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- (71) **Applicant:** THE SCRIPPS RESEARCH INSTITUTE
[US/US]; 10550 North Torrey Pines Road, La Jolla, California 92037 (US).
- (72) **Inventors:** KODADEK, Thomas; 8711 SE Somerset Island Way, Jupiter, Florida 33458 (US). DORAN, Todd; 300 South Australian Avenue, West Palm Beach, Florida 33401 (US).
- (74) **Agents:** PERDOK, Monique, M. et al.; Schwegman, Lundberg & Woessner, P.A., P. O. Box 2938, Minneapolis, Minnesota 55402 (US).
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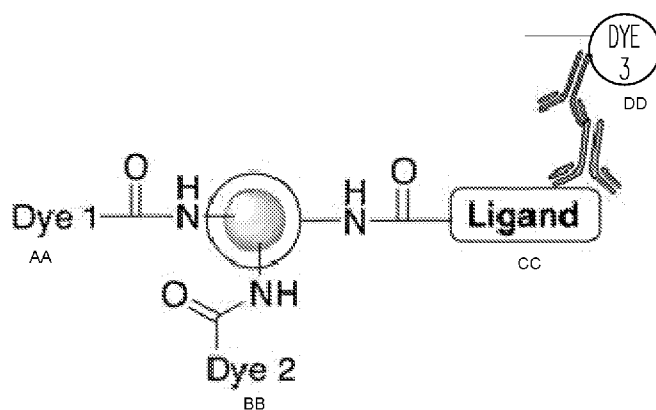
(54) **Title:** LIQUID ARRAY PLATFORM FOR MULTIPLEXED ANALYSIS OF MOLECULE-PROTEIN INTERACTIONS

Fig. 1A

(57) **Abstract:** The invention provides a method for high-throughput analytical detection of ligand-protein interactions. Polyethyleneglycol-coated amino-functionalized polystyrene microspheres are treated to block surface amino groups and separated into subpopulations, each of which is treated with two or more aminoreactive fluorescent dyes in a defined ratio, which serves to encode each subpopulation for identification in a fluorescence-activated cell sorter (FACS). Each subpopulation is then bonded via surface amino groups to a respective ligand. The protein under evaluation is associated with another fluorescent dye. Each fluorescent dye has a unique emission wavelength the intensity of which can be quantified by the FACS. The ratio of light emission of the two or more encoding dyes serves to identify each subpopulation of microspheres and the protein-associated dye indicate the binding of the respective ligand to the protein analyte.

AA
BB
CC
DD



TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, **Published:**
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LIQUID ARRAY PLATFORM FOR MULTIPLEXED ANALYSIS OF MOLECULE-PROTEIN INTERACTIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority of U.S. provisional applications Serial No. 61/771,952, filed March 4, 2013, and Serial No.
5 61/904,784, filed November 15, 2013, which applications are incorporated herein by reference in their entireties.

STATEMENT OF GOVERNMENT SUPPORT

This invention was made with government support under DK094309, awarded by the National Institutes of Health / National Institute of Diabetes and
10 Digestive and Kidney Diseases. The U.S. government has certain rights in the invention.

BACKGROUND

Multiplexed small molecule-protein binding assays are often carried out using microarray technology in which some number of small molecules is
15 spotted onto a chemically-modified planar surface, such as a glass slide.⁽¹⁻⁵⁾ The protein(s) of interest are exposed to the slide and bound material is visualized, usually via a labeled antibody that recognizes the protein(s) of interest or via a label that is covalently attached to the protein itself. Complex arrays displaying thousands of small molecules have been employed as a primary library-
20 screening platform. Ligands for transcription factors,⁽⁶⁾ antibodies⁽⁷⁻⁹⁾ and other proteins⁽¹⁰⁻¹²⁾ have been discovered in this fashion. Arrays displaying thousands of random peptides have been employed to obtain serum antibody “signatures” of possible diagnostic utility.⁽¹³⁻¹⁷⁾ Smaller arrays comprised of tens to hundreds of ligands have been used to stratify hits from larger library screens conducted
25 on some other platform. Similarly, structure activity relationships can be gleaned by array-based, multiplexed analysis of derivatives of protein- or RNA-binding ligands.⁽¹⁸⁻²²⁾ In the future, there is the hope that if one has high affinity synthetic ligands for many serum proteins involved in disease states, that arrays of these species might be employed for clinical diagnostics.

30 SUMMARY

The invention is directed in various embodiments to an improved method for analysis of multiple potential ligands in a single analytical evaluation with

respect to binding of one or more of the potential ligands to a selected protein. The invention can provide a method for identification of specific protein-binding ligands, such as antigens, receptor modulators, and enzyme inhibitors, as part of a large set of potential ligands evaluated for binding to a test protein sample.

- 5 Ligands can be synthetic compounds, such as peptide analogs, receptor agonist/antagonist analogs, enzyme transition state analogs, and the like, or can be biologically-derived antigens, receptor modulators, or enzyme substrates.

Various embodiments of the method provide means of “encoding” polyethylene glycol (PEG)-coated, amino-functionalized, polystyrene (e.g.,
10 TentaGel®) microspheres, also referred to herein as “beads,” to provide identifying (encoding) information that can be associated with each one of a set of potential specific ligands (e.g., antigens, receptor modulator candidates, enzyme inhibitor candidates) to one or more of which the test sample of the protein (e.g., antibody, receptor, enzyme) binds with specificity and/or high
15 affinity. A single sample of microspheres as used in practice of a method of the invention comprises a plurality or set of subpopulations of microspheres, each subpopulation of which is identifiable based on a defined ratio of fluorescent emission intensity from each of two or more respective fluorescent encoding dyes bonded internally thereto, wherein at least some of the microspheres of
20 each of the subpopulations have respective potential ligands from a set of potential ligands (e.g., antigens, modulators, inhibitors, and the like) also bonded thereto on the surface of each microsphere. Each subpopulation of microspheres thus has surface-bonded to the microspheres of the subpopulation one potential ligand selected from a set of potential ligands to be evaluated for binding to the
25 protein sample. A plurality of subpopulations, each subpopulation identified by the ratio of fluorescent emission intensities from the two or more fluorescent encoding dyes bonded thereto, and each subpopulation having at least some member microspheres thereof surface-bonded to a respective potential ligand selected from a set of potential ligands, are mixed together. The mixture of the
30 plurality of subpopulations includes microspheres that together display the full set of potential ligands to be evaluated in the particular analytical procedure. The resulting mixture of the plurality of the subpopulations of microspheres is then contacted with a protein that is under evaluation to test for binding some of the set of potential ligands.

The protein under evaluation can be functionalized with an additional fluorescent protein reporter dye, e.g., by means of a protein-specific entity, e.g., an antibody conjugated to the fluorescent protein reporter dye that binds an epitope of the protein not involved in binding of the ligand. The protein can be bonded to the protein-specific entity comprising the protein reporter dye at the time of testing with the mixture of subpopulations of microspheres, or the microspheres can be treated with the protein-specific entity comprising the reporter dye after contacting with the mixture of microsphere subpopulations. The protein is thus bound to or is capable of binding a protein-specific molecule bearing a fluorescent reporter dye for the protein. The two (or more) encoding dyes used to identify each subpopulation of microspheres, and the protein-bound reporter dye, each have a unique fluorescence emission spectrum, allowing each characteristic unique emission of each of the three (or more) dyes to be individually detected and quantified for each individual microsphere. The fluorescent emission of each of the three (or more) dyes can be stimulated by a single excitation wavelength or light, or by several excitation wavelengths of UV-visible light, such as in a FACS.

The mixture of subpopulations of microspheres each with a respective potential ligand, exposed to the protein under evaluation to which the protein-reporting fluorescent dye is bound before or after contacting the test protein and the mixture of subpopulations of microspheres, is then analyzed by a detection device such as a Fluorescence Activated Cell Sorting (FACS) system. Each microsphere is illuminated with appropriate UV-visible fluorescence stimulating light (of one or more excitation wavelengths) capable of inducing fluorescence in each of the fluorescent dyes, i.e., the two or more encoding fluorescent dyes, and the protein-bound fluorescent reporter dye. Each microsphere from which a fluorescence emission from the protein-bound reporter dye is detected is evaluated to determine the fluorescence emission intensity ratio for the first and second (or more) fluorescent encoding dyes, which serve to identify the particular ligand to which the protein is exhibiting binding. In this manner, a large number of potential ligands for the protein can be evaluated in parallel in a single analytical procedure to identify one or more ligands from the set of potential ligands that bind the test protein.

The size of the microspheres can be chosen to allow the use of FACS machines to quantify binding of the protein to each microsphere. This technique can be generally applied to any ligand with even modest affinity, to detect relevant ligands, diagnostically and otherwise, that bind the protein. Up to 36
5 distinct subpopulations of encoded microspheres have been cleanly separated using FACS as disclosed herein.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Overview of the multiplexed liquid array platform. A) Representation of the biphasic microsphere construct with internally-labeled
10 dyes and externally-immobilized synthetic molecule ligand. B) Illustration of bead sorting and fluorescence “reading” using a common flow cytometer requiring two excitation lasers and three detectors. C) Example FACS dot plot showing the ratiometric emission intensities of the two encoding dyes. Each subpopulation can be separated from a batch of differentially internally dyed
15 microspheres. D) Upon gating a designated subpopulation, binding can be quantified as the relative intensity of a protein-bound reporter fluorochrome. E) FACS dot plot of Pacific OrangeTM vs. Pacific BlueTM emission intensities for a batch of 24 subpopulations of encoded microspheres.

Figure 2. Standard bead preparation protocol. Beads are encoded as
20 shown below using substoichiometric amounts of Pacific BlueTM and Pacific OrangeTM dyes at differing ratios using the commercially available NHS esters. Once the encoded beads are prepared with immobilized ligand, they are mixed, transferred to a filter plate and re-equilibrated in aqueous media by washing several times in water followed by an overnight water wash. The beads are
25 treated with 2-mercaptoethanol to quench any unreacted alkyl bromides and washed several times with PBS followed by TBS-tween.

Figure 3. Comparison of Luminex® vs. TentaGel® platforms in serological measurements. A) Binding isotherms generated for the detection of anti-ADP3 IgY in chicken serum using ADP3 immobilized onto TentaGel®
30 microspheres. Binding was quantified by measuring the mean fluorescence intensity (MFI) of a PE-conjugated anti-IgY antibody. B) The same experiment was performed using ADP3 immobilized onto Luminex® microspheres.

Figure 4 . Evaluation of the multiplexed capabilities of the dye-encoded microspheres. A) Chemical structure of ADP3 with a free cysteine.

Position labels indicate the side chains substituted for the methyl scan derivatives. B) Binding isotherms generated for ADP3 and all methyl derivatives of ADP3 using fluorescence polarization. C) Dot plot of Pacific OrangeTM vs. Pacific BlueTM emission intensities for the encoded subpopulations containing ADP3 and selected methyl derivatives. D) Binding isotherms generated for each of the methyl derivatives using the microsphere immunoassay. E) Fluorescence polarization of fluorescein-labeled BBHit3 when incubated with monovalent PAFAH-1B2 or a negative control protein. F) Binding isotherms for BBHit3 binding to PAFAH-1B2 or a negative control protein using the microsphere immunoassay.

Figure 5. Separation of 36 populations. The plot illustrates bead populations sorted by FACS into 36 subpopulations exhibiting specific dye ratios.

Figure 6. Effect of polyethyleneglycol linkers. (A) Chemical structures of linkers added to 10 μ m TentaGel® microspheres between the bead and the ligand of interest. (B) Binding saturation plots for a ligand HH031 against its monoclonal antibody target, CLL169, utilizing the different linkers, varying in length and chemical composition. Mean fluorescence intensity is read from AlexaFluor 647 conjugated to anti-human IgG and read using an LSRII flow cytometer. (C) MFI for HH031 binding to 700 nM of CLL169 using the various linkers.

DETAILED DESCRIPTION

Synthetic molecule microarrays, often termed affinity arrays, consisting of many different compounds spotted in a spatially defined manner onto a planar surface such as modified glass or cellulose, have proven to be useful tools for the multiplexed analysis of small molecule- and peptide-protein interactions. However, these arrays are technically difficult to manufacture and use with high reproducibility, and require specialized equipment. While planar glass arrays of peptides or non-peptidic small molecules can be effective in these applications, their creation is technically demanding and requires sophisticated instruments, including robotic liquid handlers and spotters.

Here we report a more convenient alternative using fluorescent emission color-encoded microspheres (beads) that display a potential protein ligand on the surface. Quantitative, multiplexed assay of protein binding to up to 36 different

potential ligands can be achieved using a common flow cytometer for the readout. This technology should be useful for evaluating hits from library screening efforts, for the determination of structure activity relationships involving protein-ligand interactions, and for certain types of serological analyses.

In considering this problem, we were influenced by precedents in the fields of genomics and proteomics where “liquid arrays” have emerged as an alternative to the microarray platform. Liquid arrays employ small, polystyrene microspheres, also known as beads, as the scaffold to which the capture agent (ligand) is immobilized. Unlike the planar microarrays, where the identity of the ligand is defined spatially, liquid arrays are employed in a batch mode whereby beads displaying different ligands are added to a single sample. Therefore, an encoding strategy is required.

For example, the popular Luminex® technology (<http://www.luminexcorp.com>) employs 5.3 μm polystyrene microspheres that display antibody capture agents and are encoded by a specific ratio of two organic dyes that are physically adsorbed into the hydrophobic interior of the beads. Binding of the analyte of interest to each bead is measured by addition of a sandwich antibody tagged with a third color dye. The beads are analyzed using a proprietary flow cytometer-like instrument with lasers that measure the level of the sandwich antibody and identify the encoding ratio of dyes on each bead as they pass single file past the detector. Thus, the Luminex® system is a potentially attractive alternative to planar arrays for making multiplexed measurements of small molecule-protein interactions.

However, in its practical application, there are problems with this off-the-shelf technology in the analysis of small molecule-protein complexes. First, the encoded beads are expensive and not well suited as a platform for synthesis. Since the encoding dyes are only adsorbed in the beads, they can leach out when the beads are suspended in organic solvents in order to link small molecules, potential ligands, to their surface.

Second, like any polystyrene-based bead platform, there is a high level of non-specific protein binding. This can be tolerated if one is using high affinity capture agents such as antibodies and detecting bound analyte via a sandwich assay. But typically lower affinity synthetic ligands and direct detection of

bound proteins make this a much more serious issue with respect to sensitivity and accuracy.

Therefore, we sought to combine the advantages of the Luminex® platform with a less expensive, more organic chemistry-friendly solid support and encoding system. In this patent application we describe the development of such a system that is capable of measuring up to 36 different small molecule-protein interactions simultaneously with excellent sensitivity and accuracy using inexpensive materials and a common flow cytometer for the readout.

