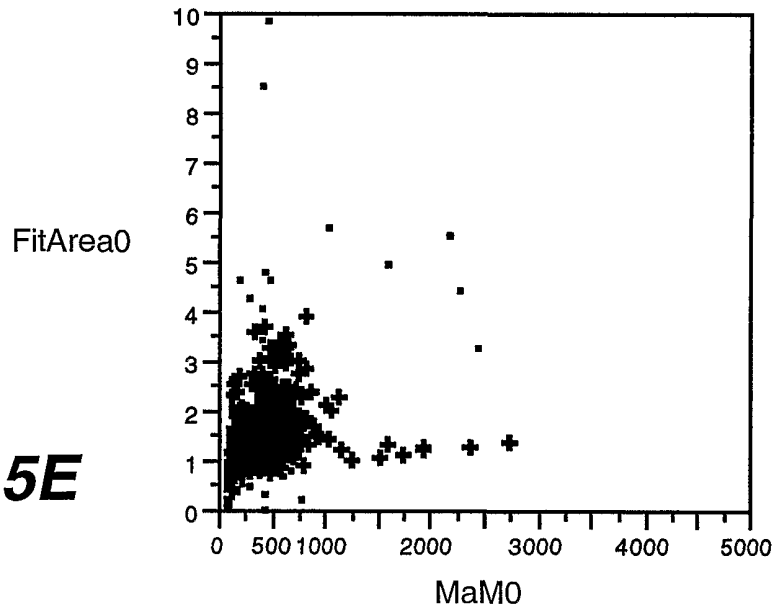


FIG._5E

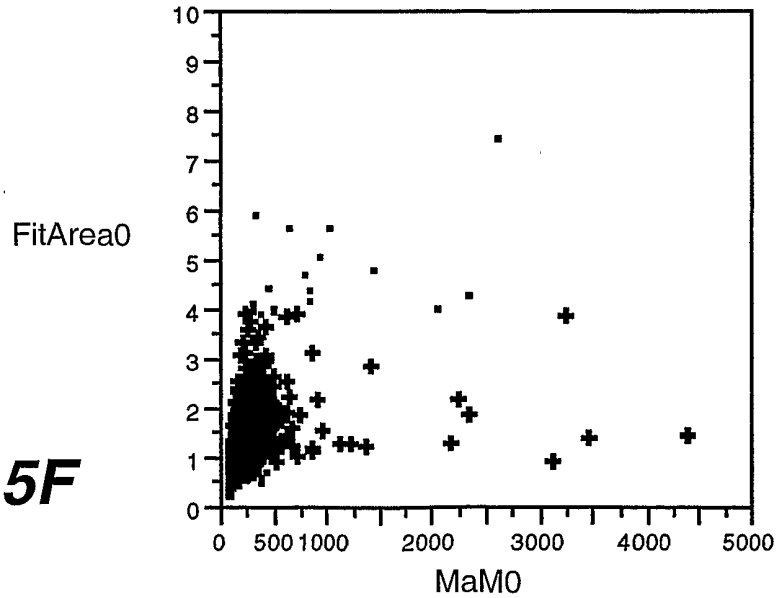
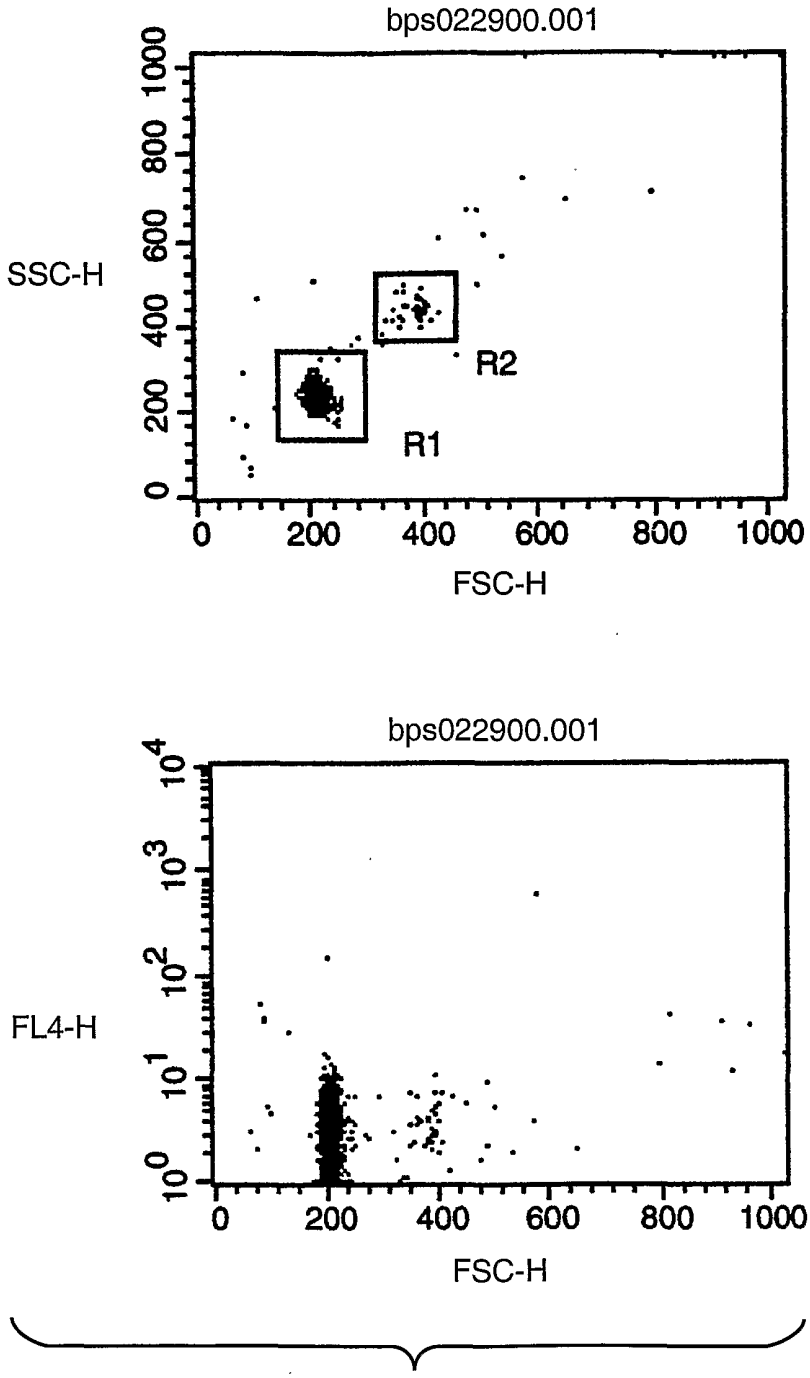


