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(54) **Title:** COMPOSITION COMPRISING AN IRON INDICATOR ATTACHED TO A MICROPARTICLE AND USES OF SAME FOR QUANTIFYING NON-TRANSFERRIN BOUND IRON (NTBI) AND CELLULAR LABILE IRON (LCI)

Fluorescence metal sensors on beads (FMSB) as probes for labile iron
concept and application

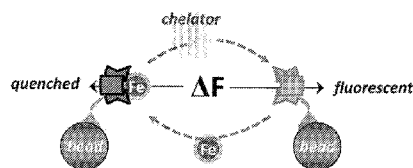


FIG. 1A

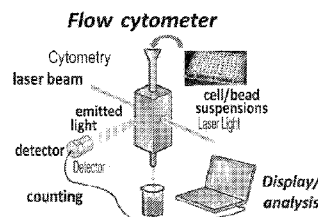


FIG. 1B

(57) **Abstract:** A composition-of-matter is provided. The composition comprising an iron indicator attached to a microparticle, wherein the iron indicator comprises an iron binding moiety and a signal generating moiety, wherein an intensity of the signal generated by the signal generating moiety is related to an amount of the iron bound by the iron binding moiety. Also provided are clinical uses of this composition.

COMPOSITION COMPRISING AN IRON INDICATOR ATTACHED TO A
MICROPARTICLE AND USES OF SAME FOR QUANTIFYING NON-
TRANSFERRIN BOUND IRON (NTBI) AND CELLULAR LABILE IRON (LCI)

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FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to a composition comprising a iron indicator attached to a microparticle and uses of same for quantifying labile iron in biological fluids, often identified with non-transferrin bound iron (NTBI) (which is considered non-labile).

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Systemic iron overload is a pathological condition characterized by persistently high levels of iron in plasma and interstitial fluids. In that condition, the levels of iron in the circulation exceed by > 70% the binding capacity of transferrin generating chemical forms identified as non-transferrin bound iron (NTBI). Although plasma NTBI might be considered a marker of systemic iron overload *per se*, more importantly, it might serve as indicator of impending organ iron accumulation and ensuing damage. This is because some forms of NTBI present in plasma over extended periods of time can infiltrate cells and cause tissue iron to attain levels that override cellular antioxidant capacities and thereby affect vital functions, leading to oxidative cell damage and death. Clinical management of systemic iron overload requires sensitive and simple diagnostic tests that reflect the level of patients' overall iron burden, but more importantly the component of iron directly implicated in cell toxicity, i.e. labile iron. The most commonly used indicators for assessing *systemic iron overload (including chelation efficacy)* have thus far been serum ferritin levels (in some cases in conjunction with transferrin saturation) and T2* MRI (and/or biopsies when possible) for assessing end organ accumulation . These indicators are mostly associated with iron accumulated over extended periods of time, with serum ferritin reflecting iron accumulated primarily in the liver and spleen. Plasma NTBI, on the other hand, may be conceived as an early indicator of systemic iron overload and some of its components as indicators of chelation efficacy.

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Nature of NTBI and pathophysiological relevance: NTBI is detected in plasma (or serum) of patients whose plasma TBI exceeds ~70% transferrin saturation, due to excessive absorption of iron from the gut (dietary iron) and/or an output of iron from the reticuloendothelial system (recycling of red blood cell iron). NTBI appears in

various chemical forms, depending on the origin and degree of iron overload, the history of blood transfusions, bone-marrow transplantation and treatment (chelation or phlebotomy or chemotherapy). At present it is not clear which of the NTBI components is of pathophysiological relevance as marker of systemic iron overload and/or as a source of tissue iron overload and ensuing toxicity. With regard to the latter, if NTBI is the major source of tissue iron overload, then one must consider the chemical nature of the permeating substrate, namely, the NTBI forms in plasma/interstitial fluids in the various pathological conditions and the various “opportunistic” membrane routes through which the NTBI forms can gain cellular access. As those routes differ among cells types, both qualitatively and quantitatively, their relative contribution to tissue iron overload will vary accordingly. This is best exemplified in the differential loading of parenchymal versus Kupfer cells in hemochromatosis versus thalassemia major or the ostensible lack of cardiac iron loading in thalassemia intermedia versus thalassemia major, despite the high levels of plasma NTBI.

Plasma NTBI is comprised of both organic and inorganic complexes, some non-specifically associated with plasma proteins. For the free complexes, the relevant plasma NTBI permeating components are either: a. the small and “free” ligand-metal, in which case ingress might require metal reduction by membrane associated reductases followed by translocation or diffusion of the free-iron via putative transporters or channels (that can handle only free or hydrated divalent ions) or b. the iron complex, in which case more complex machineries that can handle iron-ligands should be implicated. If the major permeating species are the protein-bound iron complexes, then their tissue ingress would be limited to bulk endocytotic routes (mostly adsorptive endocytosis) followed by intracellular metal release and translocation from vesicles to cytosol. Clearly, a serial or parallel combination of the above mechanisms, bulk and ionic transport, can possibly account for the handling of all NTBI forms by different tissues in the different forms of iron overload. Moreover, as mentioned above, the relative contribution of the operative ingress routes of NTBI into cells might differ in different tissues, depending on the available repertoire of transporters/channels vis a vis the NTBI forms present at a particular site.

Determination of NTBI.

NTBI appears in plasma in multiple forms, some bound to small organic ligands which, in turn, might be free (filterable) or adsorbed to proteins, some cryptic or occluded to chelators and partially accessible to cells. These forms might change in the same patient depending on the time of sampling vis a vis its patho-physiological status and current treatment regimen. Because of technical difficulties in revealing the apparently cryptic NTBI in plasma/ serum (protein-bound complexes that are not easily accessible/chelatable), most of the published NTBI assays rely on strategies that attempt to “differentially” extract NTBI from macromolecular plasma/sera components and render the extract filterable and/or detectable by analytical chelating agents. These approaches assume that NTBI extracted from plasma/serum is not derived from highly saturated TBI (plasma TBI at full saturation ranges 40-50 μ M and NTBI is generally less than 1/10th that value). In most cases, analysis is preceded by storage of frozen sera (or heparinized plasma) under conditions that, ideally, should preserve both the TBI and the NTBI components and particularly their possible deterioration or inter-conversion (during storage or analysis).

LPI (labile plasma iron) and DCI (directly chelatable iron) as redox-active and/or chelatable forms of NTBI.

The forms of plasma NTBI that are potentially toxic to cells are those that can be deposited on cell surfaces as redox-active species or excessively taken up by cells in forms that can raise the labile cell iron (LCI). As those are the labile forms that are also chelatable, they represent the primary pharmacological targets of any chelation treatment aimed at preventing undesirable iron overloading of cells. However, NTBI assays were designed to reveal all forms of non TBI, a goal attained generally by adding strong mobilizing agents that can extract all NTBI forms, the easily accessible and the “cryptic” ones, followed by filtration and chemical analysis of the extracted material (by standard colorimetric assays, ICP-MS or in combination with HPLC). LPI and DCI assays, on the other hand, were designed to reveal labile forms of iron in fluids while avoiding potential complications associated with very high concentrations of iron-mobilizing agents. The advantage of DCI and LPI assays is their ability to provide a measure for clinical efficacy of chelation/ phlebotomy in reducing/eliminating NTBI's

labile/ chelatable component. The assays are based on reading of fluorescence by high throughput devices, designed to be non laborious and suitable for all biofluids.

A potential limitation of the existing LPI and DCI assays is incomplete detection of some NTBI complexes with low redox-activity and/or limited accessibility of the iron-detector agent to some chemical forms bound/adsorbed to proteins.

Increased sensitivity has been achieved using mild extraction agents or mobilizers that minimize the interference of plasma components without affecting TBI. The inclusion of mild mobilizers also reduces the interference of albumin, citrate and uric acid while causing no release from highly saturated transferrin.

Related art:

WO 00/36422

WO 01/84161

WO 04/040252

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SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided a composition-of-matter comprising an iron indicator attached to a microparticle, wherein the iron indicator comprises an iron binding moiety and a signal generating moiety, wherein an intensity of the signal generated by the signal generating moiety is related to an amount of the iron bound by the iron binding moiety.

According to some embodiments of the invention, the iron binding moiety and the signal generating moiety are covalently attached forming a single molecule.

According to some embodiments of the invention, the iron binding moiety and the signal generating moiety are distinct molecules.

According to some embodiments of the invention, the iron binding moiety comprises deferrioxamine (DFO).

According to some embodiments of the invention, the iron indicator comprises dihydrorhodamine 123 (DHR) or carboxydimethylfluorescein (CDCF).

5 According to some embodiments of the invention, the iron indicator is a cell permeant iron indicator.

According to some embodiments of the invention, the iron indicator comprises calcein.

10 According to some embodiments of the invention, the iron indicator is selected from the group consisting of calcein, deferiprone, deferasirox and salicyl-aldehyde hydrazone (SIH).

According to some embodiments of the invention, the iron binding moiety comprises a modified apo-transferrin.

15 According to some embodiments of the invention, the iron indicator is fluorescein-apo-transferrin.

According to some embodiments of the invention, the signal generating moiety is a fluorophore.

20 According to some embodiments of the invention, the fluorophore is selected from the group consisting of Fluorescein, Rhodamine, nitrobenzofurazan, fluorogenic β -galactosidase and a green fluorescent protein.