Accordingly, the invention is directed in various embodiments to a method for determining protein binding to each of a plurality of ligands in a single analytical sample, comprising:

a) treating a population of polyethyleneglycol-coated amino-functionalized polystyrene microspheres comprising surface and internal amino groups with a amino group blocking reagent to selectively block surface amino groups, and dividing the population into a plurality of subpopulations of the microspheres; then,

b) treating each respective subpopulation of microspheres with two or more amino-reactive fluorescent encoding dyes in a respectively unique ratio to bring about reaction of internal amino groups of the microsphere with the two or more fluorescent encoding dyes, such that a microsphere of each subpopulation can be distinguished from a microsphere of each other subpopulation by a detection device that determines a ratio of a fluorescence emission intensities from each of the two or more fluorescent encoding dyes; then,

c) deblocking the surface amino groups, then treating each subpopulation of microspheres with a reactive form of a respective ligand from a set of potential ligands to be evaluated for protein binding interactions, to bring about coupling of the respective ligand to at least some of the microspheres of the respective subpopulation; then,

d) combining the plurality of subpopulations of microspheres, at least some of the microspheres of each subpopulation bearing the respective test ligand bonded thereto, and then contacting the combined microsphere subpopulations with a protein to be evaluated for protein binding interactions with the respective test ligands, and contacting the combined microsphere subpopulations with a protein reporter molecule comprising a fluorescent

reporter dye immobilized thereto, the protein binding or being capable of binding the reporter molecule, wherein each of the encoding fluorescent dyes and the protein-binding fluorescent reporter dye have a unique fluorescence emission spectrum; then,

- 5 e) analyzing each microsphere of the combined sample with the detection device to determine a ratio of fluorescence emission intensity of each of the two or more microsphere-bound encoding dyes for each microsphere in which the protein-binding fluorescent reporter dye is detected, thereby identifying each respective ligand associated with each respective protein-binding subpopulation
10 of microspheres. The analysis of each microsphere of the combined sample can be carried out individually with a fluorescence activated cell sorter (FACS).

It is understood that until each potential ligand is evaluated, it is not strictly speaking a ligand for the test protein undergoing evaluation in any specific analysis. However, as the term “ligand” is used herein, reference is
15 made to a potential ligand, or a molecular entity undergoing evaluation as a ligand, or the like, as well as to a molecular entity shown to actually be a ligand for a particular protein. It is believed that some of the “ligands” that are evaluated will prove to be true ligands of the test protein under evaluation. Other molecular entities will test negative for a particular test protein.
20 Nevertheless, for simplicity and clarity of nomenclature, these potential ligand molecules are referred to herein as “ligands”, or “capture agents”, even prior to evaluation of their ability to bind to, or capture, any particular protein undergoing evaluation by the method herein.

A microsphere (bead), composed of polyethyleneglycol
25 (polyoxyethylene) coated polystyrene, which can be porous, permeable, and the like, and can contain amino group functionalities both on the surface and in the interior of the bead. Polyethyleneglycol (PEG) coating of a polystyrene bead can be effective in reducing non-specific binding of proteins, such as by general lipophilic interactions. Examples of such microspheres include TentaGel®
30 (Rapp Polymere) microspheres. For example, an average molecular weight of the PEG coating the bead can be about 3000 Da. The coating can be a covalent bonding of PEG chains to the surfaces of the bead. The spherical shape and the monosized character of microspheres can be chosen to allow handling in automated sorters, such as FACS. Such microspheres can comprise amino

groups, both internal and external to the bead, available for reaction with amino-reactive substances. For example TentaGel® M NH₂ monosized amino brand TentaGel® microspheres can be used in carrying out methods of the present invention.

5 TentaGel® beads (Rapp Polymere GmbH) are a superior support for analyzing interactions between bead-displayed small molecules and proteins.⁽²³⁻²⁵⁾ TentaGel® beads are comprised of an amine-functionalized polystyrene core onto which is grafted a thick layer of amine-terminated polyethylene glycol (PEG). The PEG layer grossly reduces the level of non-specific protein binding to the beads. Lam and co-workers have published a protocol by which the hydrophobic interior and hydrophilic exterior of the TentaGel® beads can be modified differentially and they have used this strategy to encode synthetic molecules on the surface of the bead with internal mass spectrometry-
10 sequenceable tags.^(24, 26-28)

15 A population of the microspheres comprises multiple individual microspheres. The microspheres can be all of approximately the same shape and size, such as to facilitate handling by automated sorters of objects of that size, such as FACS. For example, the TentaGel® M NH₂ monosized amino brand TentaGel® microspheres are adapted by the manufacturer (Rapp Polymere of
20 Tuebingen, Germany) to be monosized, i.e., spheres of approximately the same diameters, which can be about 10 µm in diameter, or can be about 20 µm in diameter, or can be about 30 µm in diameter. For use in a FACS sorting and separation device, it is preferred that the beads have a relatively tight distribution of diameters. 10 µm diameter TentaGel® beads are available and are small
25 enough to pass through a standard flow cytometer, allowing for the analysis of thousands of beads per second (Figure 1).

 The microspheres are functionalized, both internally and externally, with amino groups. The amino groups are present both externally (on the surface of the microsphere) and internally (in solvent-accessible porous regions in the
30 interior of the microsphere). The reactivity or accessibility to reactive reagents can be significantly different between interior and exterior amino groups. Accordingly, the inventive method provides that surface amino groups can be selectively blocked, with a substoichiometric amount of a suitable N-blocking reagent that is subsequently removable under conditions that do not destroy the

microsphere, e.g., Fmoc-N-hydroxysuccinimide ester, e.g., in 50:50 ethyl ether / dichloromethane, or with any suitable Fmoc (9-fluorenylmethoxycarbonyl) reagent, such as are commonly used in peptide synthesis. Such a reagent can be removed later as desired with an amine such as piperidine. Other removable N-protecting groups can also be used. With the exterior amino groups blocked, e.g., as their Fmoc carbamates, the interior amino groups are still available for reaction with amino-reactive reagents.

The population of microspheres with blocked surface amino groups and unblocked internal amino groups, chosen for reaction with various ligand groups in reactive form, is divided into a plurality of subpopulations. The number of subpopulations is chosen to reflect the number of ligands in the set to be analyzed for protein binding. An outstanding feature of the method of the invention in its various embodiments is that each of a plurality of ligands can be evaluated for binding to a protein of interest in a single analytical run. This allows for high throughput evaluation of many potential ligands for a protein of interest, such as an antibody, a receptor, an enzyme, or any other protein for which evaluation of binding of a library of potential ligands is needed to identify those specific effective ligands (potential antigens, potential receptor modulators, potential enzyme inhibitors, etc.) that bind to the protein. A ligand can be a peptide analog, also known as a peptoid. A peptide analog can be a model for a binding site of a second protein to the protein undergoing evaluation. The automated operation of the FACS or similar system makes high throughput evaluation with automated data collection and analysis possible, allowing large numbers of potential ligands to be screened efficiently.

The interior of TentaGel® microspheres can be modified covalently with a particular mixture of encoding dyes in defined ratios. Due to the modification of the interior of the microspheres, the protein ligand would never be in physical proximity to these hydrophobic dyes, which could otherwise cause protein binding unrelated to the ligand. Furthermore the dyes, being covalently bound in each microsphere's interior, are unable to leach out of the microspheres during subsequent synthetic operations in organic solvents. The potential ligand for evaluation is then coupled to, or synthesized on, the surface-accessible PEG-NH₂ layer.

We first examined whether 10 μm diameter TentaGel® beads are amenable to the described biphasic bead encoding strategy. We used confocal microscopy to show that beads comprised of externally-labeled fluorescein isothiocyanate (FITC) and internally-labeled Texas Red® (Life Technologies, Thermo Fisher Scientific) dye exhibited a clear spatial separation of the two fluorochromes. Having demonstrated that 10 μm size beads can be topologically segregated, we first chose to encode 24 subpopulations of beads using our encoding strategy wherein the ratios of internally labeled Pacific Orange™ and Pacific Blue™ dyes were varied. To achieve 24 non-overlapping populations, we created 8 ratios of 3 different stock solutions of Pacific Orange and Pacific Blue activated esters. After protecting the amine groups in the hydrophilic surface layer of the beads, a small fraction of the interior amines were covalently modified with the activated esters of Pacific Blue™ and Pacific Orange™. An overview of the labeling procedure is shown in Figure 2. These dyes were chosen because each absorbs at the same wavelength (λ_{max} at about 400 nm), but have very well-separated emission maxima, Pacific Blue™ λ_{max} at about 450 nm, Pacific Orange™ λ_{max} at about 550 nm. Encoding was achieved by both altering the absolute concentration of the dye as well as the ratio between the two. As shown in Fig. 1E, the 24 differentially-encoded bead populations were readily visualized on the FACS plot. Indeed, the degree of separation indicates that there is room for a further increase in the number of differentially encoded beads, which could be achieved by using more than three stock solutions, more than eight ratios of the dyes, or both. Using this strategy, separation of 36 subpopulations was achieved, although a 24 subpopulation assay can be sufficient for many uses. Furthermore, if one wished to examine more than 24 small molecule-protein interactions in this fashion, more than one well of sample could be employed and 24 different beads could be incubated with sample in each well. Any pair, or set, of encoding dyes, can be selected by the person of ordinary skill such that the two or more encoding dyes have sufficiently distinct emission maxima that they can be differentiated by the FACS.

Given that serological measurements are particularly challenging with respect to interference from non-specific protein binding, we were interested to compare the TentaGel® beads with Luminex® microspheres in the hopes that the highly PEG-coated surface layer could both shield these nonspecific

interactions and promote specific, avidity-driven binding. We assessed the utility of the TentaGel® microspheres to detect anti-ADP3 IgY in chicken serum using immobilized ADP3. This experiment did not require bead encoding. 10 µm TentaGel® beads were first primed by acylating the amine groups with an activated ester of 3-maleimidopropionic acid. ADP3 was then attached to the microspheres via Michael addition. Chicken serum (2 mg/mL total protein) was doped with IgY from ADP3-immunized chickens, or with nonspecific IgY as a negative control. After overnight incubation, the beads were washed and hybridized with phycoerythrin (PE)-conjugated anti-IgY secondary antibody, and binding of IgY to the beads was measured by monitoring PE emission of each bead by flow cytometry (Figure 3, A and B). As expected, the microspheres incubated with chicken sera containing anti-ADP3 IgY exhibited binding saturation behavior, and a marked increase in intensity compared to the control IgY sample. Therefore, the TentaGel®-based immunoassay was able to clearly differentiate between specific and nonspecific interactions.

We proceeded to compare our liquid array platform with Luminex® microspheres to ask if the PEG layer of TentaGel® indeed exhibits a lower background signal due to the PEG surface coating. Because Luminex® microspheres are sold with a terminal carboxylic acid, the linker between the bead and ADP3 differed in the directionality of the amide bond, however all other aspects of the experiment were performed similarly. The apparent affinity of ADP3 for anti-ADP3 IgY was the same on the Luminex® platform ($K_D = 0.3 \pm 0.1$) as it was on the TentaGel® platform ($K_D = 0.2 \pm 0.1$), indicating that avidity effects are also operational on the Luminex® surface. However, a signal only modestly lower than that seen for the anti-ADP3 antibody was observed when the ADP3-modified Luminex® beads were exposed to serum lacking anti-ADP3 antibodies, presumably due to nonspecific interactions of other IgY antibodies with the Luminex® resin. These data show that the suppression of non-specific binding due to the PEG layer on the TentaGel® beads is a major advantage in this type of direct binding assay.

Each subpopulation of microspheres can be treated with a mixture of the two or more dyes in a different, distinct, molar ratio. This ratio, as detected by the FACS or by a fluorescence spectrometer, results in a ratio of intensities of the two (or more) characteristic wavelengths of light emitted under stimulatory

illumination. For example, eleven distinct subpopulations can be “encoded”, i.e., labeled for identification by ratios of emitted light intensities at characteristic wavelengths, using 10% steps in ratios of the dyes, provided the amino-reactive dyes have comparable rates of reaction with amino groups.

5 Using Pacific BlueTM (PB) and Pacific OrangeTM (PO) as exemplary dyes, one can have a first subpopulation with 0% PB / 100% PO, a second subpopulation with 10% PB / 90% PO, and so forth, up to an eleventh subpopulation with 100% PB / 0% PO tagging. Another fluorescent dye useful for carrying out a method of the invention is Alexa Fluor® 647. Each
10 subpopulation can be encoded in this manner by soaking the microspheres of that subpopulation in the two dyes in the defined ratio, e.g., in DMF, or in any organic solvent that does not react with the microsphere resin. By using a third amino-reactive dye for microsphere encoding, an even larger number of subpopulations can be encoded or enabled for identification by the method
15 described above.

 Having established the encoding capabilities of the microspheres, we next evaluated multiplexed protein detection using ligands immobilized to the surface layer of the coded beads. We chose as a target chicken IgY antibodies from an animal immunized with a synthetic molecule, a peptoid termed ADP3
20 (29) (Figure 4A). We performed a “methyl scan”⁽³⁰⁾ on ADP3 to determine what side chains in ADP3 are critical for binding to the anti-ADP3 IgY antibodies. Each side chain, in turn, in ADP3 was replaced by a methyl group (excluding cysteine). Titrations monitored by fluorescence polarization (FP) spectroscopy were performed to determine the affinity of each of these eight variants for anti-
25 ADP3 IgY. Binding was severely compromised only when the position 2 side chain or the position 7 side chain was replaced with a methyl substituent, indicating that these residues are particularly important for molecular recognition (Figure 4B). The other substitutions had either no effect or reduced binding only modestly. We also constructed an ADP3 variant that possessed a
30 methyl substituent at every position except for positions 2 and 7, which contained the original side chains. This variant still bound to the IgY antibodies, but showed a significantly reduced affinity, indicating that the sum of eliminating several minor contacts results in a large effect on binding. With these data in hand, we then evaluated if similar results could be obtained in a

single multiplexed experiment using encoded TentaGel® beads as described above.

Seven dye-encoded bead populations were modified with either ADP3-Cys or one of six derivatives. After deprotection of the surface amines, they were primed by coupling of the activated ester of 2-bromoacetic acid (Figure 4C). These six derivatives were the two single methyl derivatives with poor binding (2-Me and 7-Me), the hexamethyl derivative and three methyl derivatives that bound nearly as well as ADP3, taken from the set described above. The bead populations were mixed together and added to solutions comprised of serially diluted anti-ADP3 IgY spiked into PBS Starting Block®. After washing, Texas Red®-conjugated secondary antibody was added. After incubation and another washing step, the beads were analyzed on a flow cytometer. The trends observed for the ADP3-IgY binding affinity in the microsphere assay mirrored those observed using FP (Figure 4D). ADP3-2Me, ADP3-7ME and the hexamethyl derivative all bound the antibody with much lower affinity than ADP3 itself, while the other methyl derivatives bound the IgY with only modestly lower affinity.