FIG._5F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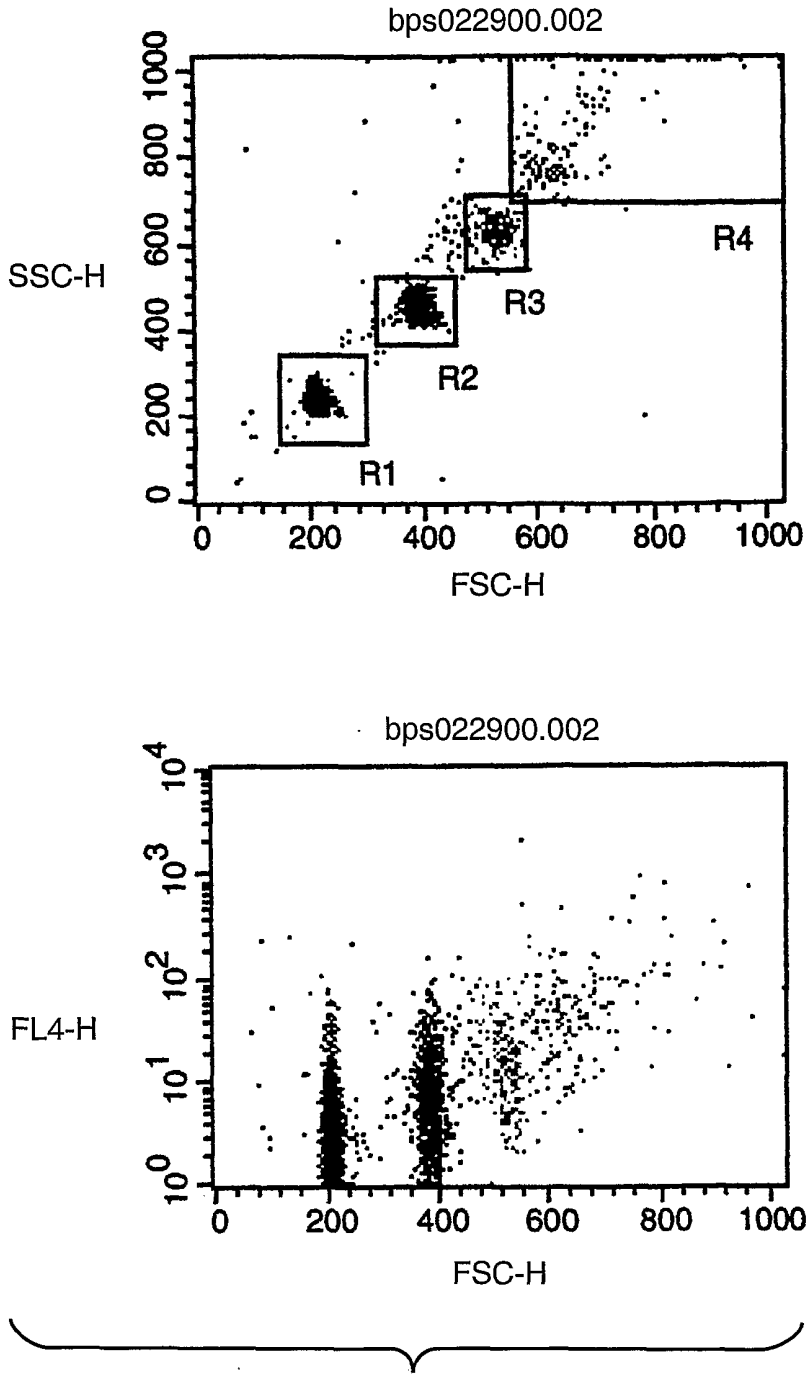
FSC-H