According to some embodiments of the invention, the iron binding moiety is selected from the group consisting of apo-transferrin, lactoferrin, ovotransferrin, deferrioxamine, phenanthroline, ferritin, porphyrin, EDTA and DPTA.

25 According to some embodiments of the invention, the iron indicator comprises an enzyme.

According to some embodiments of the invention, the enzyme is an aconitase enzyme.

According to some embodiments of the invention, the microparticle has a weight similar to, or lower than a mammalian cell.

30 According to some embodiments of the invention, the microparticle is selected from the group consisting of a polystyrene bead, a silica bead or an agarose bead.

According to some embodiments of the invention, the iron indicator is conjugated directly to the microparticle.

According to some embodiments of the invention, the iron indicator is conjugated indirectly to the microparticle.

5 According to some embodiments of the invention, the indirect conjugation is via an adhesive protein.

According to some embodiments of the invention, the adhesive protein comprises a histone or an albumin.

10 According to some embodiments of the invention, the indirect conjugation is via an extension arm.

According to some embodiments of the invention, the extension arm is composed of a dendrimer and a multifunctional bridge.

According to an aspect of some embodiments of the present invention there is provided a method of quantifying free iron levels in a sample, the method comprising:

15 (a) contacting a sample of the biological fluid with the composition-of-matter; and

(b) detecting and quantifying the signal, thereby quantifying free iron levels in the biological sample.

20 According to some embodiments of the invention, the free iron comprises non-transferrin bound iron.

According to some embodiments of the invention, the free iron comprises directly chelatable iron.

According to some embodiments of the invention, the free iron comprises labile plasma iron.

25 According to some embodiments of the invention, the iron indicator comprises fluorescein and deferrioxamine.

According to some embodiments of the invention, the fluorescein and deferrioxamine are covalently attached to form fluoresceinated deferrioxamine (FI-DFO).

30 According to some embodiments of the invention, the iron indicator comprises dihydrorhodamine 123 (DHR)

According to some embodiments of the invention, said iron indicator comprises rhodamine(R) and deferrioxamine.

According to some embodiments of the invention, said rhodamine and deferrioxamine are covalently attached to form R-deferrioxamine (R-DFO).

According to some embodiments of the invention, the method further comprising:

(c) contacting the sample with an iron binding moiety in excess with respect to the iron indicator; and

5 (d) detecting and quantifying the signal of step (C), the signal being the background signal of the chelation reaction.

According to some embodiments of the invention, the iron binding moiety in excess is identical to the iron binding moiety of the iron indicator.

According to an aspect of some embodiments of the present invention there is provided a method of calibrating free iron levels in a fluid sample, the method comprising:

(a) contacting a plurality of fluid samples with predetermined distinct free iron concentration values with the composition;

(b) detecting and quantifying the signal; and

15 (c) generating a calibration curve depicting the signal against the predetermined distinct free iron concentration values.

According to some embodiments of the invention, the method further comprising contacting the plurality of samples with an iron chelator so as to release the iron binding moiety following step (b); and detecting and quantifying a signal' generated following contact with the iron chelator, and wherein the calibration curve depicts the change in the signal vs the signal against the predetermined distinct free iron concentration values.

According to an aspect of some embodiments of the present invention there is provided a method of quantifying labile cellular iron in a biological sample, the method comprising:

25 (a) loading cells of the biological sample with a iron indicator composed of an iron binding moiety and a signal generating moiety, wherein an intensity of the signal generated by the signal generating moiety is related to an amount of the iron bound be the iron binding moiety;

(b) contacting loaded cells of step (a) with a cell permeant metal chelator so as to remove the iron from the iron binding moiety;

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(c) recording a signal generated in (a) and in (b), wherein the mean change in signal intensity is indicative of a level of labile cell iron;

(d) quantifying the change using a calibration curve generated according to the method above.

5 According to some embodiments of the invention, the iron indicator comprises calcein.

According to an aspect of some embodiments of the present invention there is provided a method of quantifying redox active iron levels in a biological fluid, the method comprising:

10 (a) contacting a sample of the biological fluid with a reducing agent, to obtain redox active iron;

(b) contacting the sample with a detector molecule selected capable of measurable activation upon contact with redox active iron reaction products, wherein the measurable activation is related to an amount of the redox active iron reaction product;

15 and

(c) quantifying the measurable activation using the composition-of-matter, thereby quantifying redox active iron levels in the biological fluid.

According to some embodiments of the invention, the reducing agent is ascorbic acid, dithionite, dithiothreitol and mercaptoacetic acid.

20 According to some embodiments of the invention, the detector molecule is selected from the group consisting of dihydrorhodamine, dihydrorhodamine, carboxy-dihydrofluorescein and dihydroresorufin.

According to some embodiments of the invention, the redox active iron reaction products are reactive oxygen species.

25 According to an aspect of some embodiments of the present invention there is provided a method of determining a presence, absence or risk of a disorder associated with abnormal levels of free iron in a biological fluid or cells of a subject, the method comprising:

30 (a) determining levels of the free iron in the biological fluid or cells of the subject according to any of the methods above; and

(b) determining in the subject based on the levels a presence, absence or risk of the disorder associated with abnormal free iron levels.

According to an aspect of some embodiments of the present invention there is provided a method of treating a subject having a disorder associated with abnormal levels of free iron in a biological fluid or cells, the method comprising:

(a) determining levels of the free iron in the biological fluid or cells of the subject according to any of the methods above;

(b) determining in the subject a presence of the disorder associated with abnormal free iron levels based on the levels obtained in (a); and

(c) treating the subject using a medicament for the disorder associated with abnormal free iron levels.

According to some embodiments of the invention, the medicament comprises an iron chelation therapy.

According to an aspect of some embodiments of the present invention there is provided a method of determining efficacy of treatment of a disorder associated with abnormal levels of free iron in a biological fluid or cells;

(a) treating a subject in need thereof using a medicament for the disorder associated with abnormal free iron levels; and

(b) determining levels of the free iron in a biological fluid or cells of the subject according to any of the methods above, wherein a change in the levels following the treating is indicative of treatment efficacy.

According to some embodiments of the invention, the medicament comprises iron chelation therapy, and whereas a reduction in the levels following the therapy is indicative of efficacious treatment.

According to some embodiments of the invention, the medicament is selected from the group consisting of oral iron supplementation, folic acid, vitamin B-12, erythropoietin, blood transfusion and hyperbaric oxygen, and whereas an increase in the levels following the therapy is indicative of efficacious treatment.

According to an aspect of some embodiments of the present invention there is provided a kit for determining a presence of a disorder associated with abnormal levels of free iron in a biological sample of a subject, the kit comprising the composition of matter.

According to some embodiments of the invention, the kit further comprising at least one of a mobilizing agent and a compound devoid of iron and being capable of binding endogenous apo-Transferrin

According to some embodiments of the invention, the compound comprises gallium or cobalt.

According to some embodiments of the invention, the mobilizing agent is selected from the group consisting of sodium-oxalate, nitrilotriacetate, ascorbate and salicylate.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying schematic drawings or data figures. With specific reference now to the drawings and figures in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings and figures makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIGs. 1A-B are schematic illustrations showing fluorescence metal sensors on beads (FMSB) as probes for labile iron. Figure 1A - is a schematic illustration showing the principle of the measurement of labile iron as DCIB (directly chelatable iron=DCI measured on beads) using particular fluorescence metal sensors (FMS) coupled covalently to beads (FMSB) and flow cytometry (FC) (Figure 1B). FMSB containing fluorescein-DFO (FDFO) or calcein green (CALG) undergo fluorescence quenching when reacting with labile iron and dequenching when treated with a strong chelator.

Plasma or other fluids containing labile Fe will react accordingly, with or without the addition of a mild metal mobilizer (e.g., nitrilotriacetic acid < 1mM or deferiprone < 30 μ M). The sample fluorescence is read by a flow cytometer (Figure 1B) after 60 minutes incubation at room temperature; a parallel (for CALG also sequential) assay is carried out with beads in the presence of excess (0.1 mM) deferrioxamine (DFO) which demonstrably prevents (for CALG also removes) Fe from binding to the respective FMS; in the last step, the iron is quantitated using a calibration curve.

FIG. 2 is a schematic illustration of the construction of the FMSB and some of its chemical properties.

FIG. 3 shows fluorescence microscopy image of a 5 μ M polystyrene-carboxyl bead onto which: a. a generation 2 amino terminal dendrimer was reacted (after activation of the carboxy bead with n-hydroxysuccinimide and EDC); b. the a aminodendrimer was subsequently reacted with fluorescein (F) isothiocyanate (ITC) and deferrioxamine –ITC=DFO-ITC (ratio 1;6). .

FIGs. 4A-B are schemes showing FMSB as probes for labile iron. Measurement of plasma NTBI as directly chelatable iron (DCI) by flow cytometry (FC). Figure 4A - Fluorescein+DFO coated beads DenB(F+D) used for quantitative assessment of DCI by flow cytometry. Figure 4B - Rhodamine+DFO coated beads DenB(R+D) used for quantitative assessment of DCI by flow cytometry.