Interestingly, the K_D values determined using the microsphere assay were lower, by about a factor of ten, than those derived from the FP data, indicating higher affinity binding on the bead support (Table 1). This trend was observed for antibody-antigen interactions over a wide range of K_D values, including the nanomolar interaction between FLAG tag and the anti-FLAG monoclonal antibody.

Table 1. Dissociation constants determined for small molecule binding probes and their targets.

Variant	K_D (FP assay, μM) ^a	K_D (TentaGel assay, μM) ^a
ADP3	4.4 ± 1.1	0.5 ± 0.2
ADP3-1Me	2.0 ± 1.5	ND
ADP3-2Me	DNS	DNS
ADP3-3Me	2.2 ± 1.6	0.4 ± 0.2
ADP3-4Me	2.3 ± 1.9	0.4 ± 0.2
ADP3-5Me	3.4 ± 0.7	1.2 ± 0.5
ADP3-6Me	8.3 ± 4.3	ND

ADP3-7Me	DNS	DNS
ADP3-8Me	3.3 ± 2.5	ND
ADP3-hexamethyl	DNS	DNS
FLAG	0.090	0.008
BBHit3	26.7 ± 11.7	17.3 ± 5.3

^aReported K_D for the ADP3 variants is an effective K_D as the IgY has not been purified with respect to antigen specificity; ND, Not Determined; DNS, Did Not Saturate.

Antibodies are bivalent molecules and thus it is possible that the beads allow for avidity-driven binding (i.e., two immobilized ADP3 molecules bind to a single IgY antibody). To test this idea, we conducted an experiment with a small molecule ligand that binds to a monovalent protein. For this, we chose the monomeric serine hydrolase PAFAH-1B2 that binds to the synthetic ligand BBHit3 with modest affinity. We monitored binding by monitoring fluorescence anisotropy of a fluorescein-labeled BBHit3 (Figure 4E). We then compared this binding affinity to the one determined by immobilizing BBHit3 to the TentaGel® platform (Figure 4F). In this case, the K_D values derived from the FP and TentaGel® assays were almost identical within experimental error (Table 1). Thus, the clustered presentation of ligands in the PEG layer of the TentaGel beads appears to afford an avidity-driven boost in binding to bivalent antibodies.

Following encoding of each subpopulation with the two or more encoding fluorescent dyes covalently bound to the internal amino groups, each distinct subpopulation is treated to bind a potential ligand to the surface amino groups. To accomplish this, the N-blocked surface amino groups are deblocked, e.g., with piperidine or the like to remove Fmoc groups, and then bonded by any of a number of methods to the ligand derivative, providing for covalent attachment of the respective ligand to the surface of at least some of the individual microspheres of each subpopulation.

Ligands can be of a widely diverse range of chemical structures, and a number of different approaches can be used to couple them to the surface amino groups. For example, a ligand can itself bear an amino-reactive group disposed in the molecule in such a way as to not interfere with binding of the ligand to the protein. Or, the resin of the microsphere, e.g., the surface amino groups, or other

functional groups disposed on the polymer backbone or the polyethyleneglycol coating of the microsphere, can be modified to provide a group other than an amino group; for example, a coupling reagent can be used that itself can react with an amino group, and possesses a second domain with a different type of reactive group, e.g., a thiol group.

Each subpopulation of microspheres, uniquely encoded with the two or more fluorescent dyes, is coupled to a respective ligand. Not every subpopulation need be coupled to a unique ligand, i.e., duplicate subpopulations can be prepared, but for the high throughput features of the invention are best used when the number of distinct ligands tested in each analytical run is maximized. Control subpopulations, i.e., those bearing no ligand, can also be part of an analytical run.

All the subpopulations to undergo evaluation in an analytical run are then combined, and are treated with the protein sample under evaluation. Those potential ligands that in fact bind the protein bring about association of the protein with the surface of that microsphere. Those ligands that do not bind the protein sample under evaluation do not bring about association of the protein with the surface of the microsphere. There can be non-specific binding of the protein to the microspheres, such as due to general hydrophobic interactions. Such non-specific binding can be evaluated by the use of control subpopulations of microspheres not bearing any ligand. It is believed by the inventors herein that the use of polyethyleneglycol coated microspheres, such as the TentaGel® microspheres, can act to reduce or minimize non-specific binding of the protein to the material of the microsphere surface.

The protein is capable of association with a protein-associated fluorescent reporter dye, which association can take place by a variety of mechanisms. For example, the protein itself can be labeled with a fluorescent reporter dye in such a manner as not to interfere with binding of an active ligand to the ligand binding site on the protein. Or, an antibody labeled with a fluorescent reporter dye can bind to an epitope of the test protein not involved in binding the ligand of the microsphere subpopulation. For example, the reporter molecule can be a protein-associate fluorescent dye (e.g. Alexa Fluor® 647) bonded to an antibody specific for an epitope disposed on the protein undergoing evaluation.

Or, a protein can have a second binding site for a known ligand, and the known ligand bonded to the protein-associated fluorescent reporter dye can be used to label the protein in a manner that does not interfere with binding to the test ligand. Thus, the protein used in the analysis is bound to or is capable of binding a reporter molecule, the reporter molecule bearing a protein-associated fluorescent reporter dye immobilized thereto, wherein each of the encoding fluorescent dyes and the protein-associated fluorescent reporter dye has a unique fluorescence emission spectrum.

The protein-associated reporter dye has a characteristic fluorescence emission that can be distinguished by the detection device (FACS) from the fluorescence emissions of the two (or more) encoding dyes used to label the microspheres of each subpopulation. Because of the distinct characteristic fluorescent emissions of the three (or more) fluorescent dyes, i.e., the two or more encoding dyes and the protein-associated dye, each microsphere, as it undergoes analysis by the FACS device, can be (a) identified as to its particular subpopulation, i.e., as to the identity of the potential ligand that that subpopulation bears, and (b) the presence or absence of the protein bound to the surface of the microsphere, i.e., whether or not the potential ligand of that microsphere subpopulation is in fact a ligand of the test protein. The automated analysis and data collection capabilities of the FACS provide for the analysis of large numbers of individual microspheres, each of which belongs to one of the plurality of subpopulations undergoing analysis. The number of individual microspheres in each subpopulation provides for replicate analyses for purposes of validation, reducing the incidence of false positives or false negatives that might result from variation in properties among individual microspheres.

FACS is well known in the art to operate by means of a beam of light (usually laser light) which can be of a single wavelength or can be of multiple wavelengths (i.e., multiple discrete wavelengths, or a broad band of wavelengths of light, such as ultraviolet (UV) or visible light for stimulation of fluorescence) is directed onto a hydrodynamically-focused stream of liquid. A number of detectors can be aimed at the point where the stream passes through the light beam: such as detectors for visible light, which can be tuned to suitable wavelengths for the signal to be detected. Each suspended microsphere can be of about 0.2 to 150 μm in diameter in normal operation, preferably about 5-50 μm .

Preferably the microspheres are of substantially uniform size. In passing through the illuminating beam (e.g., of UV-visible light), the fluorescent dyes bound in the interior or bound to the surface of the microsphere are excited by the stimulatory light into emitting light at a longer wavelength than the light source. This combination of scattered and fluorescent light is picked up by the detectors, and, by analyzing fluctuations in brightness at each detector (one for each fluorescent emission peak), it is then possible to derive information about the constituents of each individual microsphere. This information includes the encoding, revealing the identity of the ligand bound to each microsphere, and the binding or non-binding of the test protein thereto.

In the present invention, substantially every microsphere has at least two distinct fluorescent dyes bonded thereto, each having characteristic wavelength of fluorescent emission, the ratios of intensities of which emissions are used to determine the identity of the subpopulation to which the particular microsphere belongs, as described in greater detail below. In addition, the protein-associated fluorescent reporter dye, having yet another characteristic wavelength of fluorescent emission, is bonded to the protein under evaluation. Accordingly, the FACS-type detector is capable of measuring the output of fluorescent emissions at a minimum of three discrete wavelengths, and of stimulating the fluorescent dyes emitting those photons at the wavelengths of shorter-wave light necessary to do so.

We have further increased the number of subpopulations that are separable by flow cytometry to a total of 36 subpopulations, using differential internal dye concentrations for Pacific OrangeTM (PO) and Pacific BlueTM (PB). This was accomplished by applying six to eight varying ratios of the two dyes to each population of beads. The number of six to eight ratios was further multiplied by increasing the concentration of stock dye solution but keeping the ratios fixed. By using five different stock concentrations and six to eight ratios of those dye stocks, we were able to encode 36 populations of beads. Fig. 5 shows clear separation between the different bead subpopulations.

Biphasic beads were prepared as described and treated with varying ratios of a Pacific BlueTM or Pacific OrangeTM NHS ester stock solution in 200 μ L DMF. The stock solutions were prepared as described below. To obtain more than the original 24 subpopulations, we prepared two sets of Pacific BlueTM and

Pacific OrangeTM stock concentrations: 75 μ M Pacific BlueTM and 350 μ M Pacific OrangeTM applied to 3 mg of 10 μ m beads in seven different ratios (PB:PO = 0:100, 20:80, 40:60, 60:40, 75:25, 90:10, 100:0). The last row was prepared using the same ratios, but with dye-NHS ester solutions comprised of
5 200 μ M Pacific BlueTM and 1 mM Pacific OrangeTM. Ligands were immobilized onto each of the 36 populations of beads and the beads were used for measurement.

In addition to these 36 subpopulations, we have used Texas Red® as a third internal dye. The excitation and emission of Texas Red® minimally
10 overlaps with Pacific OrangeTM and Pacific BlueTM, providing a third dimension of separation. For each concentration of Texas Red® (including no incorporation), we can separate 36 additional populations.

We have also investigated the use of different linkers separating the TentaGel® PEG chain and the tested ligand of interest. We used a modest
15 affinity ligand (HH031) discovered in an OBOC screen against a monoclonal antibody called CLL169. We found that longer linker lengths provide better signal. However, no added benefit was observed using more than one 9-atom PEG linker. Moreover, nonspecific interactions were markedly reduced with the PEG-derived linkers.

20 Given that the presence of a PEG linker between the polystyrene core and ligand of interest is the major difference between Luminex® and TentaGel® beads, we investigated how different linkers added to this PEG linker might affect binding. We immobilized a series of linkers to the 10 μ m TentaGel® beads (**1–5** in Fig. 5) followed by a modest affinity ligand (HH031) discovered
25 in an OBOC screen against a monoclonal antibody called CLL169. We then probed the beads with a 2-fold serial dilution of purified CLL169 and looked at binding affinity to HH031 with respect to the different linkers. Figs. 6B and 6C show that longer linker lengths provided stronger binding. However, no added benefit was observed using more than one 9-atom PEG linker (**4** vs. **5**),
30 indicating a practical limit to the binding improvement by increasing linker length. As expected, nonspecific interactions were markedly reduced with the PEG-derived linkers when beads were probed with negative control serum.

Five populations of biphasic beads were prepared and encoded as described above. Terminal Fmoc-protecting groups were removed from the outer

bead layer using 20% piperidine (2 x 200 μ L) followed by thorough washing. With the exception of **1**, a linker derived from the following Fmoc-protected acids was incorporated onto each population using HBTU/DIEA activation for 1 h: β -alanine (**2**), aminohexanoic acid (**3**), Fmoc-8-amino-3,6-dioxaoctanoic acid (9 atom PEG, **4**), or Fmoc-18-amino-4,7,10,13,16-pentaoxaoctadecanoic acid (19 atom PEG, **5**). Following linker immobilization, the beads were washed in DMF and combined. Linker protecting groups were cleaved with 20% piperidine, washed thoroughly with DMF, and primed with bromoacetic acid as described. HH031, containing a terminal cysteine was immobilized onto each of the populations by incubation with the bromoacetylated beads overnight at 37 °C. Beads were washed, quenched, and re-introduced to aqueous media as described.

Incubations with CLL169 antibody were performed in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.05% Tween-20 using a starting concentration of 700 nM CLL169, and 2-fold serial dilutions thereof. Hybridizations were performed at 4 °C overnight and washed with TBS-T. Binding to each population was monitored by hybridizing Alexa Fluor 647-conjugated anti-IgG for 1 h at room temperature. After washing the beads with TBS-T, they were analyzed by FACS. Mean fluorescence intensity (MFI) is determined by averaging the fluorescence intensity of every bead, using a minimum of 1000 beads for each read.

In conclusion, we have demonstrated the utility of a color-coded liquid bead array system that is convenient and effective for the multiplexed, quantitative measurement of synthetic molecule-protein interactions in complex biological mixtures. At least 36 independent measurements can be made in a multiplexed fashion in a single sample. The use of PEG-coated microspheres minimizes nonspecific binding that is encountered using traditional polystyrene surfaces when high serum concentrations are used. Attractive applications of this technology will include the characterization and optimization of protein ligands that have been identified in a primary screen, and in the detection of diagnostically useful immune complexes contained within sera.

EXAMPLES

An example of encoding amino TentaGel® microspheres into subpopulations with a unique spectral signature using two fluorophore dyes is

illustrated in Figure 1. Each spectral region represents a unique ligand that can be “gated” using FACS software to independently measure the relative binding of a protein-associated fluorophore to each microsphere, which is the third fluorophore in this illustration. The third fluorophore in this example was
5 conjugated to a secondary antibody and directly correlated to analyte binding (Figure 1A). Using this strategy, we were able to measure binding of serum proteins to 24 different ligands in one sample (Figure 1B). To encode the interior of the microsphere, we topologically segregated the microsphere by soaking them in aqueous solution followed by introduction of 0.5 equivalents 9-fluorenylmethoxycarbonyl succinimidyl ester (Fmoc-Osu) in 50:50 ethyl
10 ether/dichloromethane. The remaining, interior, amines were tagged with varying ratios of pacific orange and pacific blue. After deprotecting the exterior amines, each subpopulation of coded microspheres was bromoacetylated and tagged with a unique ligand through a terminal cysteine bearing an alkyl
15 bromide-reactive sulfhydryl group. We have demonstrated the utility of this method as a multiplexing immunoassay using standard peptide epitopes (Figure 2). Three populations of microspheres, were separated and encoded with different ratios of Pacific OrangeTM and Pacific BlueTM. Each subpopulation was bromoacetylated and to each was added a common peptide epitope, either MYC,
20 HSV and a scrambled MYC variant. The epitopes were installed onto the microsphere through a C-terminal cysteine. Following peptide immobilization, the remaining unreacted alkyl bromide was quenched with 2-mercaptoethanol. The beads were mixed into a single sample and washed in water 10 times. The last water wash was performed overnight. Next, the microspheres were washed 5
25 times in tris-buffered saline containing 0.05% Tween 20 (1X TBS-T). The microspheres were blocked in PBS Starting Block® before being added to two-fold serial dilutions of a buffered solution containing a mixture of monoclonal antibodies raised against the c-MYC and HSV epitopes. The bead-antibody solution was incubated overnight at 4 °C. The microspheres were washed 3
30 times by evacuating the contents of the well and replacing with TBS-T followed by gentle pipetting of the solution. Texas Red®-conjugated secondary antibody was hybridized to the sample for 1 h at room temperature, and washed with TBS-T 3X. The microspheres were filtered to remove large particulates, sorted, and read using FACS. Thus, this work highlights the potential importance of this

technique as a multiplex-capable serological immunoassay for modest-affinity ligands.