FIG._6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10^2
10^3
10^4

FIG._7A

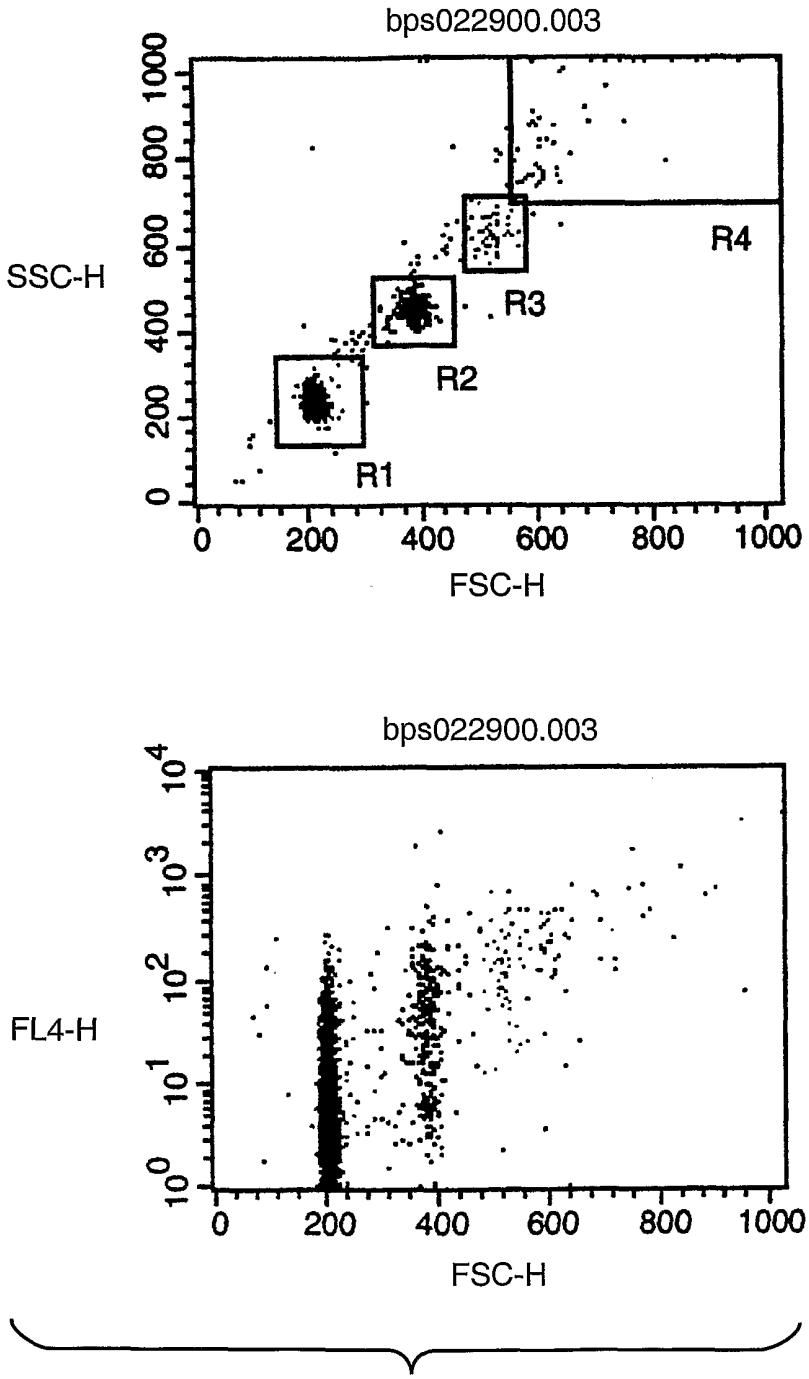
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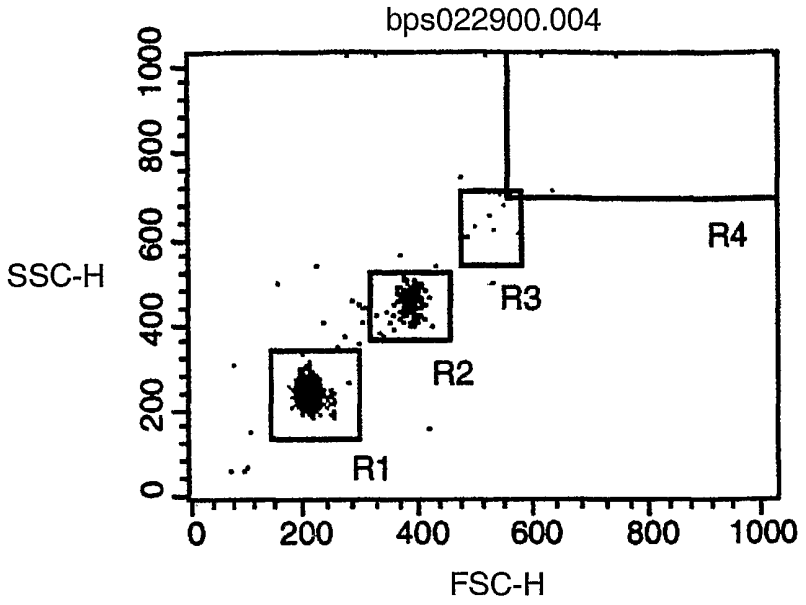