FIGs. 5A-C are schematic illustrations showing measurement of LCI in blood cells. Figure 5A – is an image of a CALG bead generated according to the teachings of the present invention. Figure 5B - In a first step, blood or other cells are loaded with calcein-green –acetomethoxy-ester (CALG-AM) for 10' at room temperature (that leads to the intracellular generation of free CALG and binding of LCI). Cells are washed with buffered saline and in the next step the sample is analyzed by flow cytometry before and after addition of a permeant chelator. Addition of the chelator causes a rise in fluorescence ΔF which is proportional to LCI. Figure 5C – demonstrates the conversion of fluorescence intensity shift ΔF into concentration of LCI using CALGB. A specific carrier molecule labeled with the fluorescence metal sensor (FMS) CALG is attached to polystyrene beads of 3-6 μ m mean diameter. These beads are used to simulate CALG loaded cells for flow cytometry.

FIG. 6 shows a schematic illustration of the method of measuring DCI and LCI according to the teachings of some embodiments of the invention.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

5 The present invention, in some embodiments thereof, relates to a composition comprising a iron indicator attached to a microparticle and uses of same for quantifying non-transferrin bound iron (NTBI) and as reference for quantifying LCI in circulating blood and other cells.

10 Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

15 Non-transferrin bound iron (NTBI) is a marker of systemic iron overload and an indicator of impending organ damage. The present inventor has previously devised compositions and assays for measuring free iron levels using indicator molecules composed of an iron binding moiety and a signal generating moiety wherein an intensity of the signal generated by the signal generating moiety is stoichiometrically related to the amount of the iron bound by the iron binding moiety.

20 However, a potential limitation of such assays is the incomplete detection of iron complexes with low redox-activity and/or limited accessibility of the iron-detector agent to some NTBI forms bound/adsorbed to proteins. This has led the present inventor to search for assay conditions that would detect free iron with higher sensitivity.

25 While reducing the present invention to practice, the present inventor has generated diverse assay conditions which make use of microparticles coated with the above-mentioned indicator molecules for recruiting chelatable iron from biofluids while concentrating the chelatable iron (by binding with high affinity) from diluted samples, overcoming media impurities, the major obstacle in presently available assays. The beads are read selectively in a flow cytometer (or equivalent instrument) with changes in signal reflecting changes in chelatable iron. The sensitivity of the method is 10-20
30 fold higher than the presently available ones and has a wide range of applications beyond plasma and serum, especially to urine and cerebrospinal fluid to which there is a

growing interest and increasing demand. The microparticle (also termed herein "bead") technology is of a special advantage in the following three modalities:

Measurement of DCI in fluids with the aid of beads and flow cytometry

(DCIB) - Figures 1A-B-Figure 4A-B. For example, the bead microspheres are constructed of derivatized polystyrene or silica (by derivatization it is meant a bead covalently bound to dendrimers (PAMAM generation 1- 3) onto which: a. fluoresceinated -DFO is coupled covalently (via the free carboxyl of fluorescein, while the DFO hydroxamates are protected via prior metal chelation) or b. either RITC (rhodamine isothiocyanate) or FITC (fluorescein-isothiocyanate) and DFO-ITC are coupled covalently via their ITC (=NCS- = isothiocyanate) groups that react covalently with bead amino groups or c. CALG is coupled (via the free carboxyl of fluorescein, while the CALG carboxyl-metal binding groups are protected via prior metal chelation). The resulting beads with fluoresceinated DFO (FDB), or with FITC + DFO-ITC (F+D)B (Figure 4A) or with RITC + DFO-ITC (R+D)B (Figure 4B) or with CALG beads (CALGB) are calibrated for iron sensitivity with solutions containing iron containing solutions of saturated plasma/serum or urine or a simple buffered salt solution (used to dilute all samples for reading purposes). Specificity: The changes in fluorescence impaired by factors in fluids are attributed to iron based on the fact that addition of the iron chelator deferoxamine (DFO) eliminates the change in fluorescence signal. For FDB or (F+D)B, only the DFO-preventable change in signal is taken as equivalent to the presence of a given concentration of iron. A minimal concentration of mobilizing agent (NTA 0.1 mM or 30 μ M deferiprone=L1) may be optionally used to increase the sensitivity of the DCIB assay by revealing possible cryptic forms of NTBI. For CALGB, only the DFO recoverable fluorescence is taken as a measure of DCI.

The same approach of using a mobilizing agent to extract all chelatable forms of iron can be used for extracting labile iron from intact cells (like red or white blood cells) with the permeable chelator deferiprone as a mobilizer (30-100 micromolar for 1-3 h) and assessing the extracted iron in the medium (after cell separation) by DCI in a fluorescence plate reader or DCIB [F+D)B or (R+D)B] in a flow cytometer.

This procedure makes use of the high iron affinity of desferrioxamine, which enables ready transfer of deferiprone-bound iron to the detector moiety used in DCI or DCIB.

Measurement of LPI in fluids (LPIB) by flow cytometry. The beads or microspheres are constructed as described using either DHR (dihydrorhodamine) or dichloro-dihydrofluorescein, coupled to protein and the protein coupled to the beads. A possible variation on this technique would involve incubation of the protein-bound probe with the bio-fluid followed by attachment of the probe to beads. An example could be soluble avidin-conjugated probe, which is incubated with the bio-fluid and then quantitatively trapped on biotinylated beads after completion of the reaction. LPI on beads (LPIB) is detected as a time dependent rise in fluorescence prompted by ascorbate and blocked by desferrioxamine. The rise in fluorescence after a predetermined period of time (20 minutes at room temperature) is proportional to the level of labile iron.

Measurements of LCI in cells by flow cytometry (Figures 5A-C). The labile cell iron content is indicative of cumulative events of exposure to LPI. Those are also manifested in circulating blood cells as a raise in LPI. LPI is determined in cells with the probe calcein CAL (green or blue, CALG and CALB, respectively) which is loaded into cells as its acetomethoxy-precursor CAL-AM. The intracellular CAL probe that is formed in cells from CAL-AM interacts with labile iron and undergoes quenching commensurate with the LCI levels. This is depicted for attached cells based on the outlined protocol. . When analyzed by flow cytometry, a plot of fluorescence intensity (FL1-H) is obtained with distributions obtained before and after the addition of the permeant chelator SIH (Figure 5B). The right shift in median fluorescence (upper panel) reveals the level of labile cell iron (LCI). The mean change in fluorescence intensity ΔF elicited by a permeant chelator (SIH or others like DFP or DFR) on blood samples containing erythrocytes and reticulocytes preloaded with CAL-AM is obtained from the difference in median values (from plots as those shown above for fluorescence associated with each population determined with appropriate instrument settings. The key question is how to convert the fluorescence changes elicited by chelator ΔF into actual LCI concentrations. For that purpose, CALG coated beads titrated with given amounts of labile iron are made and measured then the flow

cytometer before and after addition of a strong chelator that can displace the CALG-bound iron (Figure 5C). The titration of the beads with iron is depicted in Figure 5C. The lower left panel depicts the fluorescence distributions after addition of Fe and the upper those after addition of chelator (100 μ M of deferiprone, another cell permeant iron chelator). A plot of the weighted reciprocal change in fluorescence vs Fe was linear for a wide range of Fe concentrations, allowing conversion of shifts in fluorescence of CAL-laden cells elicited by chelators into concentrations.

Figure 6 provides a general description of quantifying DCI and LCI using by flow cytometry, as applied to CALG.

Requirements:

High FMS density on polymers/beads; for the FMS calcein-green (CALG) (comparable to concentration attained in cells)

FMS on beads must remain quantitatively accessible to iron (for quenching) and to chelators (for dequenching)!

a specific combination of carriers was found suitable for coating derivatized beads with the appropriate FMS

Principle (see Figure 6):

Labile iron binds to fluorescent bead and quenches its fluorescence (F) by an amount ΔF .

The changes in fluorescence (ΔF) are stoichiometric, namely 1:1 with Fe binding and the latter proportional to the the # of atoms of labile iron in solution.

For all FMS applied, ΔF should be prevented/recovered in the presence of a strong but specific Fe chelator (e.g. excess DFO for measurements of directly chelatable iron (DCI) in fluids and for calibration of measurements of labile cell iron (LCI) with CALG using CALG-beads.

Thus, the present teachings which will be described in details below, allow accurate measuring of pathological levels of labile iron.

Thus, according to an aspect of the invention there is provided a composition-of-matter comprising an iron indicator coating a microparticle, wherein the iron indicator comprises an iron binding moiety and a signal generating moiety, wherein an intensity of the signal generated by the signal generating moiety is related to an amount of the iron bound by the iron binding moiety. The iron binding moiety and the signal

generating moiety might be contiguous in the same molecule (i.e., covalently attached to form a single molecule) or distinct molecules bound on separate, but proximal sites of a polymer or a multimer such as a dendrimer (see Figure 3).

The phrase “iron binding moiety” refers to an iron chelator, which binds to or combines with iron ions, including all synthetic and natural organic compounds known to bind iron, and any molecule of biological origin, or by-product or modified product of a molecule of biological origin, such as proteins, sugars or carbohydrates, lipids and nucleic acids, and any combination thereof, that may bind iron ions.

Examples of iron binding proteins include but are not limited to transferrin, apo-transferrin, lactoferrin, ovotransferrin, p97-melanotransferrin, ferritin, Ferric uptake repressor (FUR) proteins, calcineurin, acid phosphatase, ferredoxin. .