Reagents

5 All chemicals and solvents were purchased from commercial suppliers and used without further purification. HPLC grade solvents were used for purifications. All steps involving water utilized distilled water filtered through a Barnstead Nanopure filtration system (Thermo Scientific)

Confocal microscopy.

10 10 mg of 10 μ m TentaGel® microspheres were equilibrated in water overnight. After swelling, the beads were topologically segregated (*vide supra*). Texas Red® sulfonyl chloride (25 μ g, 40 nmol) dissolved in 250 μ L DMF was added to the beads and shaken overnight. After washing the resin 5 times in DMF, the unreacted interior amines were capped by acylation using 20% acetic anhydride in DMF. The beads were washed (5 x 300 μ L DMF) times and the
15 exterior amines were deprotected in 20% piperidine. After washing the beads (5 x 300 μ L DMF), a solution of fluorescein isothiocyanate (FITC, 250 μ g, 0.64 μ mol) in DMF was added to the beads and shaken overnight. The beads were washed in DMF (3 x 300 μ L) the water (5 x 300 μ L) and brought up into 100 μ L water. 5 μ L of the microsphere solution was co-spotted with 5 μ L ProLong®
20 Gold antifade reagent (Life Technologies) onto a glass microscope slide, sealed with a cover slip and dried overnight. Confocal microscopy was performed on an Olympus Fluoview 1000® confocal microscope at 100X magnification and analyzed using the F10-ASW 3.0 software.

TentaGel® microsphere encoding.

25 TentaGel® microspheres (Rapp Polymere GmbH), were topologically segregated using the method of Lam and coworkers.⁽³¹⁾ 10 μ m microspheres (0.05 g, 0.23 mmol/g) were incubated in water overnight. After centrifuging at 500 x g for 1 min, the supernatant was decanted. N-(9-Fluorenylmethoxycarbonyl) succinimide (Fmoc-Osu, 1.9 mg, 0.5 eq.) dissolved
30 in 2.5 mL of a diethyl ether/dichloromethane (DCM) 50:50 mixture was added to the microspheres in a 5 mL centrifuge tube. The microspheres were shaken vigorously and vortexed and sonicated for 30 s each and rotated for 20 min thereafter. The microspheres were collected by centrifugation (500 x g) and washed 4 times in DMF (NOTE: In some cases, it may be necessary to add DMF

to the diethyl ether/DCM solution to help pellet the microspheres during the first centrifugation step). After washing in DMF, the microspheres were re-equilibrated in DMF for 3 h. Failure to re-equilibrate the beads in DMF resulted in heterogeneous internal dye concentrations as indicated by a large range of microsphere internal dye intensities. The washed, equilibrated resin was aliquoted (2 mg) subpopulations into black centrifuge tubes and diluted to 200 μ L volume with anhydrous DMF. Stock concentrations of the activated Pacific BlueTM and Pacific OrangeTM NHS esters (Life Technologies) were prepared at 3 μ M and 15 μ M, respectively, and added in eight different ratios such that the total volume of all added dye solutions was 10 μ L. The 8 ratios were obtained by addition of the following ratios of Pacific OrangeTM and Pacific BlueTM to the microsphere aliquots, respectively: 0:100, 5:95, 10:90, 30:70, 50:50, 70:30, 90:10, 100:0. DIPEA (1 μ L, 0.005 mmol) was added to the microsphere suspension to facilitate acylation. To produce up to 16 populations, the same eight ratios were applied using 9 μ M Pacific BlueTM and 45 μ M Pacific OrangeTM as the fluorochrome stock concentrations. To achieve 24 populations, 27 μ M and 135 μ M Pacific OrangeTM were added in the same ratios. It is necessary to optimize the ratios when purchasing new lots of fluorophore NHS esters. After addition of the dyes, the microspheres were vortexed for 30 s and rotated overnight. After washing the beads thoroughly, the unreacted interior amines were capped with 20% acetic anhydride for 20 min. Washing was best accomplished by centrifuging the microspheres, pouring off the supernatant, applying fresh DMF and pipetting the solution up and down 5–10 times. Washing was repeated 5 times after each step.

25 Peptoid and azapeptoid synthesis.

Peptoids and azapeptoid BBHit3 were synthesized on Rink Amide resin (0.32 mmol/g) using previously described protocols.^(23, 32-34) The resin (0.1 g, 0.032 mmol) was swelled in DMF for 2 hours prior to use. 9-Fluorenylmethoxycarbonyl (Fmoc) was removed by 20% piperidine and washed thoroughly in DMF. N- α -Fmoc-S-*p*-methoxytrityl-L-cysteine, (0.1 g, 0.16 mmol) was coupled to the resin using *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU, 0.061 g, 0.16 mmol) and diisopropylethylamine (DIPEA, 0.06 mL, 0.32 mmol). Fmoc was deprotected with 20% piperidine and washed thoroughly (3 x 3 mL DMF). The growing

chain was bromoacetylated using 1 mL 2 M 2-bromoacetic acid (BAA) and 1 mL 2.5 M diisopropylcarbodiimide (DIC). The mixture was shaken at 37 °C for 10 min and washed thoroughly. Primary amines and acyl hydrazide sub-
monomers were added to the bromoacetylated resin as 1 M solutions in DMF
5 and shaken at 37 °C for 1 h. 1-(*t*-butoxycarbonyl)-diaminobutane and glycine *t*-butyl ester were used as protected primary amines. Treatment of MMT-protected cysteine with 2% TFA in DCM (5 x 2 min) gave the free sulfhydryl, upon which fluorescein-5-maleimide was conjugated. For fluorescein conjugation, the resin was neutralized with 10% DIPEA, washed (5 x 2 mL DMF), and incubated with
10 a 5 mM solution of fluorescein-5-maleimide in DMF for 3 hours at room temperature. Peptoids were simultaneously deprotected and liberated from the resin by incubating in a cocktail of TFA:H₂O:TIS (95:5:5) for 2 hours. Oligomers were purified on a Vydac reverse-phase C18 column (Grace), freeze dried, and stored without further modification. Peptoid identity was confirmed
15 by matrix-assisted laser desorption ionization-time of flight mass spectrometry.
Fluorescence polarization.

Probe concentrations were determined using the absorbance of fluorescein at 495 nm ($\epsilon_{280} = 78,000 \text{ M}^{-1}\text{cm}^{-1}$) using a Nanodrop UV-vis spectrophotometer (Thermo Scientific). FP experiments were performed in 384-
20 well half-area, medium bind microtiter plate (Greiner Bio-One) using a probe concentration of 10 nM and two-fold serial dilutions of the target protein or antibody in PBS. Measurements were performed on a 2104 EnVision Multilabel Plate Reader (PerkinElmer) using 450 excitation and 515 nm emission filters. Fitting of the saturation curves to obtain K_D values was accomplished using
25 Prism (GraphPad Software, Inc.) with a one-site saturation with Hill slope model. K_D values are the average of three independent runs with standard deviation reported.

Probe conjugation to encoded TentaGel® microspheres via thioalkylation.

After encoding the microspheres, the beads were incubated in 20%
30 piperidine in DMF to afford the free external amines. The terminal amines were bromoacetylated by incubation with 150 μL BAA (2 M in DMF) and 150 μL DIC (2.5 M in DMF) for 15 minutes at 37 °C. After washing (5 x 300 μL DMF), the ligand of interest (0.46 μmol , 2 eq.) containing a terminal cysteine was dissolved in a 50:50 mixture of DMF and PBS (2.5 mg/mL) and applied to the

bead suspension. Thioether formation was permitted to occur overnight at 37 °C. The beads were washed 3 times in DMF and quenched in aqueous media. These transformations did not result in any apparent chemical bleaching.

Quenching of TentaGel® microspheres.

5 The microspheres were transferred to a 96-well filter plate and washed (10 x 300 µL H₂O) and equilibrated in water overnight. The microspheres were quenched with 150 mM 2-mercaptoethanol in PBS and washed five times in PBS. Beads were equilibrated in TBS containing 0.05% Tween 20 (TBS-T) for 1 h. The quenched microspheres were transferred to a centrifuge tube where they
10 were blocked in PBS containing 50% Starting Block® for 1 hour (200 µL total volume). Beads were immediately used for assays or stored in the blocking solution in the dark at 4 °C. Beads left in the dark did not exhibit any photobleaching for >10 days (Supplementary Figure S7).

ADP3 variant K_D determination using encoded microspheres.

15 A stock solution containing 14 µM IgY from a chicken immunized with ADP3 (Creative Biolabs) was prepared in PBS containing 50% Starting Block® and serially diluted into 150 µL of the blocking solution in a MultiScreen® Solvint 96-well filter plate (EMD Millipore). Seven subpopulations of 10 µm TentaGel microspheres containing ADP3 variants were encoded, quenched, and
20 blocked as described, and then distributed (3 µL) among the IgY solutions. The beads were incubating overnight at 4 °C with gentle shaking, washed (3 x 300 µL TBS-T) and hybridized for 1 h at room temperature with Texas Red®-conjugated anti-chicken secondary antibody (Thermo Scientific) as a 1:200 dilution in the blocking buffer. After washing the beads (3 x 300 µL TBS-T), the
25 Texas Red® mean fluorescence intensity (MFI) was monitored by fluorescence-activated cell sorting (FACS). We found no evidence of internal dye or reporter fluorophore self-quenching, indicating minimal spectral overlap.

Fluorescence-activated cell sorting (FACS).

 FACS was performed on an LSRII (BD Biosciences) using violet and red
30 lasers. Emission intensities were monitored at 450 nm (Pacific Blue™), 550 nm (Pacific Orange™), and 615 nm (Texas Red®). At least 500 microspheres were collected for each analysis and the MFI of the Texas Red® emission intensity was calculated using FlowJo software (Tree Star, Inc.). Data reported are representative of two independent experiments. Fitting of the saturation curves

to obtain K_D values was accomplished using Prism (GraphPad Software, Inc.) using a one-site saturation model with Hill slope. All FACS experiments were performed in triplicate, and saturation plots display a single representative experiment. K_D values are an average of the three experiments with standard deviation reported.

Determination of K_D for the BBHit3 and PAFAH-1B2 interaction.

BBHit3 was immobilized onto uncoded 10 μ m TentaGel® microspheres via thioalkylation and quenched as described above. A 100 μ M stock solution of His-tagged PAFAH-1B2 or p53 in 50% PBS Starting Block® was two-fold serially diluted seven times in PBS containing 50% Starting Block® and added to a 96-well filter plate. Microspheres preblocked in PBS Starting Block® for 1 h were added to the serially diluted protein solutions. The plate was gently shaken overnight at 4 °C. The beads were washed (3 x 5 min TBS-T) and incubated with anti-His-tag secondary antibody (Sigma Aldrich) diluted 200-fold in 50% Starting Block® for 1 h at room temperature. After washing the beads (3 x 5 min TBS-T), AlexaFluor® 647-conjugated secondary antibody (Life Technologies, 1:200 dilution in the blocking buffer) was added to the beads and incubated for 1 h at room temperature. The beads were washed and binding of the targets was analyzed by FACS.

Conjugation of ADP3 to uncoded TentaGel® microspheres via Michael addition.

TentaGel® microspheres (0.002 g, 0.23 mmol/g) were equilibrated in DMF for 2 h. The terminal amines were capped with 3-maleimidopropionic acid NHS ester (0.01 g, 0.038 mmol) dissolved in DMF. After washing (5 x 300 μ L DMF), ADP3-Cys (0.6 mg, 0.46 μ mol) dissolved in 200 μ L 50:50 PBS/DMF was added to the bead suspension and shaken overnight at 37 °C. The beads were washed 3 times in DMF and quenched in aqueous media (see below).

Conjugation of ADP3 to Luminex microspheres.

ADP3 was immobilized onto Luminex xMAP® microspheres (Luminex Corporation) using a protocol obtained from the company with minor modifications. Briefly, 5.0×10^6 microspheres were transferred to a 1.5 mL centrifuge tube and pelleted by centrifugation at 9000 x g for 2 minutes. The supernatant was removed, and the pellet was resuspended in 100 μ L distilled H₂O by vortex and sonication for 20 s. The microspheres were pelleted by

centrifugation and the supernatant was removed using a pipette. The microspheres were resuspended in 80 μ L of 100 mM monosodium phosphate, pH 6.2, by vortex and sonication for 20 s. 10 μ L of 50 mg/mL Sulfo-NHS in H₂O was added to the microspheres and vortexed. This was immediately
5 followed by the addition of 10 μ L of a 50 mg/mL solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) diluted into H₂O. The suspension was gently vortexed and rotated for 20 minutes at room temperature, with occasional vortexing. The microspheres were pelleted by centrifugation, and the supernatant removed. The pellet was resuspended in 250 μ L PBS (10 mM
10 Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, pH 7.2) and vortexed and sonicated for 20 s. This washing step was repeated once. After concentrating and resuspending the microspheres in 100 μ L PBS, they were diluted into 150 μ L PBS containing *N*-(2-aminoethyl)maleimide trifluoroacetate salt (1 mg, 0.004 mmol) and this suspension was shaken overnight at room temperature. The
15 microspheres were washed twice in 250 μ L PBS. A 2.5 mg/mL solution of ADP3 in PBS was added to the maleimide-activated beads and the reaction mixture was shaken overnight at 37 °C. After conjugation, the beads were quenched with 150 mM 2-mercaptoethanol, washed thoroughly (5 x 300 μ L PBS) , and blocked with 50% PBS Starting Block® (Thermo Scientific) for 1 h.
20 The microspheres were used immediately without further modification or stored in the blocking buffer at 4 °C. No attempts were made at optimizing the conjugation protocol.

Serological assays.

Stock solutions were prepared containing either 14 μ M IgY from a
25 chicken immunized with ADP3, or 14 μ M nonspecific control IgY (Santa Cruz Biotechnology, Inc.) spiked into chicken serum (2 mg/mL total protein, Sigma Aldrich) and 50% Starting Block®. The stock solutions were two-fold diluted into 150 μ L PBS containing 50% Starting Block® in a 96-well filter plate. The quenched TentaGel® or Luminex® microspheres containing immobilized ADP3
30 were added to each well in 3 μ L aliquots. After incubating the microspheres overnight at 4 °C, the beads were washed 3 times with TBS-T, and hybridized for 1 h at room temperature with a phycoerythrin (PE) anti-chicken secondary antibody (Santa Cruz Biotechnology, Inc.) diluted 200-fold in the blocking

buffer. The microspheres were washed (3 x 300 μ L TBS-T), and the extent of PE binding to each microsphere was measured by FACS.