FIG._7B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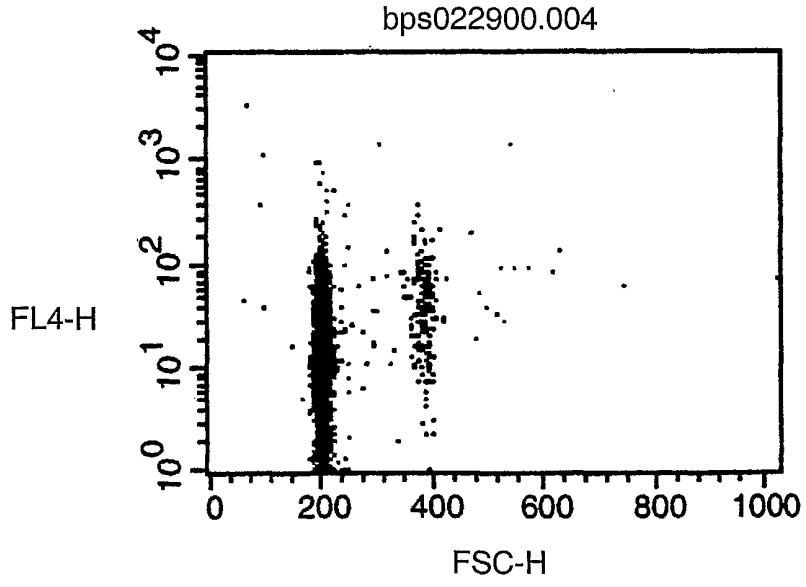
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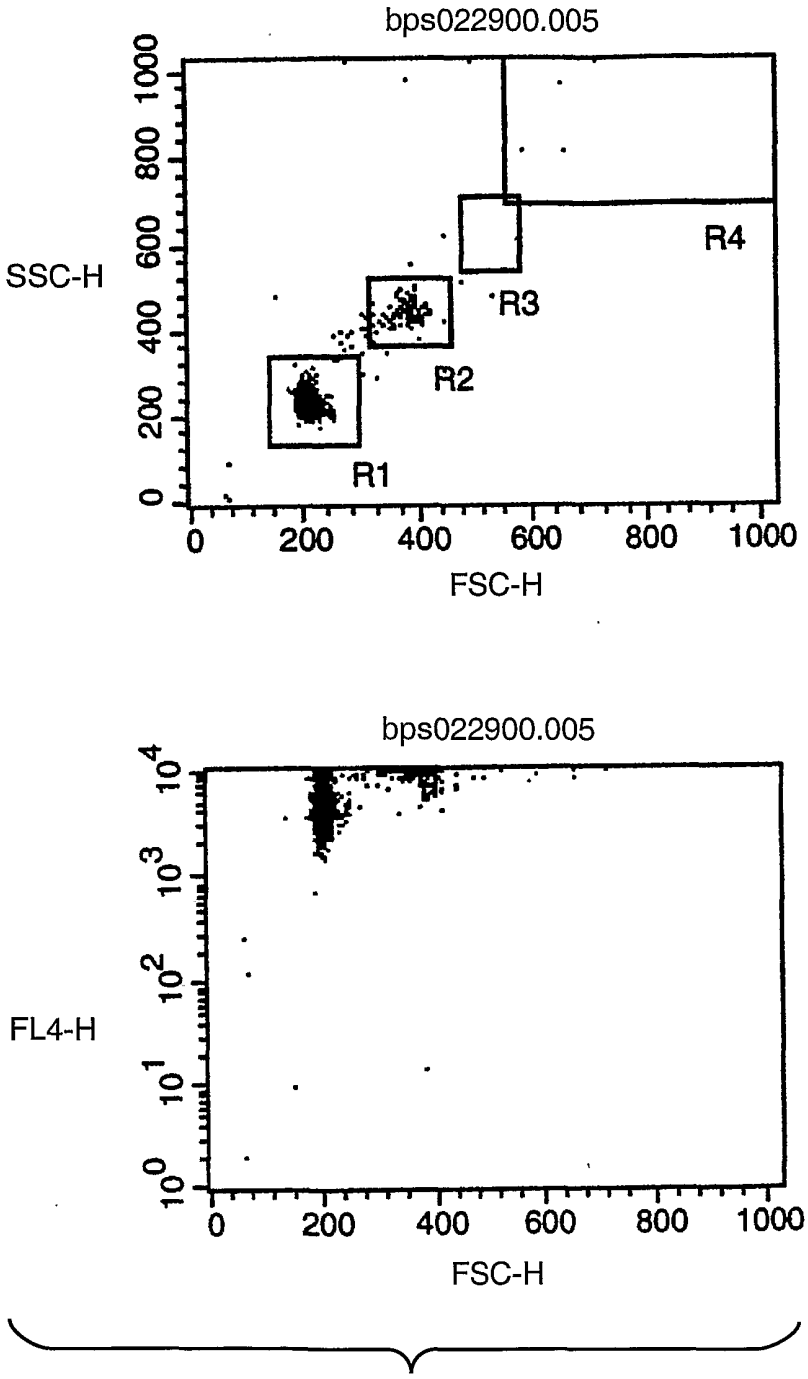
FIG._7C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10^4