According to a specific embodiment, the iron binding moiety is transferrin (Tf), since iron binding to Tf or apo-Tf is significantly faster than to other known iron binding molecules, which enhances the probability that free iron (including NTBI) will bind the indicator molecule rather than to endogenous apo-Tf.

Chemical moieties which are suitable for use as the iron binding moiety of this aspect of the present invention include iron chelators. The phrase “an iron chelator” refers to a molecule comprising nonmetal atoms, two or more of which atoms are capable of linking or binding with a iron ion to form a heterocyclic ring including the metal ion.

Examples of iron chelators include but are not limited to desferrioxamine (DFO), phenanthroline, ethylene diamine tetra-acetic acid (EDTA), diethylene triamine-pentaacetic acid (DTPA), N,N'-bis[2-hydroxybenzoyl]ethylene diamine-N,N'-diacetic acid (HBED) and the like. Other examples of iron chelators and related compounds are provided in U.S. Pat. NOs. 4,840,958, 5,480,894 4,585,780, 5,925,318 and in Hider (1996) *Acta Heamatologica* 95:6-12.

The signal generating moiety of the indicator molecule, described herein above, is selected such that the intensity of signal generated therefrom is related to the amount of the iron which is bound to the iron binding moiety. According to a specific embodiment, the intensity of the signal is stoichiometrically related to the iron bound by the iron binding moiety.

One of the properties of ionic iron is its inherent ability to affect the fluorescence properties of fluorophores when in atomic or molecular contact, usually resulting in the quenching of the fluorescence signal [Lakowicz, J.R. (1983) Principles of fluorescence spectroscopy, Plenum Press, New York, pp.266 ff.]. Hence, according to a specific embodiment, the signal generating moiety is a fluorophore, which can be quantified via its fluorescence, which is generated upon the application of a suitable excitatory light. The use of a fluorophore as the signal generating moiety allows the generation of a direct correlation between changes in fluorescence and NTBI concentration. According to some embodiments of the invention, the fluorophores are selected fluorescent in most channels of the flow-cytometer.

A non limiting list of commercially available fluorophores suitable for use as the signal generating moiety of the present invention along with approximate absorption (Abs) and fluorescence emission (Em) is provided in Table 1 below. The listed fluorophores are available from Molecular Probes (www.molecularprobes.com).

Table 1

<i>Fluorescence color (Abs/Em)</i>	<i>Fluorophore</i>
Blue (346/442)	Alexa Fluor 350
Blue (365/460)	Marina Blue
Blue (410/455)	Pacific Blue
Yellow-Green (433/539)	Alexa Fluor 430
Green (494/518)	Fluorescein-EX
Green (485/517)	CALCEIN
Green (495/519)	Alexa Fluor 488
Green (496/524)	Oregon Green 488
Green (511/530)	Oregon Green 514
Yellow (532/554)	Alexa-Fluor 532
Orange (556/573)	Alexa-Fluor 546
Red-Orange (555/580)	Tetramethylrhodamine
Red-Orange (570/590)	Rhodamine Red-X
Red-Orange (578/603)	Alexa Fluor 568

Red (595/615)	Texas Red-X
425/531	Lucifer Yellow
544/570	BODIPY TMR
493/503	BODIPY 493/503
499/508	BODIPY 499/508
507/515	BODIPY 507/515
478/541	NBD
555/580	Sulforhodamine

Alternatively, a fluorophore can be a protein belonging to the green fluorescent protein family including but not limited to the green fluorescent protein, the yellow fluorescent protein, the cyan fluorescent protein and the red fluorescent protein as well as their enhanced derivatives.

Optionally, the signal generating moiety of the indicator molecule can be an enzyme which when in the presence of a suitable substrate generates chromogenic products. Such enzymes include but are not limited to alkaline phosphatase, β -galactosidase, β -D-glucuronidase and the like.

It will be appreciated that a naturally occurring molecule such as an enzyme can comprise the indicator molecule of the present invention, wherein following iron binding a measurable conformational and/or a functional alteration is effected. For example, the aconitase enzyme is activated following iron binding [Klausner, R.D. et al. . (1993) Cell 72:19-28].

According to a specific embodiment of this aspect of the present invention the indicator molecule is DFO e.g., fluoresceinated deferrioxamine (FI-DFO).

According to a specific embodiment of this aspect of the present invention the indicator molecule is 5-4,6-dichlorotriazinyl aminofluorescein (DCTF)-apo-transferrin i.e., FI-aTf.

According to a specific embodiment, the iron indicator is a cell permeant iron indicator. This is beneficial for measuring cellular iron.

According to a specific embodiment, the iron indicators is selected from the group consisting of calcein, deferiprone, deferasirox (Exjade) and salicyl-aldehyde hydrazone (SIH).

According to a specific embodiment, the iron indicator is calcein (calcein-AM).

The indicator molecules of the present invention can be synthesized using well known chemical synthesis procedures. Detailed protocols describing how to use reactive fluorophores are available in www.dotmolecularprobesdotcom.

For example, amine-reactive fluorophores (i.e., including a reactive group such as dichlorotriazinyl, isothiocyanate, succinimidyl ester, sulfonyl chloride and the like) can be used to modify proteins, peptides and various synthetic molecules with primary amines (see www.dotprobesdotcom/media/pis/mp00143.pdf).

Following conjugation, unconjugated fluorophore is removed, usually by gel filtration, dialysis, HPLC or a combination of these techniques. The presence of free fluorophore, particularly if it remains chemically reactive, can greatly complicate subsequent analysis with the indicator of the present invention.

According to a specific embodiment, the indicator molecules of the invention are characterized by a high fluorescence yield yet retain the critical parameters of the unlabeled iron binding moiety (i.e., iron binding).

It will be appreciated though, that oftentimes, highly labeled conjugates are likely to precipitate or bind nonspecifically. It may therefore be necessary to have a less-than-maximal fluorescence yield to preserve function or binding specificity.

As described herein above, the indicator molecule of the present invention can be a chimeric protein including a protein fluorophore (e.g., GFP) linked to an iron binding protein. Such a chimeric protein can be produced via well known recombinant techniques.

It will be further appreciated that commercially available indicator molecules can also be used according to this aspect of the present invention. For example, calceins are commercially available from Molecular Probes Inc, FL-DFO, synthesized by reacting FITC with desferrioxamine (available from Evrogen, Moscow, Russian Republic) and the respective isothiocyanate derivatives of DFO (DFO-ITC) and DTPA (DTPA -ITC) are obtainable from Macrocyclics Inc (Dallas, TX, USA), RITC and derivaives are available from Fluka (Sigma-aldrich Co St louis MO).

According to a specific embodiment, the molecule comprises DFO.

According to a specific embodiment, the iron indicator comprises dihydrorhodamine 123 (DHR) or carboxydihydrofluorescein (CDCF).

According to a specific embodiment, the iron indicator comprises calcein.

According to a specific embodiment, the molecule comprises a modified apo-transferrin.

According to a specific embodiment, the molecule comprises fluorescein-apo-transferrin.

5 According to a specific embodiment, the fluorophore is selected from the group consisting of Fluorescein, Rhodamine, nitrobenzfurazan, fluorogenic β -galactosidase and a green fluorescent protein.

As mentioned the indicator molecule coats a microparticle.

10 As used herein the term "microparticles" or "beads" refers to particles having a mean diameter 0.1 to 100 μm (e.g., 5-10 μm). Microparticles have a much larger surface-to-volume ratio than at the macroscale, rendering the compositions of the invention highly advantageous in terms of affinity to free iron.

According to a specific embodiment, the microparticles comprise microspheres.

15 The microparticle should preferably be made of such material and be of such size as to stay suspended, with minimal agitation if necessary, in solution or suspension (i.e., once the sample is added). It should preferably not settle any faster than cells of interest in the sample, as further described herein below. The material from which the microparticles are made should be such as to avoid clumping or aggregation, i.e., the formation of doublets, triplets, quadruplets and other multiplets. This can be minimized
20 by using mild detergents such as Triton X-100 (0.05 %).

Microparticles may be used in accordance with the present teachings include, but are not limited to fixed human red blood cells, coumarin beads, liposomes, cell nuclei and microorganisms. However, particularly advantageous examples of microparticles that may be used in the invention include microbeads, such as agarose
25 beads, polyacrylamide beads, polystyrene beads and silica gel beads.

Other microparticles for use in the methods and compositions described here include plastic microbeads. While plastic microbeads are usually solid, they may also be hollow inside and could be vesicles and other microcarriers. They do not have to be perfect spheres in order to function in the methods described here. Plastic materials such
30 as polystyrene, polyacrylamide and other latex materials may be employed for fabricating the beads, but other plastic materials such as polyvinyl chloride,

polypropylene and the like may also be used. According to a specific embodiment, the microparticles are polystyrene beads, silica beads or agarose beads.

According to a specific embodiment, the microparticle has a molecular size of 4-10 μm similar to, or lower than most mammalian cells.

5 The significance of using analytical beads of sizes similar to those of mammalian cells is first because beads must have $> 2\text{-}3\text{ }\mu\text{m}$ diameter for proper flow cytometry (FC) analysis; and second for obtaining changes in fluorescence intensity per particle/cell under equivalent conditions.

10 The indicator molecules coat the microparticles. Preferably, the coating is homogeneous. Attachment or conjugation of the indicator molecule to the microparticle is effected such that the functionality of the indicator molecule is unharmed (that is, without compromising neither the signal intensity nor the binding to the metal binding moiety).