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All patents and publications referred to herein are incorporated by reference herein to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference in its entirety.

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The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be
25 understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

30

CLAIMS

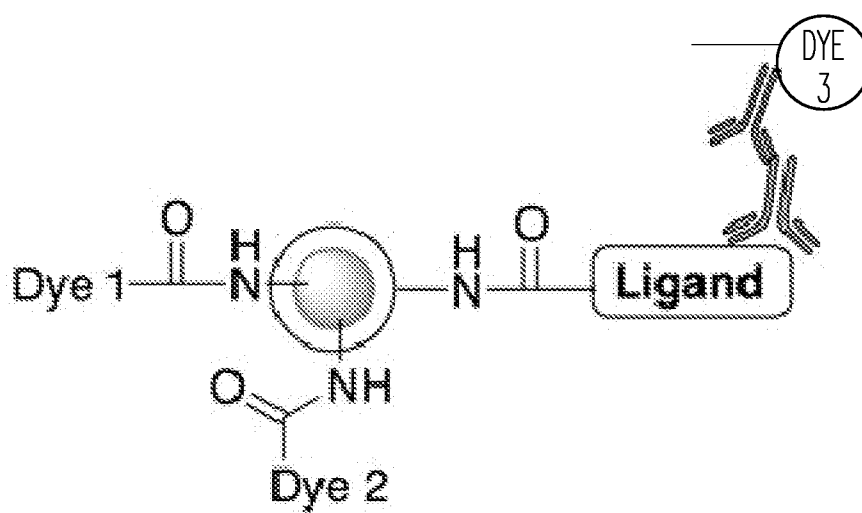
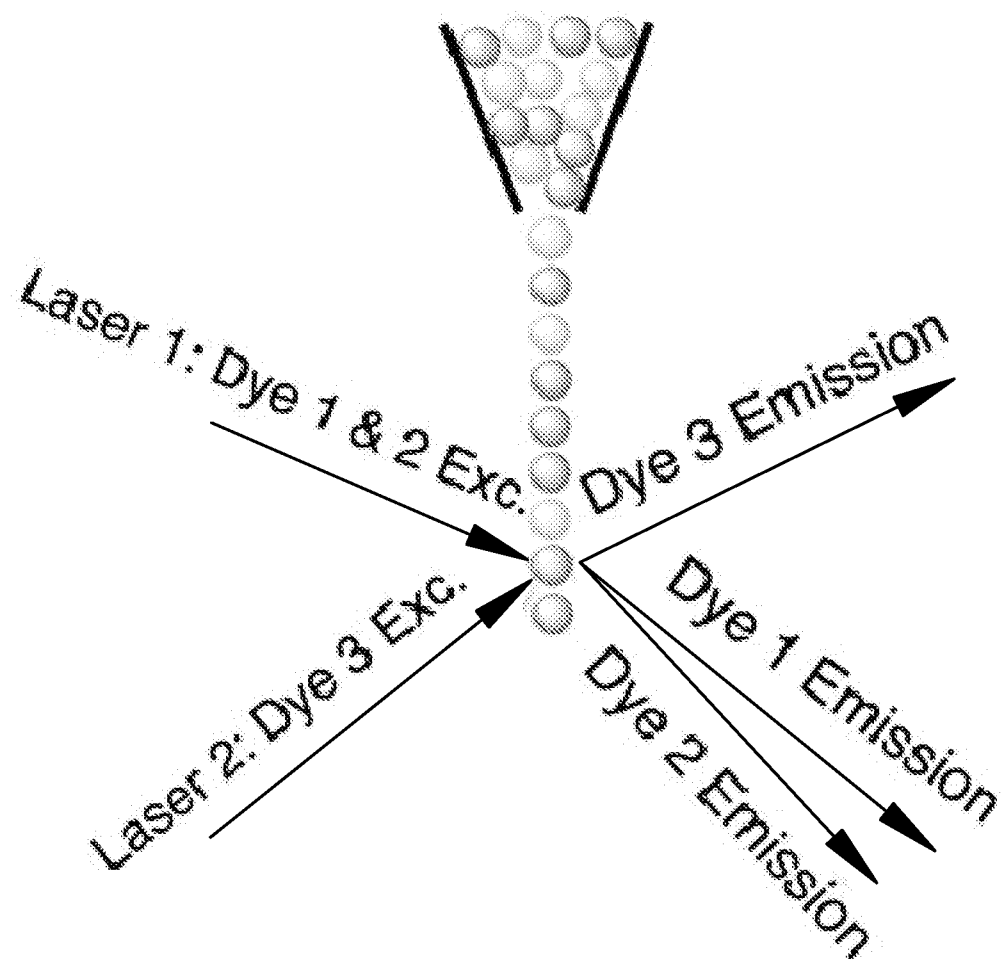
What is claimed is:

1. A method for determining protein binding to each of a plurality of ligands in a single analytical sample, comprising:
 - a) treating a population of polyethyleneglycol-coated amino-functionalized polystyrene microspheres comprising surface and internal amino groups with a amino group blocking reagent to selectively block surface amino groups, and dividing the population into a plurality of subpopulations of the microspheres; then,
 - b) treating each respective subpopulation of microspheres with two or more amino-reactive fluorescent encoding dyes in a respectively unique ratio to bring about reaction of internal amino groups of the microsphere with the two or more fluorescent encoding dyes, such that a microsphere of each subpopulation can be distinguished from a microsphere of each other subpopulation by a detection device that determines a ratio of a fluorescence emission intensities from each of the two or more fluorescent encoding dyes; then,
 - c) deblocking the surface amino groups, then treating each subpopulation of microspheres with a reactive form of a respective ligand from a set of potential ligands to be evaluated for protein binding interactions, to bring about coupling of the respective ligand to at least some of the microspheres of the respective subpopulation; then,
 - d) combining the plurality of subpopulations of microspheres, at least some of the microspheres of each subpopulation bearing the respective test ligand bonded thereto, and then contacting the combined microsphere subpopulations with a protein to be evaluated for protein binding interactions with the respective test ligands, and contacting the combined microsphere subpopulations with a protein reporter molecule comprising a fluorescent reporter dye, the protein binding or being capable of binding the protein reporter molecule, wherein each of the encoding fluorescent dyes and the protein-binding fluorescent reporter dye have a unique fluorescence emission spectrum; then,
 - e) analyzing each microsphere of the combined sample with the detection device to determine a ratio of fluorescence emission intensity of each of the two

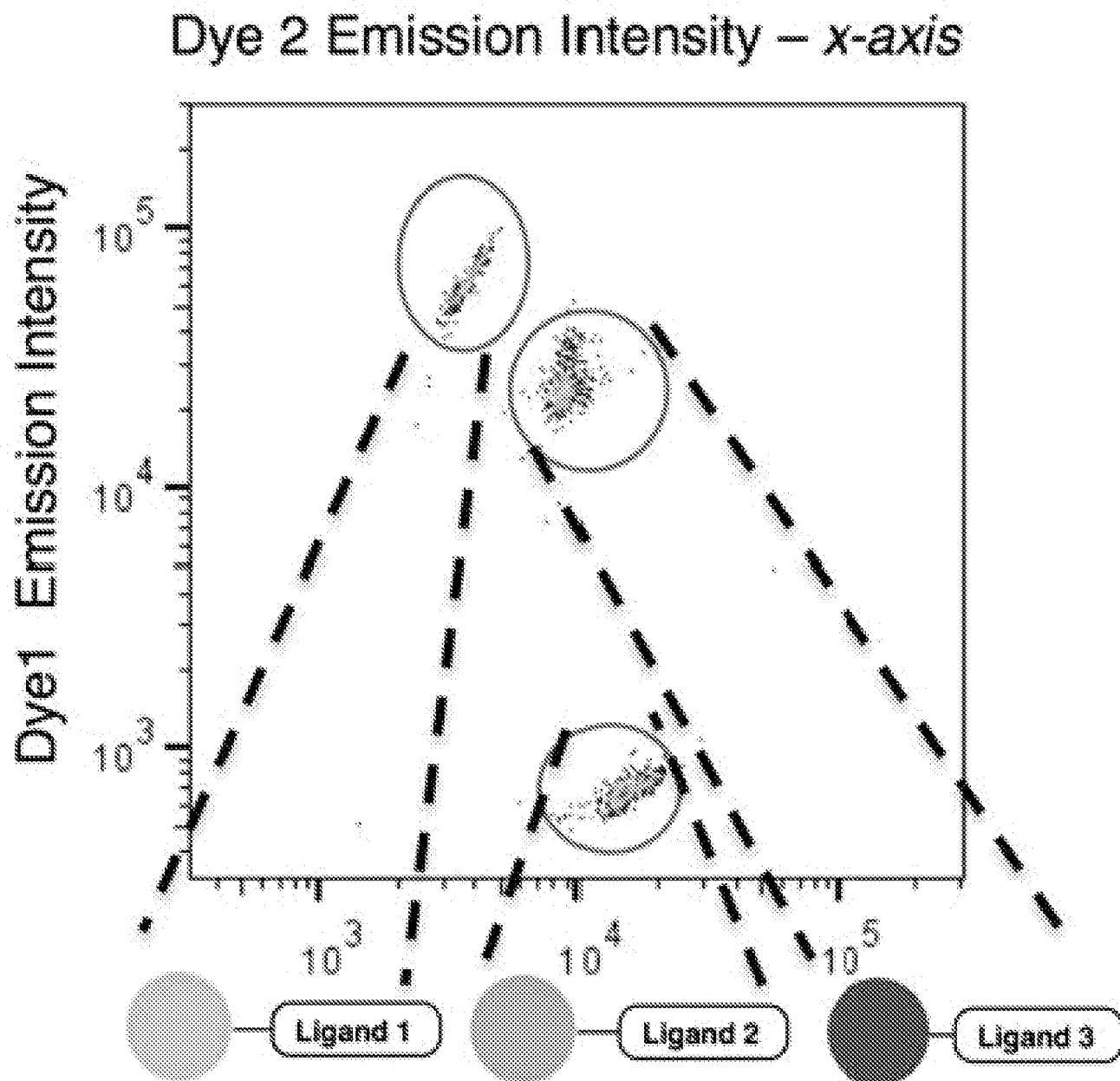
or more microsphere-bound encoding dyes for each microsphere in which the protein-binding fluorescent reporter dye is detected, thereby identifying each respective ligand associated with each respective protein-binding subpopulation of microspheres.

2. The method of claim 1, wherein the detection device comprises a fluorescence activated cell sorter (FACS).
3. The method of claim 1, wherein each of the plurality of ligands is a potential antigen, receptor modulator, or enzyme inhibitor with respect to protein undergoing evaluation.
4. The method of claim 1 wherein a ligand is a peptide analog.
5. The method of claim 4 wherein the peptide analog is a model for a binding site of a second protein to the protein undergoing evaluation.
6. The method of claim 1, wherein the protein undergoing evaluation is an antibody, a receptor, or an enzyme.
7. The method of claim 1, wherein the amino group blocking reagent is an amino-reactive Fmoc compound.
8. The method of claim 1, wherein the two encoding dyes are Pacific OrangeTM and Pacific BlueTM.
9. The method of claim 1, wherein three encoding dyes are used and the three encoding dyes are Pacific OrangeTM, Pacific BlueTM, and Texas Red®.
10. The method of claim 2, wherein the detection device comprising a fluorescence-activated cell sorter uses a single fluorescence-inducing wavelength of light.

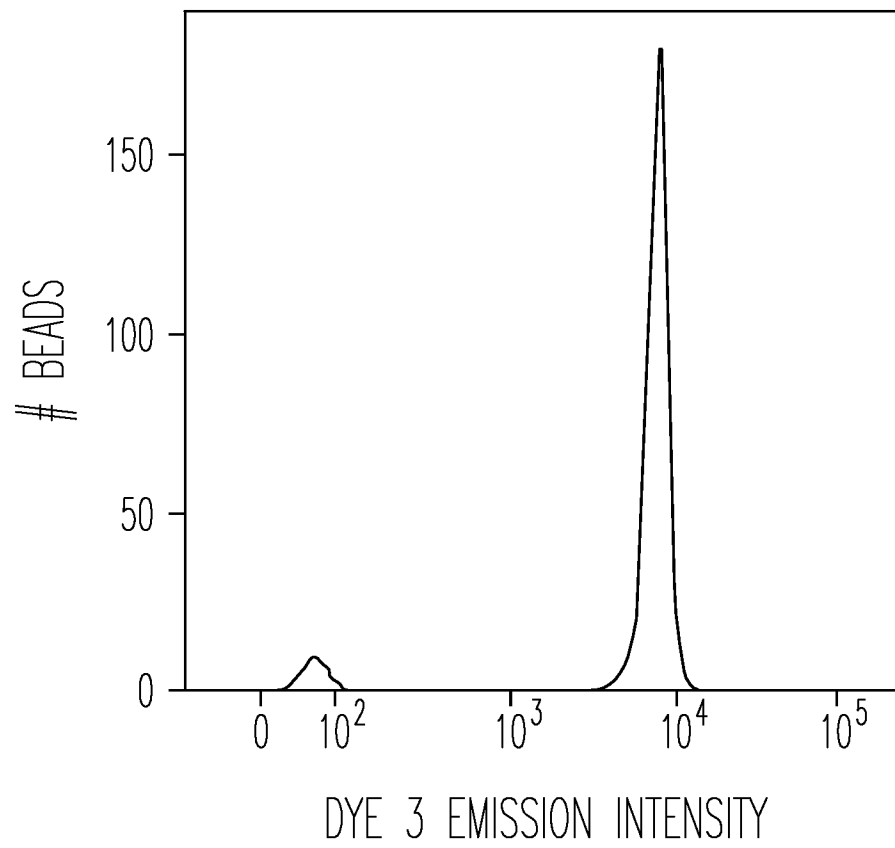
11. The method of claim 1, wherein there are two encoding dyes, and up to 36 distinct subpopulations that can be distinguished by the ratio of fluorescent emission intensities from the two encoding dyes.
12. The method of claim 1, wherein the reporter molecule bearing a protein-associate fluorescent dye is an antibody specific for an epitope disposed on the protein undergoing evaluation.
13. The method of claim 1, wherein the reporter molecule bearing a protein-associated fluorescent dye is a second ligand for a second binding site disposed on the protein undergoing evaluation.
14. The method of claim 1, wherein the reporter molecule bearing a protein-associated fluorescent reporter dye comprises Alexa Fluor® 647.
15. The method of claim 1, wherein the polyethyleneglycol-coated amino-functionalized polystyrene microsphere exhibits less non-specific protein binding than does a comparable amino-functionalized polystyrene microsphere that is not polyethyleneglycol-coated.
16. A protein-binding ligand identified from a set of potential protein-binding ligands by the method of any one of claims 1-15.