FIG._7D

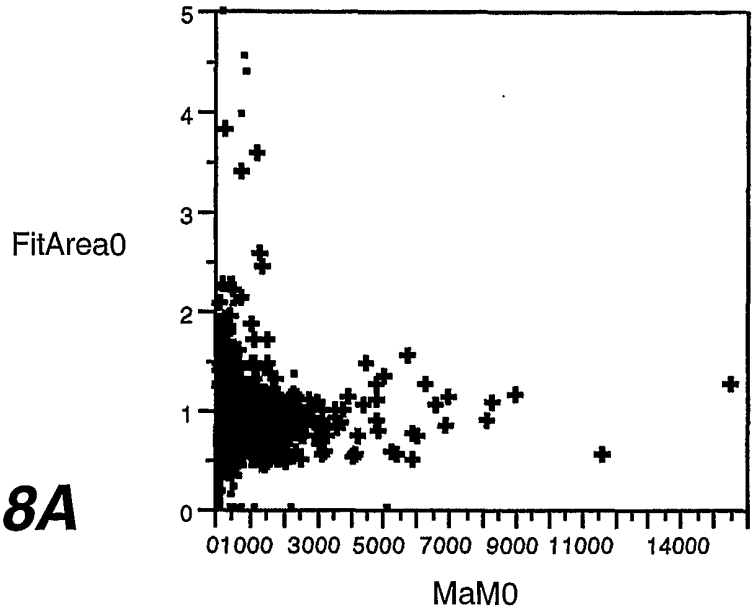


FIG._8A

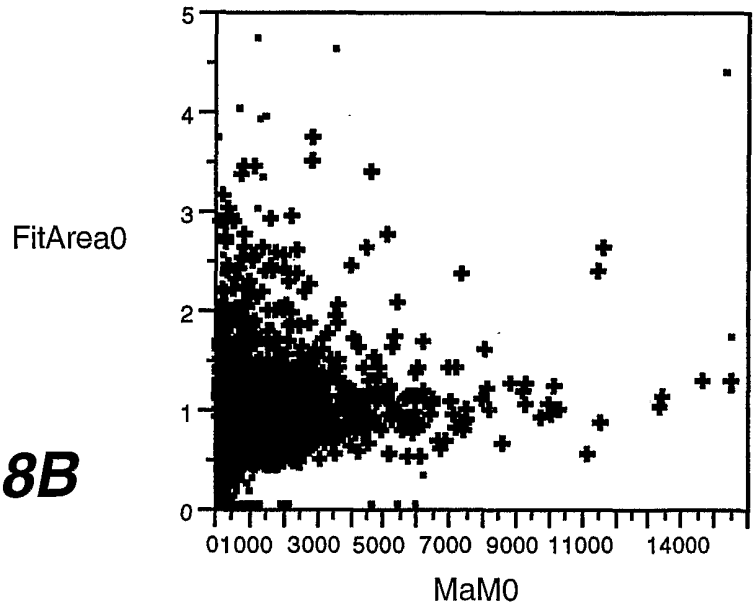


FIG._8B

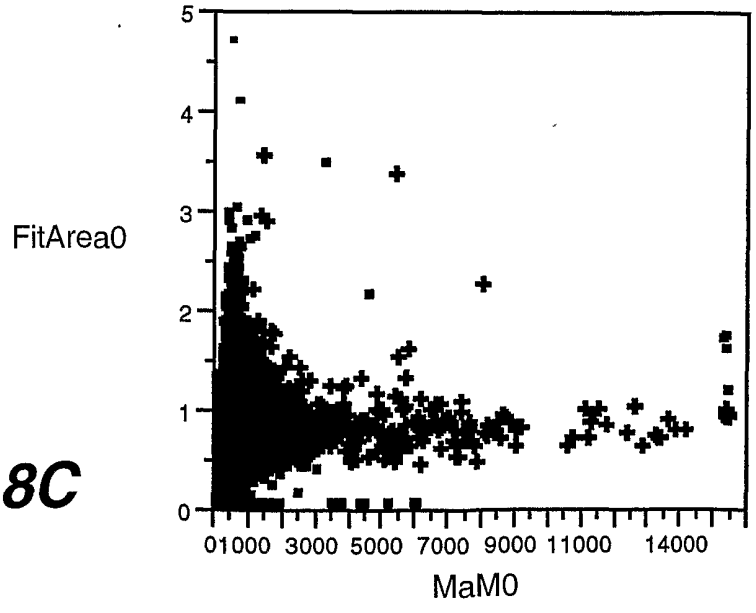


FIG._8C

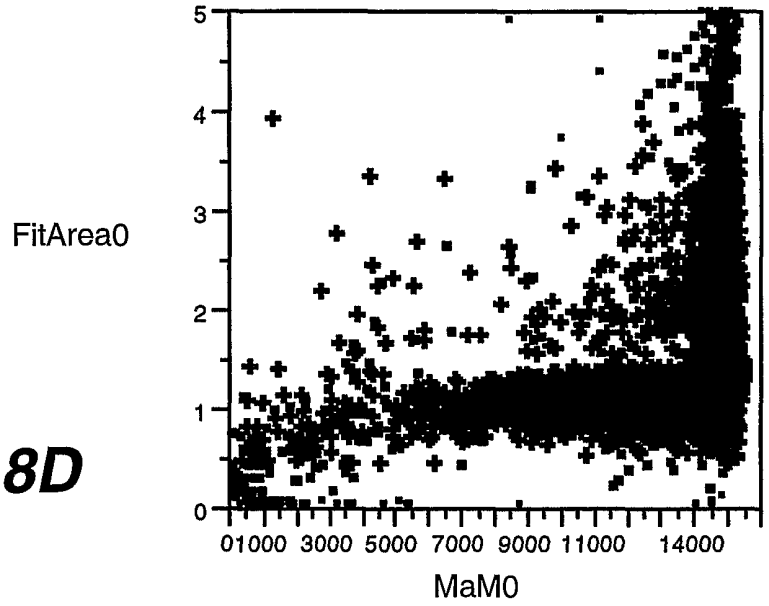