15 The following is a non-limiting example of bead construction, with reference being made to Figure 2. The microparticle to which the iron indicator is bound may be composed of a polymeric microparticle bead onto which the iron indicator is covalently attached. The binding may be direct or via a spacer layer bound to the polymer. In order to provide for signal quenching which results from specific binding of labile iron to the bead the following parameters should be met:

- 20 (i) high iron indicator density on the microparticle ($>10,000$ per particle);
- (ii) the iron indicator on the microparticle should remain quantitatively accessible to iron for quenching and, where applicable, for the iron binding moiety provided in excess for dequenching (important for elucidating the background signal);
- (iii) a specific combination of carriers should be used for coating derivatized
- 25 beads with the appropriate iron indicator, so as to attain what is explained above [(ii)]. . For example, a spacer (linear or branched-as in polyethylene glycols, dendrimers or globular proteins) should be introduced between the bead and the iron indicator.

According to a specific embodiment, the iron indicator is conjugated directly to the microparticle.

30 According to a specific embodiment, the iron indicator is conjugated indirectly to the microparticle.

According to a specific embodiment, the indirect conjugation is via an adhesive protein.

According to a specific embodiment, the adhesive protein comprises a histone or an albumin, (serum albumin, ovalbumin, lactalbumin).

5 According to a specific embodiment, the iron indicator is conjugated indirectly to the microparticle via an extension arm.

According to a specific embodiment, the extension arm is composed of a dendrimer or a multifunctional bridge.

Figure 2 describes specific configurations (non-limiting) of conjugating the iron
10 indicator to the microparticle.

A specific coat labeled with the iron indicator is covalently coupled to the surface of (COOH or NH₂-derivatized) polystyrene (or silica) 4-10 μ m mean diameter bead. In a specific embodiment the iron indicator consists of probes like calcein green (CALG) or fluorescein + deferrioxamine (F+DFO), which are bound covalently to the
15 coated beads (either separately or as F-DFO conjugate) Alternatively, the iron indicator comprise rhodamine (R)+ deferrioxamine (R+DFO), which are bound covalently to the coated beads (either separately or as R-DFO conjugate).

As used herein the term "separately" means that each of the iron binding moiety and the fluorophore are individually bound to the bead without prior covalent
20 conjugation of same.

Polymer bead: Polystyrene, silica or the like (4-10- μ m diameter) with terminal primary amino or carboxyl groups.

Extension arm:

a. dendrimer (PAMAM generation 1-3) or polyethylene glycol (linear or
25 branched) coupled covalently to pre-activated microparticle, or

b. multifunctional bridge (lysine or orthogonally-protected diaminobutyric acid derivative with hindered Dde variant ivDde.) coupled to bead-dendrimer via N-(α and γ protected) -COOH

FMS platforms (coat layer):

30 protein/polypeptide (ovalbumin or the like) onto which CALG is coupled via the COOH- not involved in metal binding (the -COOH groups involved in metal binding are protected during coupling with excess Co) or

F or R and DFO are covalently attached to dendrimer NH₂ sites (via their –NCS or ITC- derivatives FITC or RITC and DFO-ITC) or

F or R and DFO are covalently attached (sequentially) to stepwise deprotected lys (pre-coupled to dendrimer)

5 Compositions of the present invention can be widely used to directly and sensitively detect free iron levels in biological fluids and inside cells, the latter also termed "labile cellular iron (LCI)".

Typically, normal cellular iron levels are between about 0.2-1.5 μ M. Thus abnormal cellular levels are typically below about 0.2 μ M or above about 1.5 μ M.

10 As used herein the phrase "free iron levels" refers to non-transferrin bound iron (i.e., NTBI).

As used herein the term "NTBI" refers to directly chelatable iron (DCI) which is accessible to exogenous iron chelators; mobilizer-dependent chelatable iron (MDCI) which is accessible to exogenous iron chelators upon addition of mobilizing agents; and
15 labile plasma iron (LPI) including redox active iron, which redox activity is eliminated upon addition of exogenous iron chelators.

Thus, according to another aspect of the invention, there is provided a method of quantifying free iron levels in a sample. The method comprising:

(a) contacting a sample of the biological fluid with the composition-of-matter
20 of the present invention; and

(b) detecting and quantifying the signal thereby quantifying free iron levels in the biological sample.

According to a specific embodiment, the free iron comprises non-transferrin bound iron.

25 According to a specific embodiment, the free iron comprises directly chelatable iron.

According to a specific embodiment, the free iron comprises labile plasma iron.

According to a specific embodiment, the iron indicator comprises fluoresceinated deferrioxamine (FI-DFO).

30 According to a specific embodiment, the iron indicator comprises calcein green (CALG).

According to a specific embodiment, the iron indicator comprises dihydrorhodamine 123 (DHR)

The phrase “biological sample refers to a biological fluid such as blood, serum, plasma, lymph, bile fluid, urine, saliva, sputum, synovial fluid, semen, tears, cerebrospinal fluid, bronchioalveolar large fluid, ascites fluid, pus and the like. The sample may be free of cells or comprise cells. Methods of quantifying intracellular free iron levels are further described herein below.

Thus, a sample of the biological fluid is contacted with the composition-of-matter of the present invention. Contacting is effected under conditions suitable for binding of the iron-binding moiety of the indicator molecule to free iron.

It will be appreciated that fluorescence detection sensitivity is oftentimes compromised by background signals, which may originate from endogenous sample constituents or from unbound or nonspecifically indicator molecules (i.e., background fluorescence). To compensate for background fluorescence, parallel samples are preferably incubated with excess unlabeled chelator molecule, which scavenges all iron in the sample.

Thus, the method further comprises:

(c) contacting the sample with an iron binding moiety (unlabeled) in excess to the iron indicator; and

(d) detecting and quantifying the signal of step (C), the signal being the background signal of the chelation reaction.

According to a specific embodiment, the iron binding moiety in excess is identical to the labeled iron binding moiety (i.e., of the iron indicator).

The background signal is typically deduced from the signals obtained in all the samples tested.

The signal is thus detected and quantified to determine the levels of free iron in the biological fluid of the subject.

Since the indicator molecules are immobilized onto microparticles, as described above, signal detection may be effected by any suitable instrumentation such as a flow cytometer e.g., FACS.

The intensity of signal produced may be analyzed manually or using a computer program.

Any abnormality in the levels of free iron in the sample is indicative of the presence of a disorder.

In general, free iron quantification is preferably effected alongside a look-up table or a calibration curve so as to enable accurate iron determination. Methods of
5 calibrating iron levels are further described herein below.

Embodiments of the method of this aspect of the present invention include the use of iron mobilizing agents and removal of endogenous apo-transferrin from the sample, to accurately determine the levels of free iron in the sample.

As described hereinabove, free iron is often found in low and high molecular
10 weight complexes such as with citrate and albumin, respectively. To accurately determine free iron levels, the fluid sample obtained from the subject is preferably treated with a mobilizing agent, described hereinabove, prior to contacting with the compositions of the present invention. Examples of mobilizing agents include but are not limited to sodium oxalate, nitrilotriacetate, ascorbate and salicylate.

15 It will be appreciated that the mobilizing reagent employed must balance the ability to detect maximal free iron levels, without contributing to iron release from iron-containing transferrin in a given sample.

Preferably, 10 mM sodium oxalate is used as the mobilizing reagent according to this aspect of the present invention.

20 It will be appreciated, though, that other mobilizing agents or other combinations thereof can be used providing that they cause quenching of an indicator molecule of the present invention which is not quenched in the absence of a mobilizing reagent while causing no quenching of samples which contain no free iron such as normal serum samples or human transferrin saturated with various concentrations of
25 iron.

Another embodiment of the method of this aspect of the present invention includes the exclusion of endogenous apo-transferrin and/or iron free transferrin from the sample prior to free iron determination.

Apo-transferrin is universally found in human sera, except in cases of extreme iron-
30 overload where the transferrin is 100 % iron-saturated. Therefore the detection of free iron may be rendered more difficult once the sample contains nearly normal levels of apo-Transferrin. The use of Fl-aTF as a probe equalizes the probability that the

mobilized iron will bind to the indicator molecule or to endogenous apo-Transferrin in the sample.

Exclusion of endogenous apo-transferrin can be effected by incubating (i.e., pre-clearing) the sample with anti-apo-transferrin antibodies, such as solid phase coupled
5 anti-transferrin antibodies available from Pharmacia, Uppsala and Bio-Rad Laboratories, Hercules, CA. Additionally or alternatively anionic beads such as MacroPrep® High S support beads available from Bio-Rad Laboratories, Hercules, CA can be used to exclude apo-transferrin from the sample.

Preferably, exclusion of apo-transferrin is effected by co-incubating the sample
10 with an apo-transferrin binding metal other than iron such as Gallium and Cobalt. These metals mimic iron and bind to the indicator molecule of the present invention, preventing their reaction with iron [Breuer and Cabantchik Analytical Biochemistry 299, 194-202 (2001)].

However, when using such metals, measures are taken not to use indicator
15 molecules which are affected by such metals. Hence, a preferably used indicator molecule is FI-aTf, described hereinabove, which is not affected by Gallium due to a biochemical mechanism which is yet to be determined. This apparent insensitivity to Gallium gives the FI-aTf indicator an iron-binding advantage over the endogenous Apo-transferrin, overcoming most of its interference.