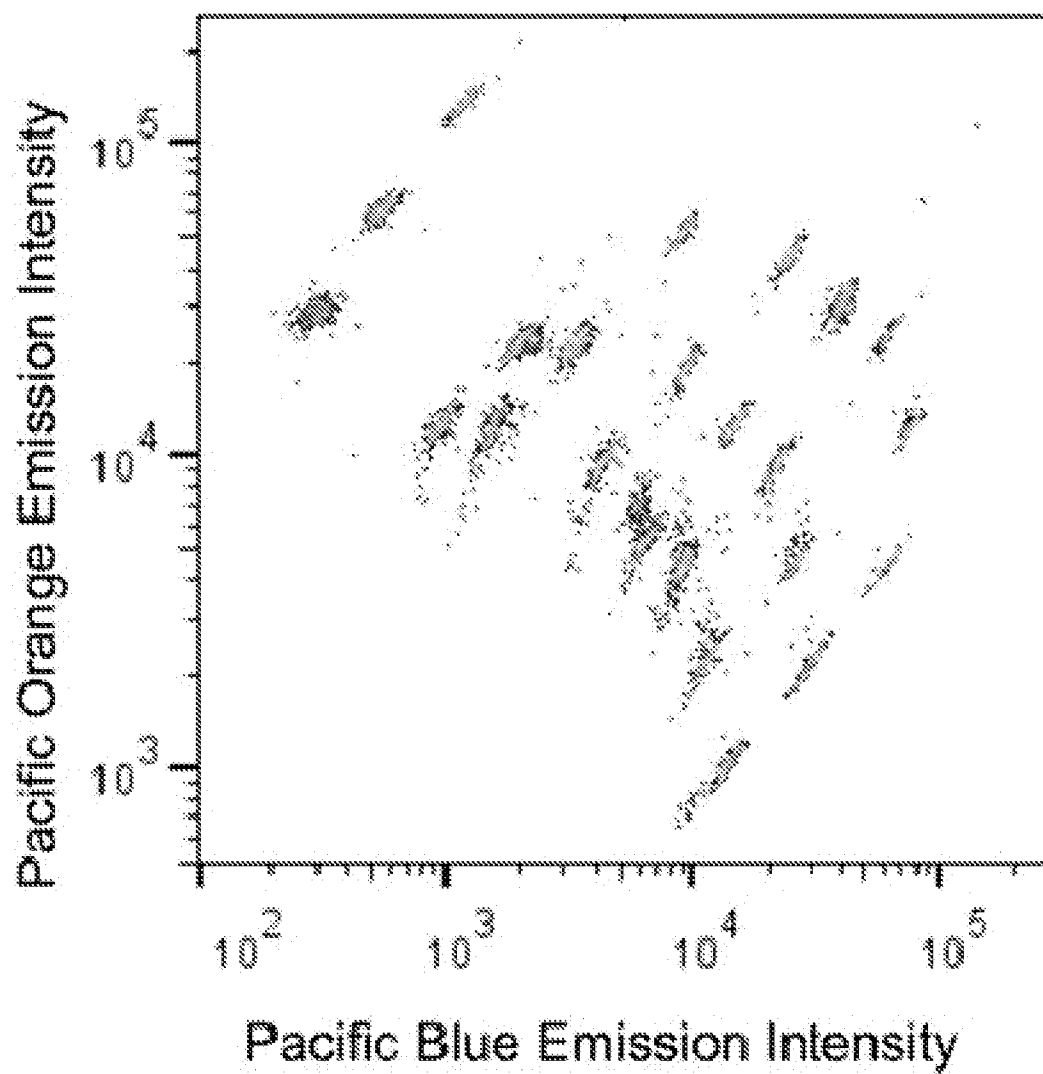
*Fig. 1A**Fig. 1B*

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*Fig. 1C*

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*Fig. 1D*

*Fig. 1E*

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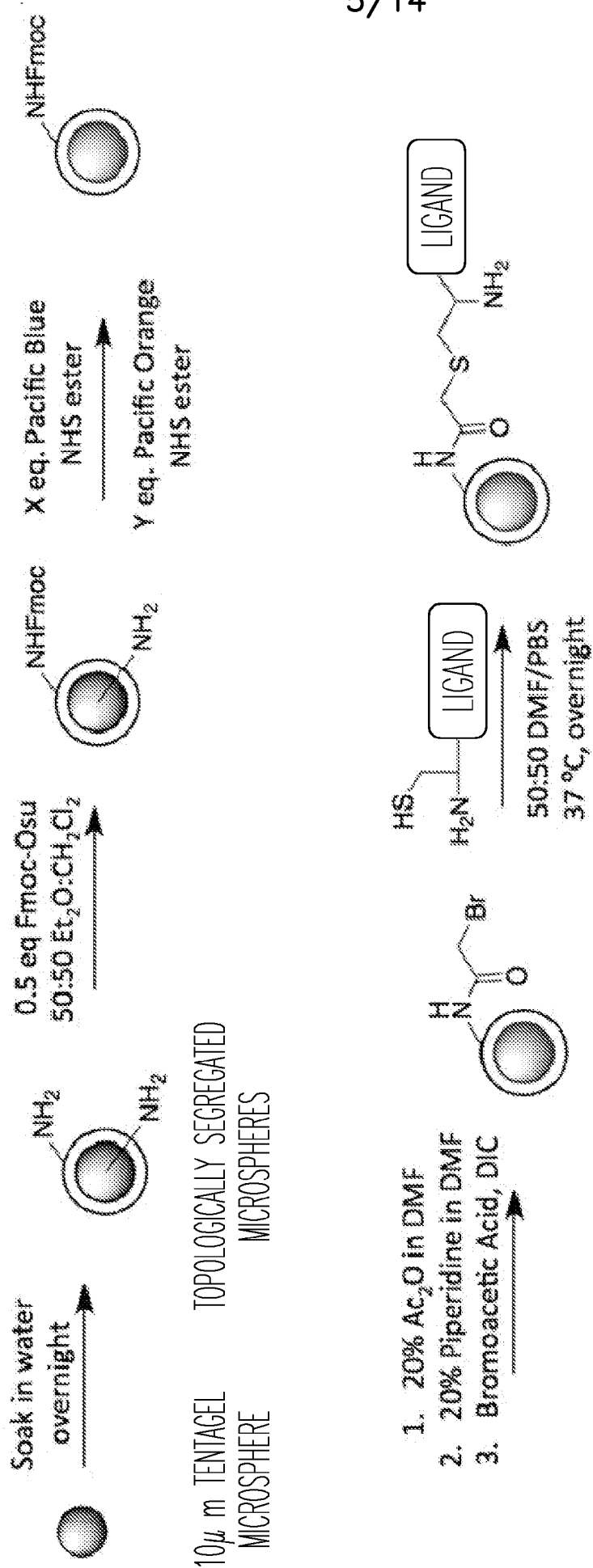