FIG._8D

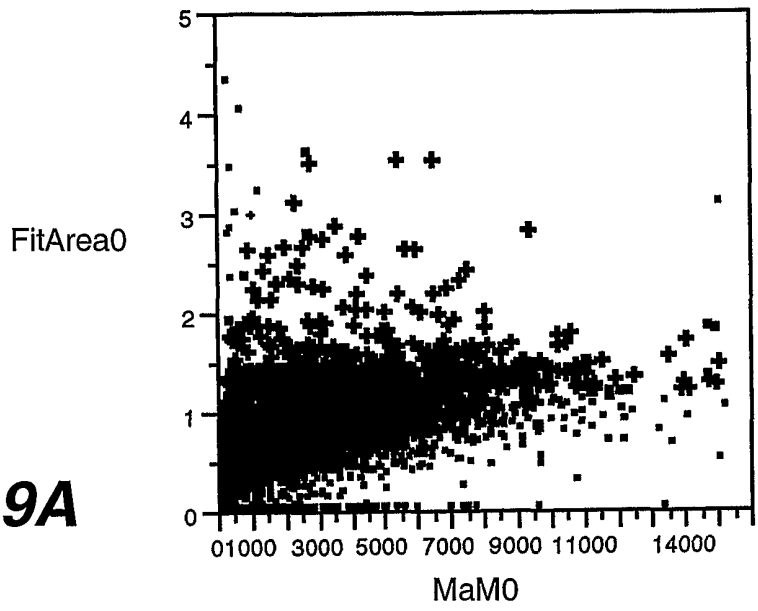


FIG._9A

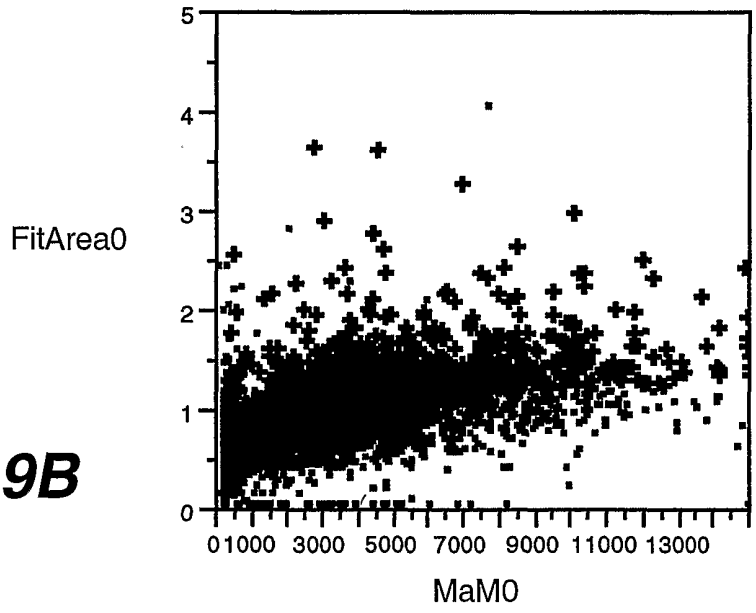


FIG._9B

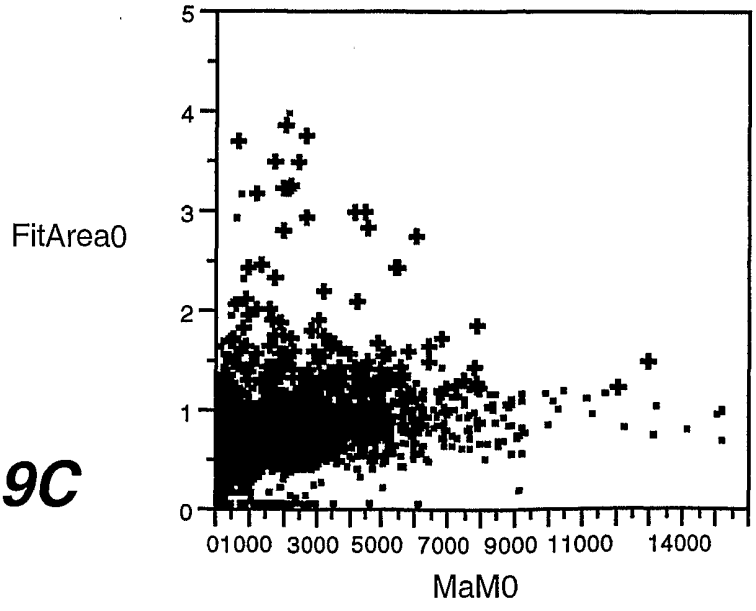
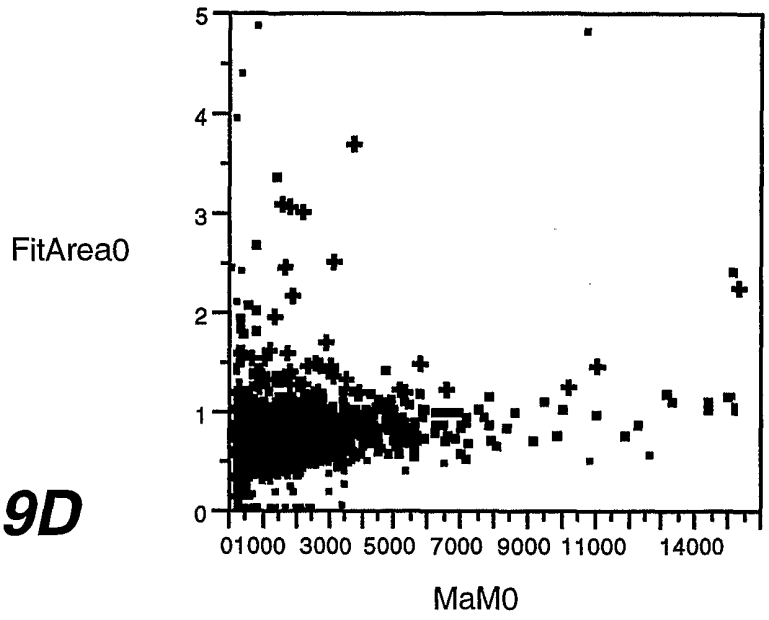