20 As described hereinabove NTBI also refers to labile plasma iron (LPI) including redox active iron, which redox activity is eliminated upon addition of exogenous iron chelators.

Redox active iron [i.e., ferrous iron] is highly damaging when labile [Herbert (1994) Stem Cells 92:1502-1509]. Ferric iron [i.e., Fe(III)] is a relatively nontoxic form
25 of iron. However, ferrous iron [i.e., Fe(II)] plays a significant role in the generation of oxygen species (ROS), excess of which has been proven to be extremely harmful to the health of individuals. Free radical toxicity is produced primarily by the hydroxy radical ($\cdot\text{OH}$). Most of the $\cdot\text{OH}$ generated in vivo comes from iron-dependent reduction of H_2O_2 [Halliwell (1986) Arch. Biochem. Biophys. 46:501-14]. It is well established that
30 redox active iron and its reaction products (i.e., ROS) promote numerous diseases including cancer, diabetes, heart diseases and liver diseases [Halliwell, B. and

Guttridge, J.M. (1995). Role of free radicals and catalytic metal ions in Human Disease: An overview. Meth. Enzymol 186:1-85].

Accordingly, it is essential to quantify redox active iron in a biological fluid.

Thus, according to yet another aspect of the present invention there is provided a method of quantifying redox active iron in a biological fluid. The method comprising:

(a) contacting a sample of the biological fluid with a reducing agent, to obtain redox active iron;

(b) contacting the sample with a detector molecule selected capable of measurable activation upon contact with redox active iron reaction products, wherein said measurable activation is related to an amount of said redox active iron reaction product; and

(c) quantifying said measurable activation using the composition-of-matter of the present invention, thereby quantifying redox active iron levels in the biological fluid.

Thus, as mentioned, first, a sample of the biological fluid is contacted with a reducing agent. The reducing agent is selected capable of reducing free iron from ferric to ferrous form. Suitable reducing agents according to this aspect of the present invention include, but are not limited to, ascorbic acid, dithionite, mercaptoacetic acid, dithiothreitol.

Preferably, a physiological concentration of ascorbic acid is used according to this aspect of the present invention.

Reduced iron is capable of reacting with any oxygen species dissolved in the sample to generate redox active iron reaction products (e.g., ROS).

Reducer-treated sample is then contacted with a detector molecule, which can be measurably activated upon interaction with the newly generated redox active iron reaction product. The detection molecule is selected such that its activation is related to the amount of the redox active iron reaction products. Specific examples include, but are not limited to, dihydrorhodamine, carboxy-dichloro-dihydrofluorescein, dihydroethidium and dihydroresorufin.

Finally, activation of the detection molecule is quantified, thereby quantifying redox active iron levels in the biological fluid.

Preferably, the detector molecule is a molecule which undergoes a spectrophotometric/fluorescence change following interaction with the redox active iron reaction products, such as ROS. Examples include but are not limited to dihydrorhodamine-123 (DHR), which converts to fluorescent rhodamine, dichloro-
5 dihydrofluorescein, which converts to dichlorofluorescein, dihydroresorufin which converts to resorufin and the like.

Quantification of detector molecule activation is effected according to the methodology described herein above.

It will be appreciated that to specifically identify redox active iron reaction
10 products which are generated by the catalysis of iron alone, a parallel sample is subjected to the treatment described hereinabove and an excess of iron chelator. Any increase in fluorescence reflects background ROS generation.

Labile cellular iron (LCI) is also an important pathological measure.

As used herein "labile plasma iron" refers to a component that is redox active and
15 chelatable in native plasma or serum or plasma diluted in salt solutions or serum following mobilization with a mild extracting agent.

Thus, the present invention further provides for a method of quantifying labile cellular iron in a biological sample, the method comprising:

- (a) loading cells of the biological sample with an iron indicator composed of
20 an iron binding moiety and a signal generating moiety (i.e., microparticle-free), wherein an intensity of said signal generated by said signal generating moiety is related to an amount of said iron bound by said iron binding moiety;
- (b) contacting loaded cells of step (a) with a cell permeant metal chelator so as to remove the iron from the iron binding moiety;
- 25 (c) recording a signal generated in (a) and in (b), wherein the mean change in signal intensity is indicative of a level of labile cell iron;
- (d) quantifying the change using a calibration curve generated as described below.

Cells in which cellular iron is recorded include but are not limited to, red blood
30 cells, white blood cells and cells taken from biopsies.

According to a specific embodiment, the iron indicator is first contacted with the reaction buffer and only then the diluted sample is added.

Rationale: The initial incubation is meant to bind all the contaminating iron in solutions prior to addition of the biological test-solution.

In a specific embodiment, the microparticles are added to a 240 microliter solution of buffered saline (+/- 50 micromolar DFO) and preincubated for 0.5-1 hr at room temperature, with frequent mixing. A 60 microliter volume of plasma/or serum/or unknown sample/ or calibration solution with a given concentration of Fe (0-20 micromolar original concentrations will yield final 0-5 micromolar in the 300 microliter volume= 1:5 dilution of the test sample) is added and incubated for an additional hour with mixing.

Aliquots are then taken for FACS analysis .

Figures 5A-B provide specific and non-limiting assay conditions for measuring LCI (Labile cell iron).

The iron indicator is added as a non-fluorescent precursor that permeates the cells and releases a fluorescent indicator that binds labile cell iron (LCI) and undergoes quenching. Addition of a strong permeant chelator (identical or different from that in the iron indicator) removes the iron from the metal indicator causing cell fluorescence F to rise, so that:

$$\Delta F = F_{MS-Fe} \equiv LCI$$

Measuring fluorescence on a flow cytometer before and after addition of chelator allows determination of ΔF .

Using beads coated with the fluorescent iron indicator, a plot of ΔF versus iron concentration produces a hyperbolic curve for the entire range of iron concentration and a linear curve (Figure 5C) for a given range (depends on the concentration of the microparticles added to the assay medium).

The relationship between ΔF and [Fe] for a given number of microparticles can be used for converting ΔF obtained with cells. This is done using a calibration curve. Thus the instant application further provides for a method of calibrating free iron levels in a fluid sample, the method comprising:

(a) contacting a plurality of fluid samples with predetermined distinct free iron concentration values (e.g., serial dilutions of iron at a dilution index of e.g., 10) with the composition of matter of the invention;

(b) detecting and quantifying the signal; and

(c) generating a calibration curve depicting the signal against the predetermined distinct free iron concentration values.

According to a specific embodiment, the method of calibrating further comprises contacting the plurality of samples with an iron chelator so as to release said iron binding moiety following step (b); and detecting and quantifying a signal' generated following contact with said iron chelator, and wherein the calibration curve depicts said change in the signal versus the signal' against the predetermined distinct free iron concentration values.

It will be appreciated that the compositions of the present invention can be included in a diagnostic or therapeutic kit. For example, indicator sets including one or more of the following components described hereinabove (e.g., the composition comprising indicator molecules in solution or attached to microparticles along with a mobilizing agent, a non-labeled iron chelator, an apo-transferrin binding metal other than iron, an anti-apo transferrin antibody or anionic beads), can be packaged in a one or more containers with appropriate buffers and preservatives and used for diagnosis or for directing therapeutic treatment.

The compositions of the present invention can be widely used to directly and sensitively detect NTBI which persists in sera of patients, even with low transferrin saturation.

Thus, according to still another aspect of the present invention there is provided a method of determining presence, absence or risk of a disorder associated with abnormal levels of free iron in a biological fluid or cells of a subject.

These can be classified under diagnosis of a disorder associated with abnormal levels of free iron in a biological fluid or cells.

As used herein the term "diagnosis" or "diagnosing" refers to determining presence or absence of a pathology (e.g., a disease, disorder, condition or syndrome associated with abnormal levels of free iron in a biological fluid or cells), classifying a pathology or a symptom, determining a severity of the pathology, monitoring pathology progression, forecasting an outcome of a pathology and/or prospects of recovery, determining a risk for the pathology and screening of a subject for the pathology.

Examples of disorders and conditions which are associated with abnormal levels of free iron include, but are not limited to, hemolytic diseases hemoglobinopathies,

thalassemia, thalassemia major, anemia, sickle cell anemia, aplastic anemia, megaloblastic anemia, myelodysplasia, diseases which require repeated transfusions, diseases which require dialysis, hereditary hemochromatosis, cancer, heart diseases, Myelo Dysplasia Syndrome (MDS), iron poisoning and rheumatoid arthritis.

5 The present teachings further provide for a method of treating a subject having a disorder associated with abnormal levels of free iron in a biological fluid or cells, the method comprising:

 (a) determining levels of the free iron in the biological fluid or cells of the subject according to any of the methods described above;

10 (b) determining in the subject a presence of the disorder associated with abnormal free iron levels based on said levels obtained in (a); and

 (c) treating the subject using a medicament for the disorder associated with abnormal free iron levels.

 According to a specific embodiment the medicament comprises an iron chelation
15 therapy.

 According to a specific embodiment, the iron chelation therapy comprises Deferoxamine (Desferal, DFO), Deferiprone (Ferriprox, L1), Exjade (Deferasirox), Deferasirox, FB50701 (Ferrokin Bioesciences) or a combination of same e.g., Desferal and Deferiprone.