Fig. 2

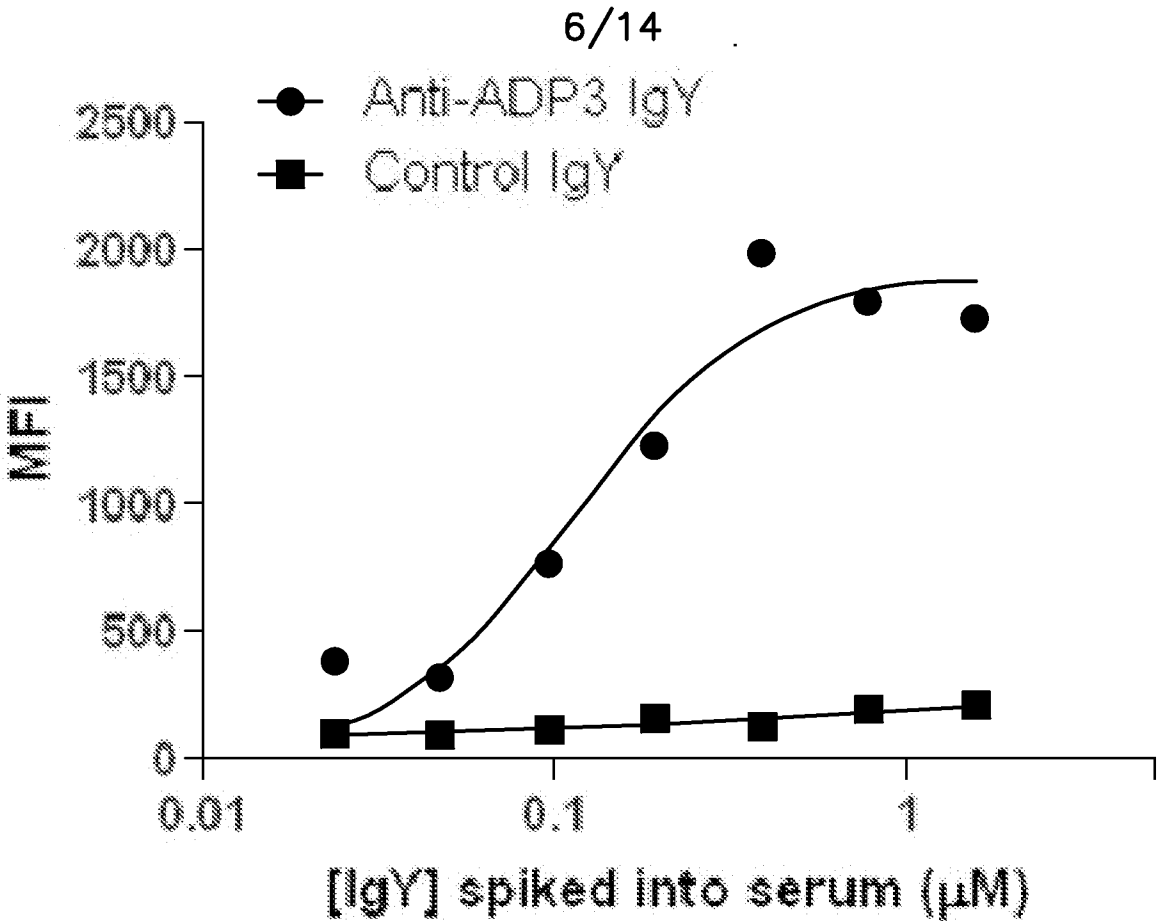


Fig. 3A

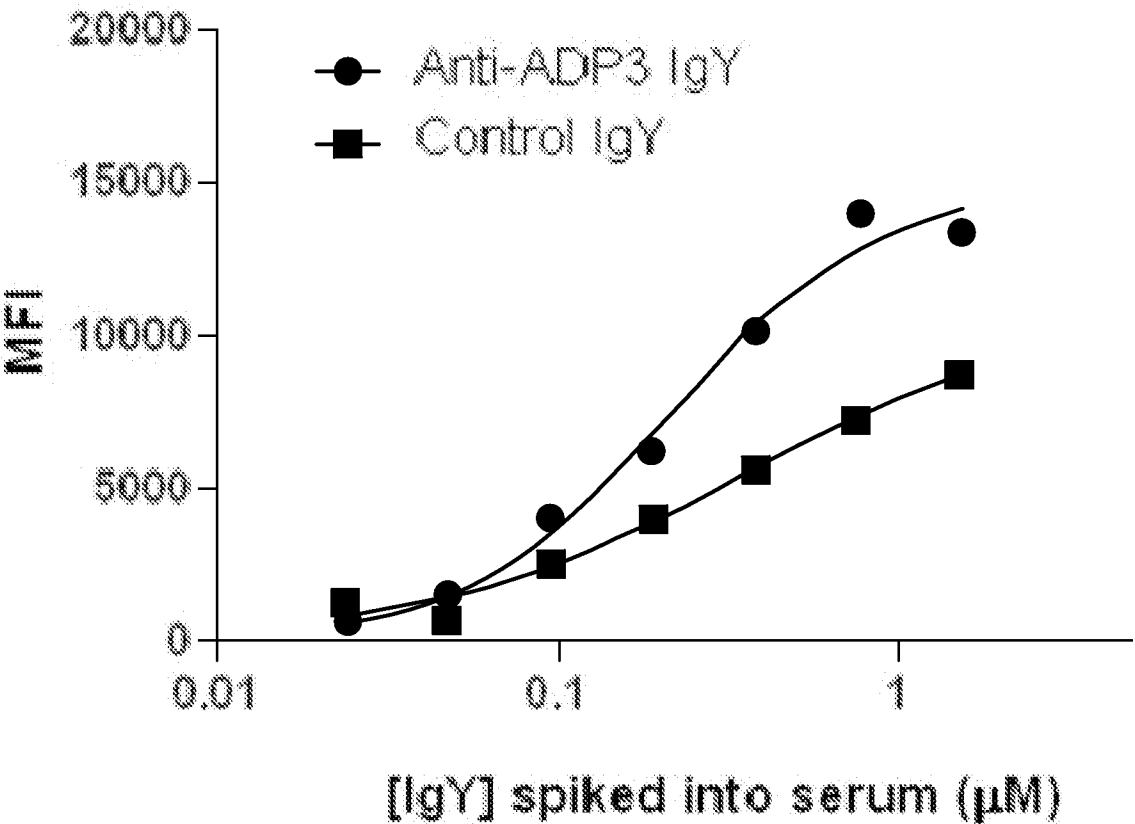


Fig. 3B

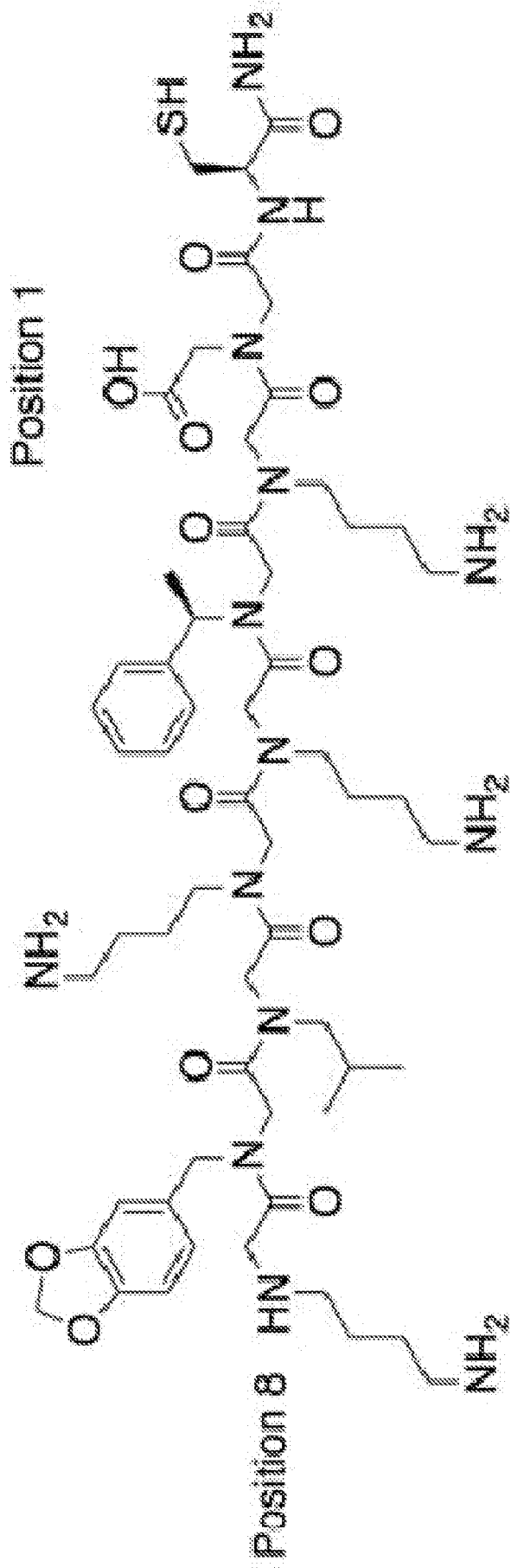
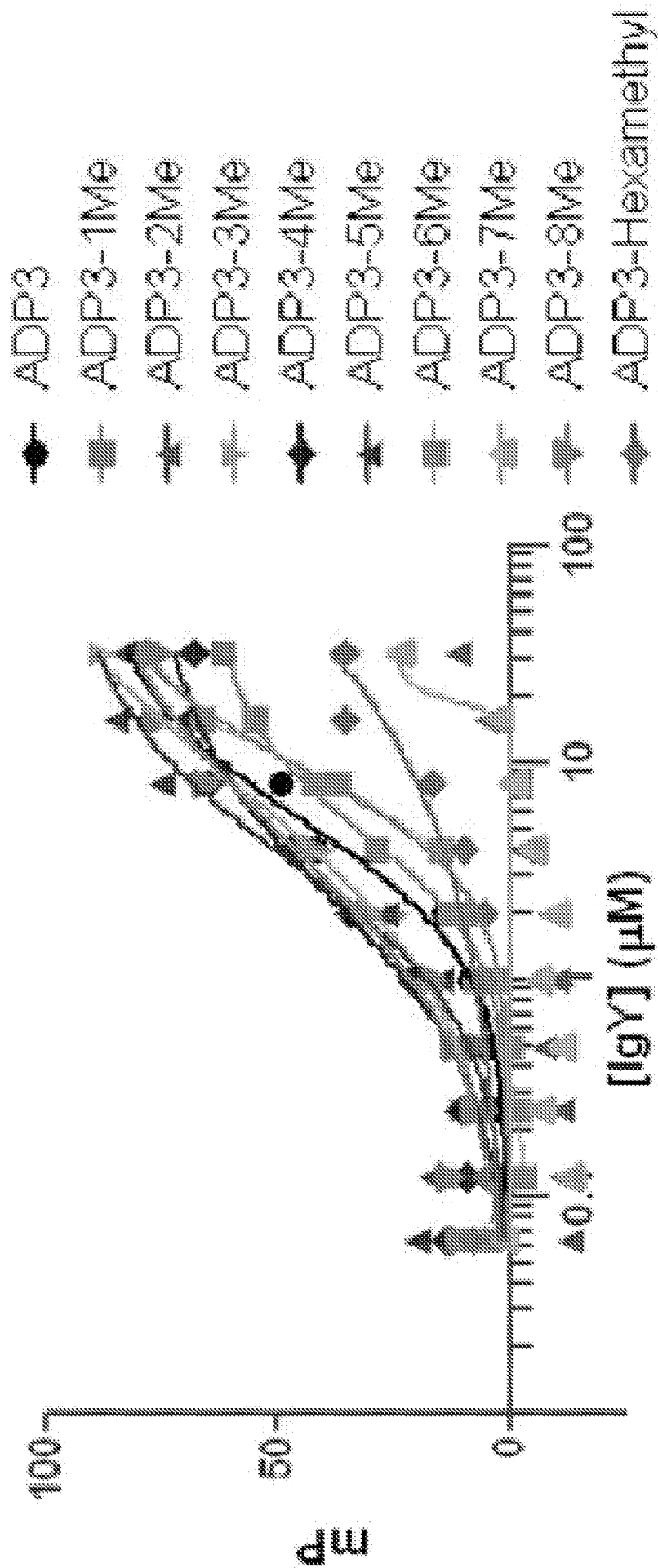
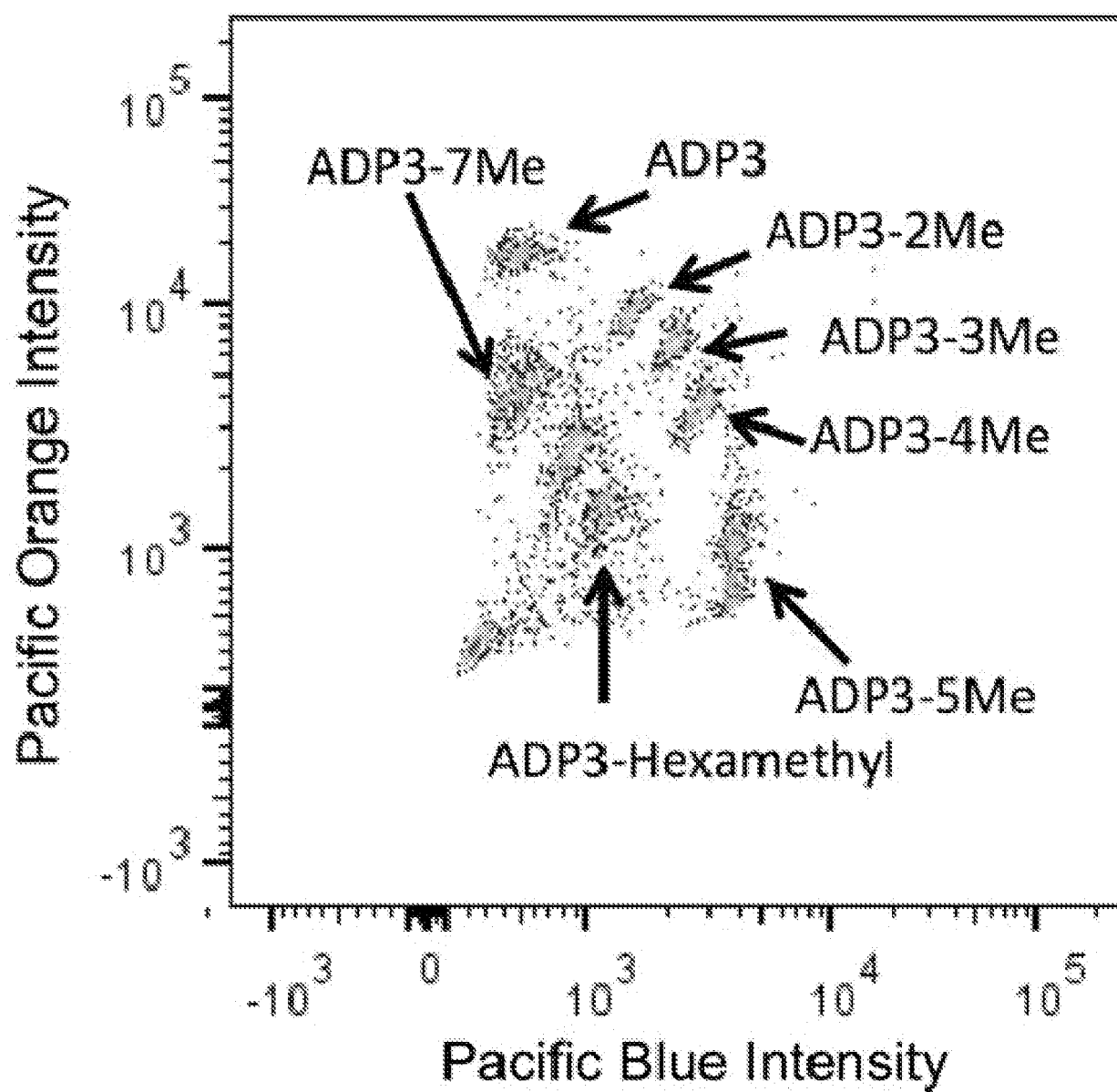


Fig. 4A

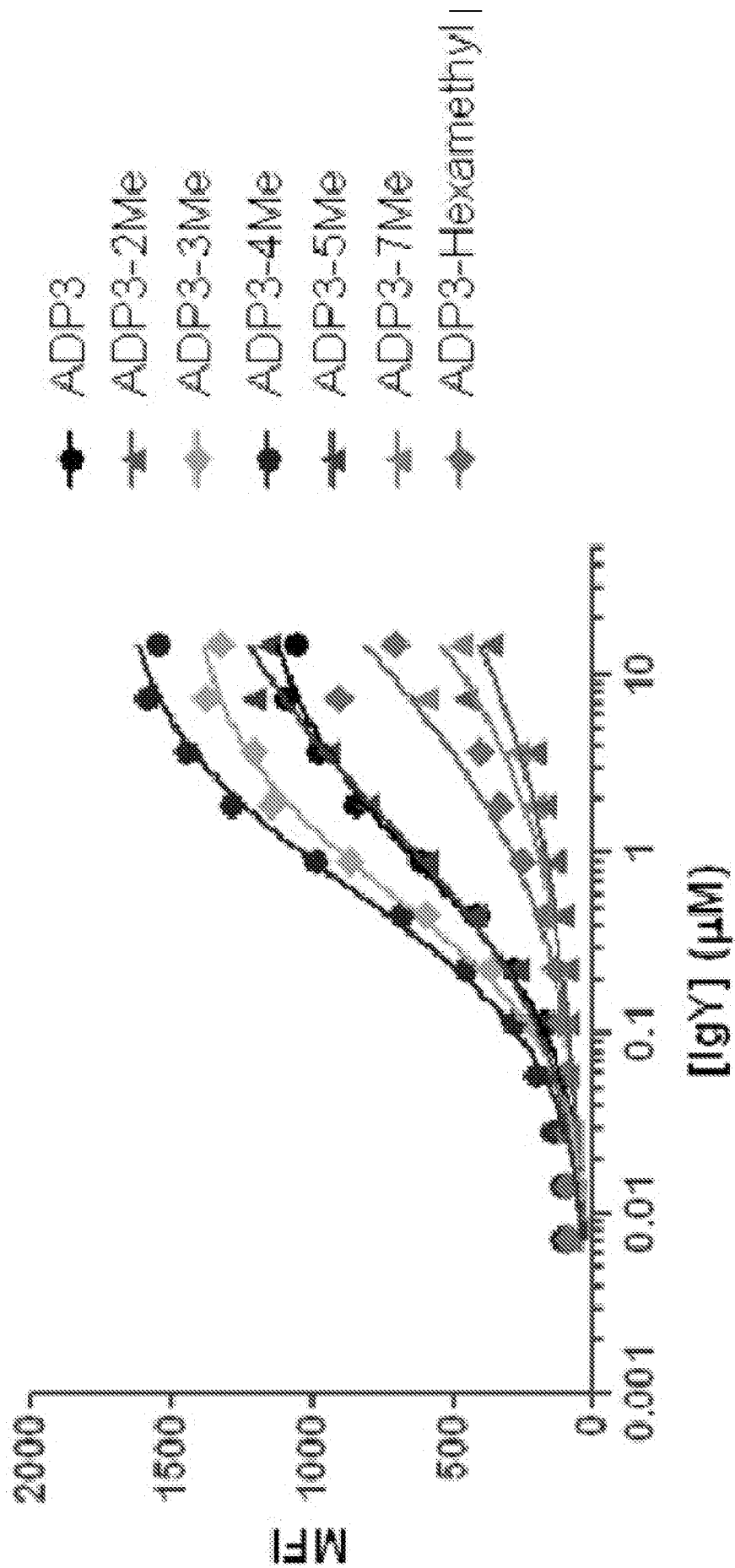
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*Fig. 4B*

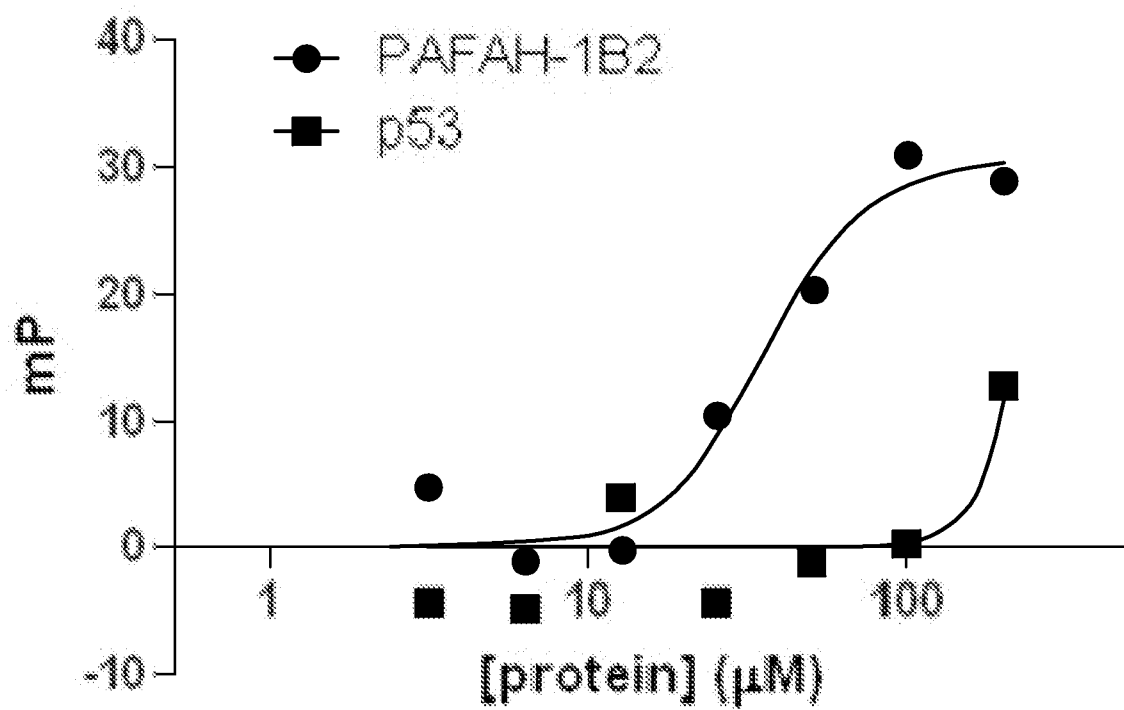
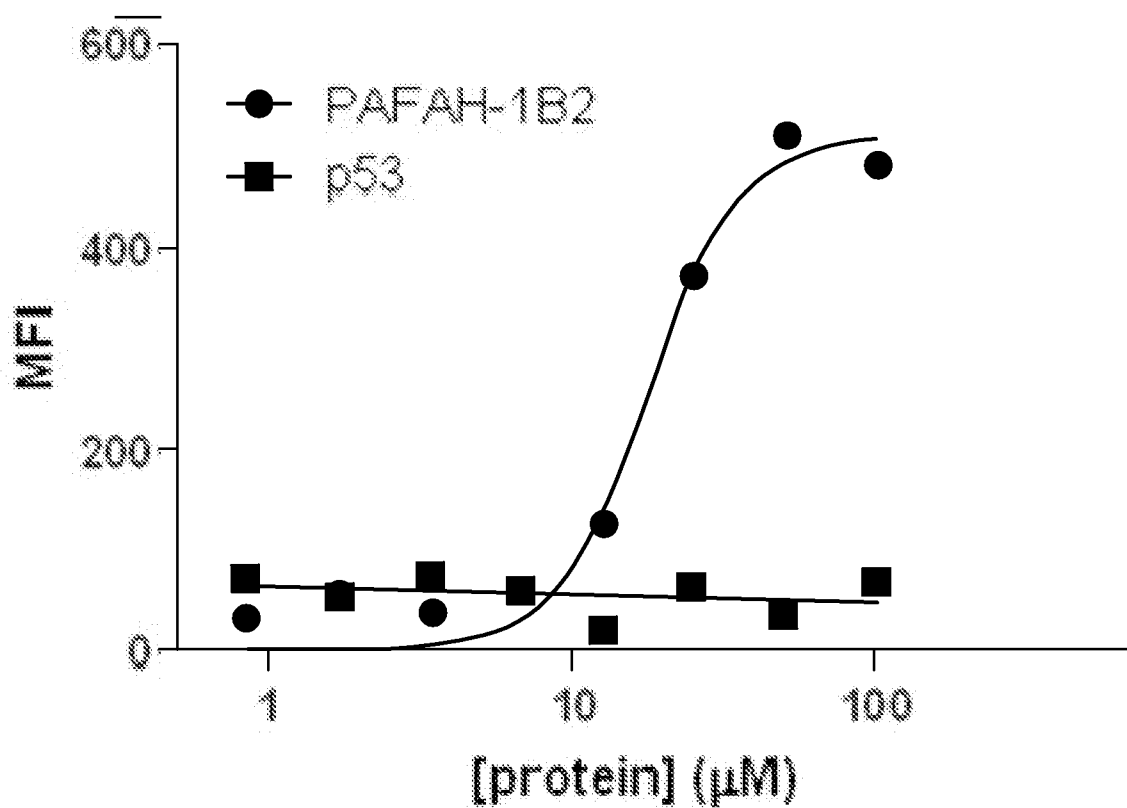
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*Fig. 4C*