FIG._9C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MaM0

FIG._9D

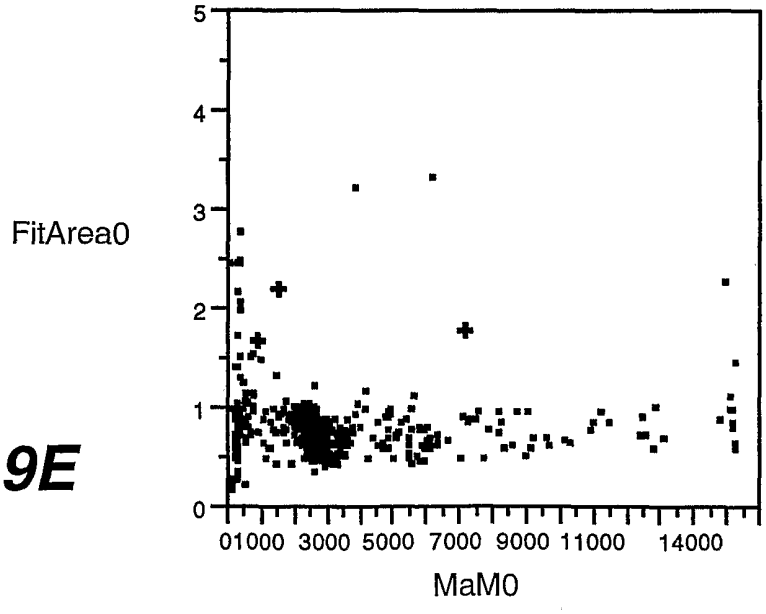
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FIG._9E