20 The present teachings further contemplate a method of determining efficacy of treatment of a disorder associated with abnormal levels of free iron in a biological fluid or cells, the method comprising;

 (a) treating a subject in need thereof using a medicament for the disorder associated with abnormal free iron levels; and

25 (b) determining levels of the free iron in a biological fluid or cells of the subject according to any of the methods of claims 24-40, wherein a change in the levels following the treating is indicative of treatment efficacy.

 According to a specific embodiment, the medicament comprises iron chelation therapy, and whereas a reduction in the levels following the therapy is indicative of
30 efficacious treatment. For example, normal LPI/plasma NTBI levels are below about 0.2 μ M, the sought after goal in the treatment of hyper-transfused patients (e.g. thalassemia) or phlebotomized samples (hemochromatosis).

According to another specific embodiment, the medicament is selected from the group consisting of oral iron supplementation, folic acid, vitamin B-12, erythropoietin, blood transfusion and hyperbaric oxygen, and whereas an increase in said levels following said therapy is indicative of efficacious treatment.

5 Preferred individual subjects according to the present invention are mammals such as canines, felines, ovines, porcines, equines, bovines, humans and the like. Methods of obtaining body fluids or cells from mammals are well known in the art. It will be appreciated that the source of the fluid varies between the different disorders identified.

10 Quantification of free iron levels is effected as described hereinabove.

Determining iron levels originating from a biological sample of a patient is preferably effected by comparison to a normal sample, which sample is characterized by normal levels of free iron (i.e., no detectable free iron). If free iron levels exceed normal values, the subject is informed of the diagnosis.

15 The availability of an accurate and non-invasive free iron quantification method is also useful in disease management. The diagnostic method of the present invention, can aid a medical professional diagnosing of a patient even with low levels of free iron and instructing the patient on the type of diet to maintain in terms of iron content and iron availability for adsorption, which can result in iron overload.

20 It is expected that during the life of a patent maturing from this application many relevant chelators, particle, fluorophores, etc. will be developed and the scope of the aforementioned terms is intended to include all such new technologies *a priori*.

As used herein the term "about" refers to $\pm 10\%$.

25 The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to".

The term "consisting of means "including and limited to".

30 The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or

"at least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases "ranging/ranges between" a first indicate number and a second indicate number and "ranging/ranges from" a first indicate number "to" a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals there between.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

As used herein, the term "treating" includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical or aesthetical symptoms of a condition or substantially preventing the appearance of clinical or aesthetical symptoms of a condition.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided

separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

5 Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

10 Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the
15 literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific
20 American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994);
25 Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262;
30 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and

Translation" Hames, B. D., and Higgins S. J., Eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader.

10 All the information contained therein is incorporated herein by reference.

Preparation of polystyrene beads coated with iron-sensitive probes (specific conjugation protocols are provided infra)

15

Example 1

CALGB

Beads coated with calcein green (CALG)

Preparation of CALG coated polystyrene beads.

Step 1. Covalent attachment of calcein (CALG) to ovalbumin: A complex of CALG with Cobalt (CA:Co) is generated by mixing 2 mM CALG A (Sigma, Molecular Probes) with 4 mM CoCl₂ in a solution containing 50 mM MES, 100 mM NaCl, pH 6.5. Ovalbumin (OVA) in powder form is added and allowed to dissolve, giving a final concentration of 0.1 mM. Finally, dry EDC is added in 2 separate portions at 20 min intervals to give a final concentration of 25 mM. Total reaction time is 40 min. The reaction mixture is then passed through a Sephadex G-25 column pre-equilibrated with PBS and the conjugate OVA-CALG-Co is eluted in the void volume. This results in a 2-fold dilution of the input OVA, giving a final OVA concentration of 0.05 mM. The concentration of CALG-Co in the OVA-CALG-Co conjugate is determined by absorbance at 490 nm using a calibration curve of free CA-Co. The ratio OVA:CALG is typically 1:2.

Step 2. Preparation of dendrimer-coated polystyrene beads (Den-B).

Polystyrene beads (5% w/v, Spherotech) with surface carboxyl groups, diameter (4-6.2 μm), are washed once in DDW, once in MES buffer pH 6 and reacted with n-hydroxysuccinimide sulfonic acid (NHS) (3 fold molar excess)+ 20-fold excess of EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide)] for 2 h and then washed with distilled water (centrifugation and resuspension) and then resuspended with vigorous stirring in NaHCO_3 buffer containing PAMAM dendrimer, generation 2 or -3 (16-32 amino groups per dendrimer; 10% w/v) final concentration 1 mM and reacted for 2 h at room temperature, then washed with 4 volumes of PBS containing 0.05 % Triton-X-100 followed by sonication.

Step 3. Binding of OVA-CALG-Co to dendrimer polystyrene beads (Den-B).

The Den-B beads (washed once in DDW and packed by centrifugation are supplemented with a volume of OVA-CALG-Co solution (0.05 mM OVA) equivalent to 2 volumes of the original bead suspension and allowed to passively adsorb to the beads by mixing for 10 min. To the mixture of beads and OVA-CA-Co is added (10% w/v, MW 14,215; 64 amino groups) to a final concentration of 0.07 mM and the mixture is vigorously mixed for 10 min followed by bath sonication.. To the mixture of beads+ OVA-CA-Co is then added the bis-suberimidate crosslinker BS3 to 1 mM and the mixture is vigorously mixed for 20 min at room temperature. BS3 is then neutralized by addition of L-lysine to a final concentration of 50 mM and further incubation for 20 min. The mixture is then diluted with 4 volumes of PBS containing 0.05 % Triton-X-100 and washed once in PBS/ Triton-X 0.05%. The beads are then re-suspended in PBS/ Triton-X 0.05%/ EDTA 2 mM/ DTPA 2 mM and mixed for 20 min, to remove the Cobalt. The beads are then washed 3 times with PBS/ Triton-X 0.05%, resuspended in the same buffer at ~ 2.5 % w/v. The beads are sonicated in a bath sonicator to break up clumps and passed through a 30 μm filter mesh to remove aggregates. At this stage the beads are ready for use. The beads examined by epifluorescence microscopy are depicted in the figure.

Step 4. Assessing responsiveness of CALGB to iron. The beads are added to a final concentration of ~0.025% w/v to 1 ml aliquots of HBS containing Fe:NTA (ratio 1:2) at Fe concentrations ranging from 0 to 10 μ M. After 30 min incubation with occasional mixing, in the dark, the beads are analyzed by flow cytometry. The change in mean fluorescence intensity (ΔF in arbitrary flow cytometry units) elicited by a given concentration of [Fe] in the presence of a given number of beads (counted by the flow cytometry) is depicted in the main graph (with the hyperbolic non-linear best fit) and the linearized Hanes Woolf's plot (Figure 5C inset):

$$\frac{[Fe]}{\Delta F} = \frac{[Fe]}{\Delta F_m} + \frac{K_{1/2}}{\Delta F_m},$$

where ΔF_m represents the maximal attainable change in fluorescence by a given preparation of beads.

Example 2

Specific formulations of Den-B beads

I. Den-B (F+D)

Den-B Beads coated with Fluorescein (F) + Deferrioxamine (D)

The Den-B Beads are reacted with fluorescein isothiocyanate FITC (0.1 mM in 50 mM NaHCO_3 (pH 8.5-9.0) buffer for 2 h at room temperature, washed in the same buffer and subsequently reacted with deferrioxamine isothiocyanate (DFO-ITC, from Macrocytics Inc, Dallas TX) 0.2 mM for 2 h at room temperature, washed with 0.05 % Triton x-100 in PBS, PBs, sonicated (bath sonicator).

The Den-B (F+D) beads were used to determine the concentration of standard labile iron in solution in the absence and presence of 50 μ M DFO and the fluorescence intensity was read in a flow cytometer and the difference between $\pm$ DFO ΔF was determined and used for constructing the calibration curve shown in Figure 4A.

Example 2A

Den-B (R+D)

Den coated with Rhodamine (R) + Deferrioxamine (D): Den (R+D)

Reaction: The Den (original Dendrimer 3, Aldrich, MW=6909; 32 NH_2 groups; conc. 29mM) were suspended as to yield a solution of 0.1 mM NH_2 groups in 50 mM NaHCO_3 (pH 8.5-9.0) buffer and reacted with rhodamine isothiocyanate RITC (from Fluka) + DFO-ITC ((from Macrocytics) (~0.1 mM each, added from stock DMSO

solutions, final DMSO 2%) under vortexing at 37°C. for 20 min, (centrifuge 13000 rpm for 10s to remove percipitates) and repeat the reaction twice more, the last reaction time 2 hrs. Final assumed reaction conc : Den=0.15 mM (NH₂ groups) ; DFO-ITC=0.3 mM; RITC =0.375 mM

5 Purification: 1 ml of Den(R+D) solution are passed through 18 ml Sephadex-G25 column pre equilibrated in PBS and eluted with PBS while collecting the void volume and the sample stored frozen.