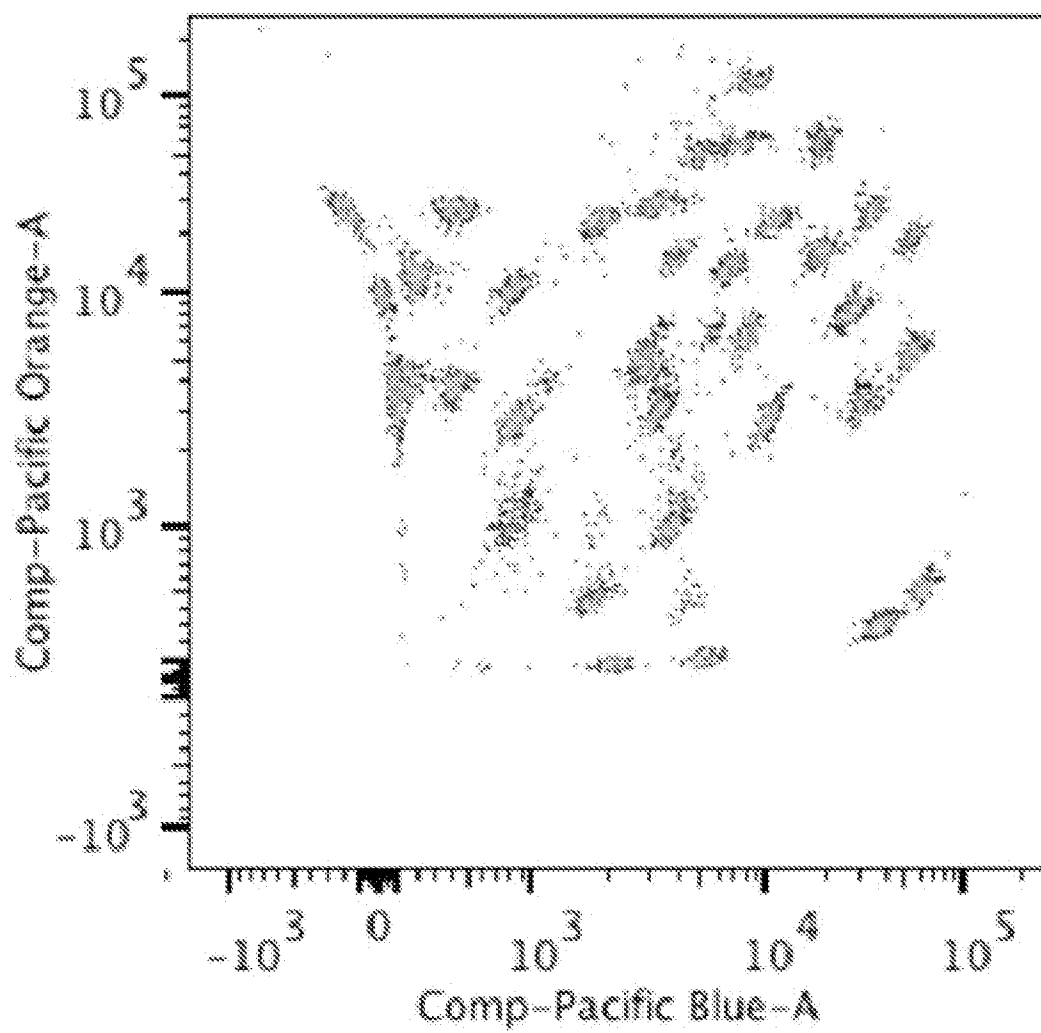
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*Fig. 4D*

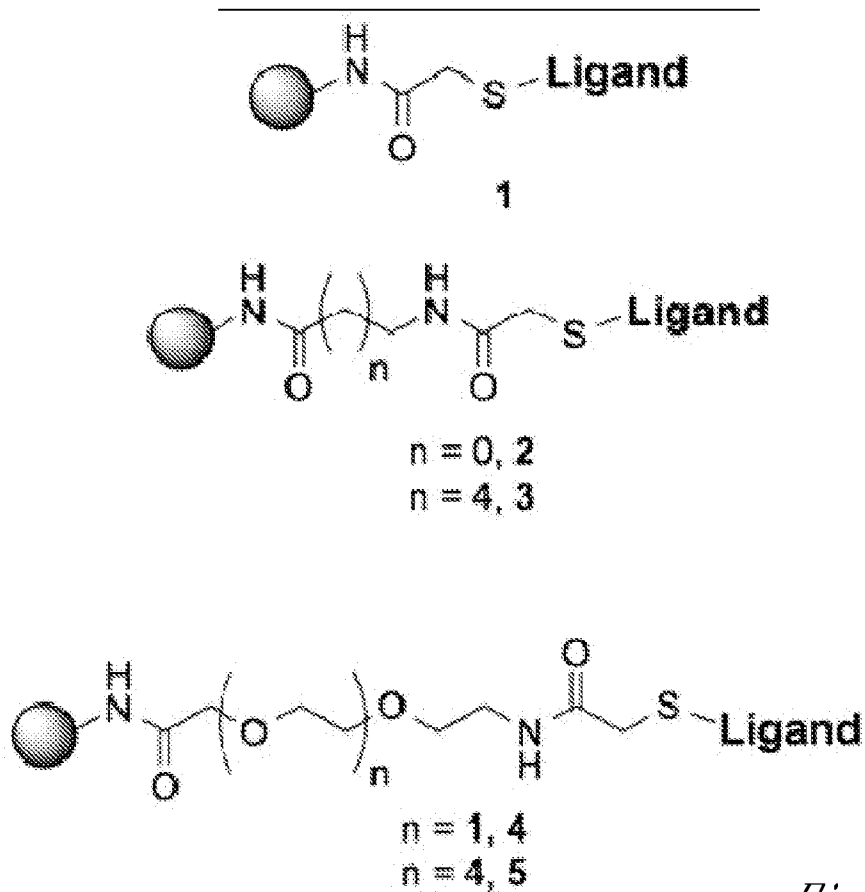
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*Fig. 4E**Fig. 4F*

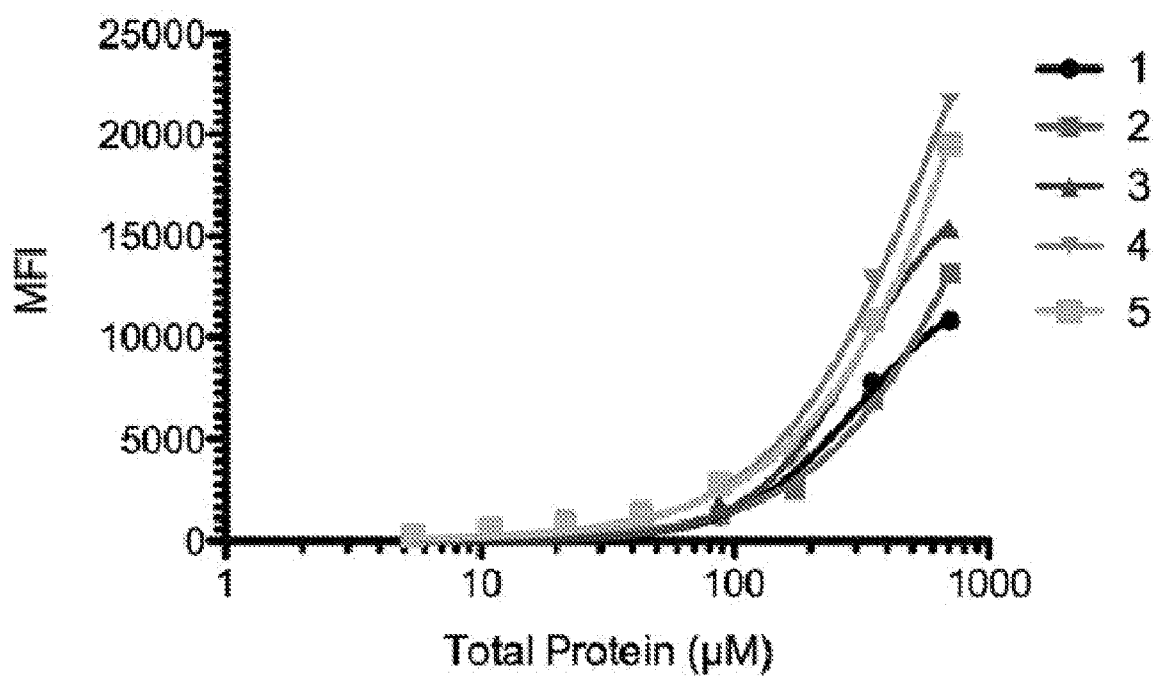
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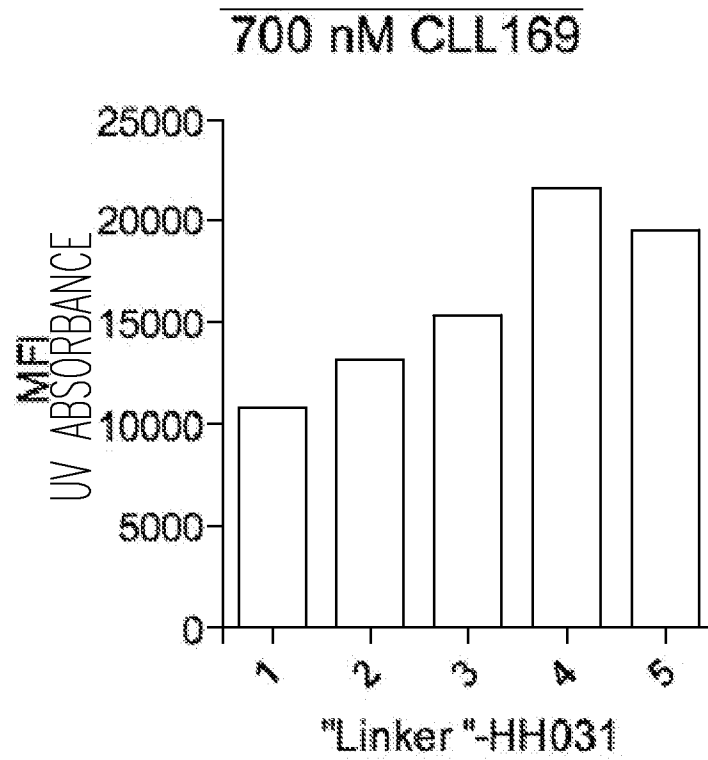
*Fig. 5*

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*Fig. 6A*

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*Fig. 6B*

*Fig. 6C*

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01N 33/53; G01N 33/53; G01N 31/00 (2014.01)

USPC - 435/7.8,7.1,125;436/15

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC: 435/7.8,7.1,125;436/15

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Electronic Database Searches: Thomson innovation (US Grant, AU Innov, CA App, US App, AU Grant, FR App, EP Grant, AU App, DE Util, EP App, GB App, DE Grant, WO App, CA Grant, DE App, JP App, KR Grant, KR App, Other, JP Util, KR Util, CN Util, JP Grant, CN Grant, CN App, VN Grant), Google, Sciencedirect. Terms: determin* protein binding, polystyrene,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2003/0143570 A1 (Abrams et al.) 31 July 2003 (31.07.2003) entire document especially para [0004]-[0005]; [0041]; [0046]	1-16
A	US 2007/0003925 A1 (Nolte et al.) 04 January 2007 (04.01.2007) entire document especially abstract; para [0006]-[0007]	1-16
A	US 2007/0166725 A1 (McBride et al.) 19 July 2007 (19.07.2007) entire document especially para 22]-[0023]; [0061]	1-16
A	US 2004/0023249 A1 (Balch) 05 February 2004 (05.02.2004) entire document especially para [0025]-[0026]; figure 1	1-16
A	US 2001/0029049 A1 (Walt et al.) 11 October 2001 (11.10.2001) entire document	1-16
A	US 2012/0010107 A1 (Griffiths et al.) 12 January 2012 (12.01.2012) entire document	1-16
A	US 2003/0124029 A1 (Webb et al.) 03 July 2003 (03.07.2003) entire document	1-16

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search

23 May 2014 (23.05.2014)

Date of mailing of the international search report

13 JUN 2014

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