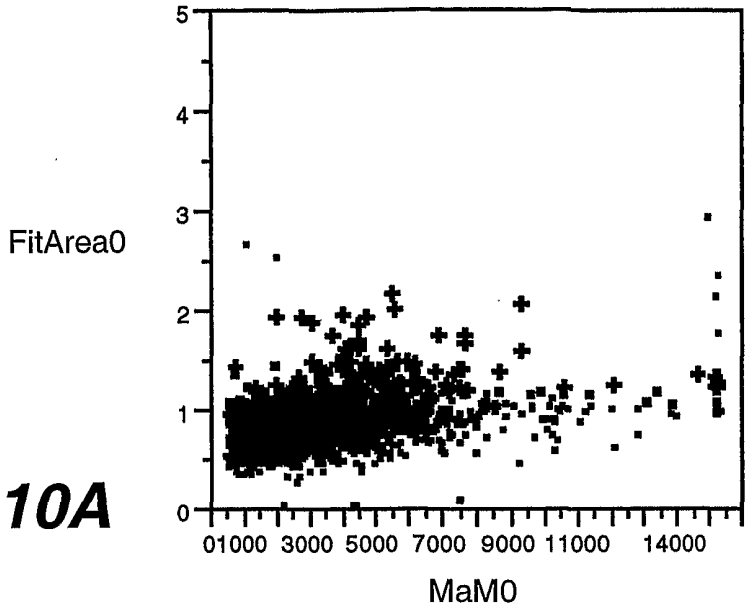

FIG._10A

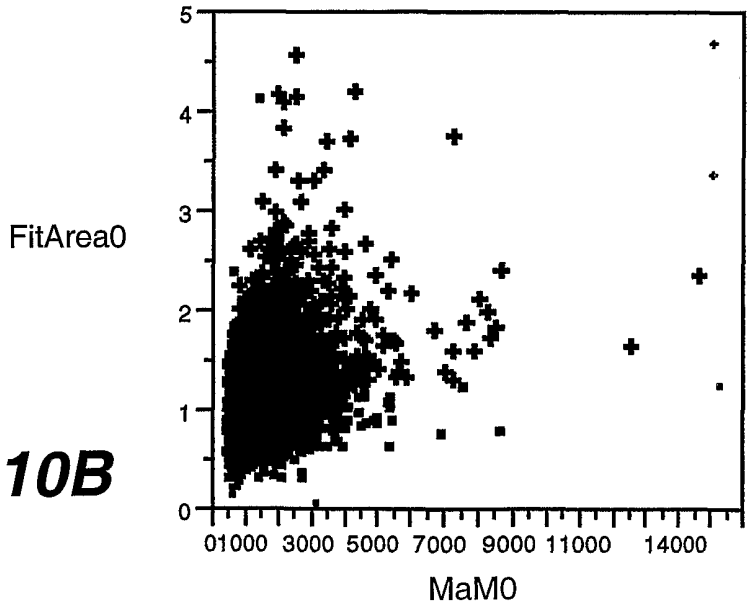

FIG._10B

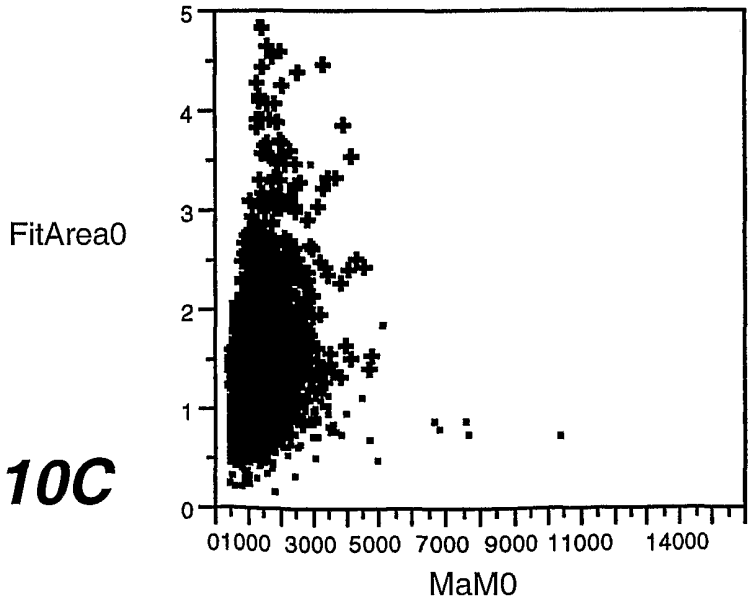

FIG._10C

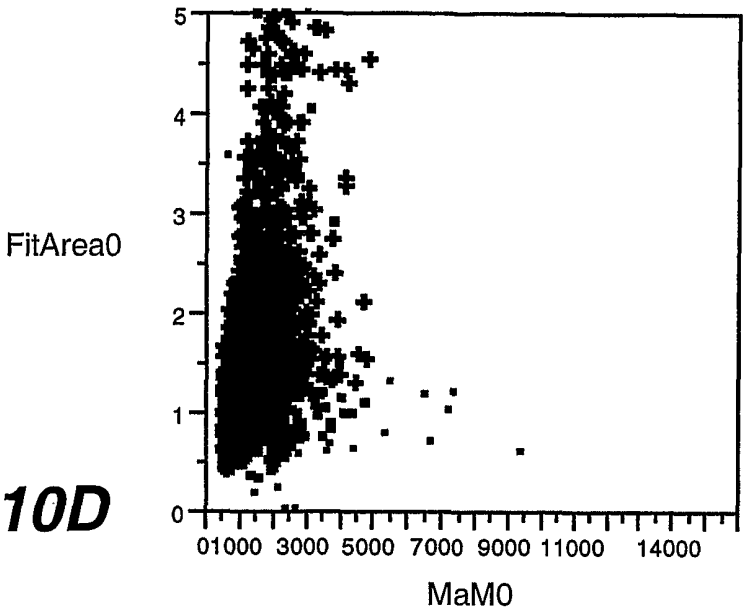

FIG._10D

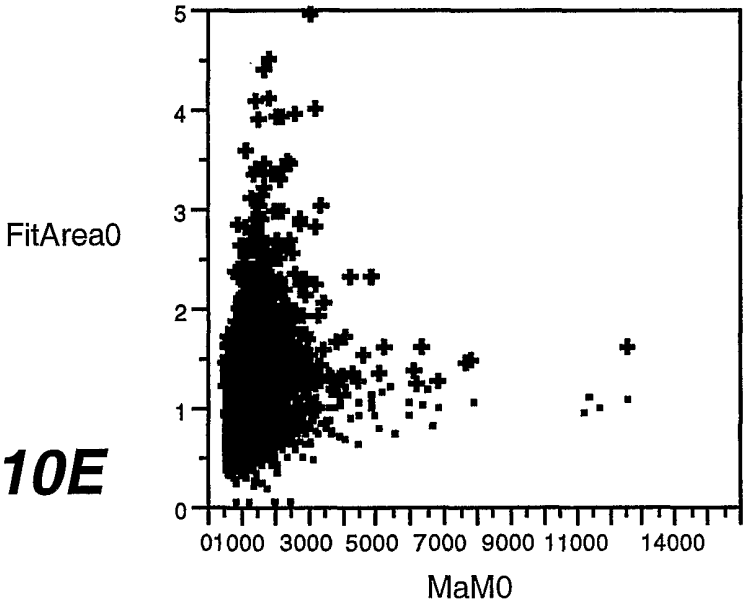

FIG._10E

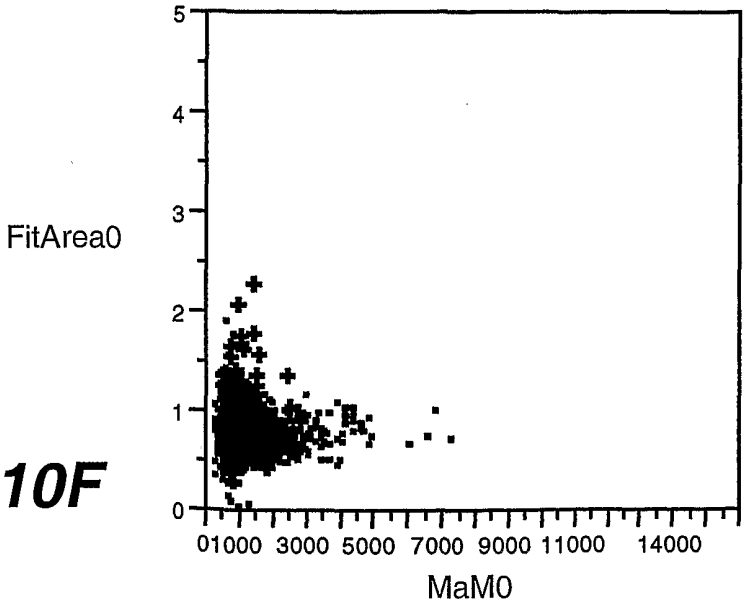

FIG._10F