 Coupling of Den (R+D) to beads to yield Den B (R+D). 0.1ml of amino-coated polystyrene beads (Spherotech; 6.2um diam) are added to eppendorf tubes and washed
10 twice distilled water (by centrifugation at 10K rpm x 1 min). A 100 µl of Den (R+D) are added together with 50 ul Ovalbumin (Sigma 45mg/ml in DDW), incubate for 5 min, and centrifuged 10K rpm 10 sec, and the supernatant is aspirated. These steps are repeated twice more (we referred to these steps as coating of the beads with dendrimer with the aid of ovalbumin as additional crosslinking bridge)) . After the last coat, the
15 suspension (150µ l) is transferred to 2 ml Eppendorf and subjected to crosslinking (while vortexing) by addition of 17 µl of BS3(Bis(sulfosuccinimidyl) suberate; Pierce-Thermo Scientific, cat# 21580; prepared .Fresh 10 mM in DDW). The reaction of crosslinking is carried out on rotatory mixer for 1hr rm temp in the dark. 1 ml PBS+0.05% Triton-X(PBS-T) are added together with 60 µl of Lysine 0.5M in DDW
20 pH 7.3 (to stop the reaction and neutralize extra reagent) for 20 min room temp dark. Additionally, 1 ml 20 mM acetic Acid pH 3.5 + 0.05% Triton-X are added for 10 min at room temperature in the dark, the crosslinkwed beads are centrifuged, washed with 20 mM Acetic Acid pH 3.5 + 0.05% triton-X and finally resuspended in 300 ul of HBS+0.05% Triton-X (HBS-T). The beads are finally sonicated for 2 min in HBS-T
25 and stored aftyre addition of ethanol (50% final) for long term storage at 4°C.

 Figure 4B shows fluorescence intensity dispersity FL2 (560->610 nm) of Rhodamine+DFO coated beads DenB(R+D) analyzed by flow cytometry. Titration of DenB(R+D) with the indicated concentrations of Fe as analyzed by flow cytometry (tested 1 month apart). The relative change in fluorescence ΔF produced by the
30 indicated concentration of Fe is plotted in the inset (semilog scale). The figure further shows linear correlation in the comparison of NTBI values of 10 thalassemia patients

obtained by the plate reader method of DCI (based on Fl-DFO) versus those obtained by the flow cytometric method based on DenB(R+D).

Example 3

Den-B (FD)

Beads coated with Fluorescein-Desferrioxamine

The procedure is similar to that for Den-B (F+D). FD or FDFO [N-(fluorescein-5-thiocarbamoyl)-desferrioxamine] (produced by Evrogen Ltd., Moscow, Russian Republic) is first protected by complexing with iron (1:1 ratio with FeSO_4). Ovalbumin-F-DFO (ovaFD) is formed by reacting: 2.8 mg of ovalbumin with 20-fold excess of EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide], N-hydroxysuccinimide sulfonic acid in 3 fold molar excess) and 1 mM FDFO:Fe at pH 6.5 for 2 h, followed by gel filtration. Unreacted components and Fe were removed by dialysis against 10 mM acetic acid; 1 mM EDTA; 150 mM NaCl, pH 4.5 followed by dialysis against saline. Aminopropyl-containing polystyrene beads (diameter 6.2 μm) are coated with Fluorescein-Desferrioxamine by the same procedure as described above for CALGB,

Determination of protein: CAL-G ratio

15 μl of 4 mM CAL-G-Co + 285 μl of PBS are added to a 1st well of 96- well plate. Serial dilution in PBS (150 μl per transfer) are made in 8 steps (2 fold dilution each step). The light absorption peak of CAL-G-Co is determined. Absorbance of dialyzed samples containin FMS are compared to calibration curve so as to determine the concentration of FMS.

Example 4

Determination of labile iron using fluorescence metal sensors on beads (FMSB)

Figure 1A- Labile iron (Fe) binds to a fluorescent bead that carries a fluorescent metal sensor (FMS) and quenches its fluorescence (F) by an amount ΔF . The change in fluorescence (ΔF) is stoichiometric, namely 1:1 with Fe binding and the latter proportional to the number of atoms of labile iron in a given solution.

For all FMS applied, the relevant ΔF is that which is affected (blocked) by the presence of a strong/ specific Fe chelator:

for directly chelatable iron (DCI) in fluids, the chelator deferrioxamine (DFO) is added in excess.

5 for measurements of labile cell iron (LCI) with CALG, a permeant chelator such as deferiprone, deferasirox or salicyl-aldehyde hydrazone (SIH) is added in excess.

Figure 1B - Labile iron (Fe) binds to a fluorescent bead that carries a fluorescent metal sensor (FMS) and quenches its fluorescence (F) by an amount ΔF .

The change in fluorescence (ΔF) is stoichiometric, namely 1:1 with Fe binding and the
10 latter proportional to the number of atoms of labile iron in a given solution.

For all FMS applied, the relevant ΔF is that which is affected (blocked) by the presence of a strong/ specific Fe chelator:

for directly chelatable iron (DCI) in fluids, the chelator deferrioxamine (DFO) is added in excess

15 for measurements of labile cell iron (LCI) with CALG, a permeant chelator such as deferiprone, deferasirox or salicyl-aldehyde hydrazone (SIH) is added in excess

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations
20 will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same
25 extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

WHAT IS CLAIMED IS:

1. A composition-of-matter comprising an iron indicator attached to a microparticle, wherein said iron indicator comprises an iron binding moiety and a signal generating moiety, wherein an intensity of said signal generated by said signal generating moiety is related to an amount of said iron bound by said iron binding moiety.
2. The composition-of-matter of claim 1, wherein said iron binding moiety and said signal generating moiety are covalently attached forming a single molecule.
3. The composition-of-matter of claim 1, wherein said iron binding moiety and said signal generating moiety are distinct molecules.
4. The composition-of-matter of claim 1, wherein said iron binding moiety comprises deferrioxamine (DFO).
5. The composition-of-matter of claim 1, wherein said iron indicator comprises dihydrorhodamine 123 (DHR) or carboxydimethylfluorescein (CDCF).
6. The composition-of-matter of claim 1, wherein said iron indicator is a cell permeant iron indicator.
7. The composition-of-matter of claim 6 wherein said iron indicator comprises calcein.
8. The composition-of-matter of claim 6, wherein said iron indicator is selected from the group consisting of calcein, deferiprone, deferasirox and salicylaldehyde hydrazone (SIH).
9. The composition-of-matter of claim 1, wherein said iron binding moiety comprises a modified apo-transferrin.

10. The composition-of-matter of claim 1, wherein said iron indicator is fluorescein-apo-transferrin.

11. The composition-of-matter of claim 1, wherein said signal generating moiety is a fluorophore.

12. The composition-of-matter of claim 11, wherein said fluorophore is selected from the group consisting of Fluorescein, Rhodamine, nitrobenzfurazan, fluorogenic β -galactosidase and a green fluorescent protein.

13. The composition-of-matter of claim 1, wherein said iron binding moiety is selected from the group consisting of apo-transferrin, lactoferrin, ovotransferrin, desferrioxamine, phenanthroline, ferritin, porphyrin, EDTA and DPTA.

14. The composition-of-matter of claim 1, wherein said iron indicator comprises an enzyme.

15. The composition-of-matter of claim 14, wherein said enzyme is an aconitase enzyme.

16. The composition-of-matter of claim 1, 4, 5 or 7, wherein said microparticle has a weight similar to, or lower than a mammalian cell.

17. The composition-of-matter of claim 16, wherein said microparticle is selected from the group consisting of a polystyrene bead, a silica bead or an agarose bead.

18. The composition-of-matter of claim 1, wherein said iron indicator is conjugated directly to the microparticle.

19. The composition-of-matter of claim 1, wherein said iron indicator is conjugated indirectly to the microparticle.

20. The composition-of-matter of claim 19, wherein said indirect conjugation is via an adhesive protein.

21. The composition-of-matter of claim 20, wherein said adhesive protein comprises a histone or an albumin.

22. The composition-of-matter of claim 19, wherein said indirect conjugation is via an extension arm.

23. The composition-of-matter of claim 22, wherein said extension arm is composed of a dendrimer and a multifunctional bridge.

24. A method of quantifying free iron levels in a sample, the method comprising:

(a) contacting a sample of the biological fluid with the composition-of-matter of claim 1; and

(b) detecting and quantifying said signal, thereby quantifying free iron levels in the biological sample.

25. The method of claim 24, wherein said free iron comprises non-transferrin bound iron.

26. The method of claim 24, wherein said free iron comprises directly chelatable iron.

27. The method of claim 24, wherein said free iron comprises labile plasma iron.

28. The method of claim 26, wherein said iron indicator comprises fluorescein and deferrioxamine.

29. The method of claim 26, wherein said iron indicator comprises rhodamine(R) and deferrioxamine.

30. The method of claim 28, wherein said fluorescein and deferrioxamine are covalently attached to form fluoresceinated deferrioxamine (FI-DFO).

31. The method of claim 29, wherein said rhodamine and deferrioxamine are covalently attached to form R-deferrioxamine (R-DFO).

32. The method of claim 27, wherein said iron indicator comprises dihydrorhodamine 123 (DHR).

33. The method of claim 24, further comprising:

(c) contacting said sample with an iron binding moiety in excess with respect to said iron indicator; and

(d) detecting and quantifying said signal of step (C), said signal being the background signal of the chelation reaction.

34. The method of claim 33, wherein said iron binding moiety in excess is identical to said iron binding moiety of said iron indicator.

35. A method of calibrating free iron levels in a fluid sample, the method comprising:

(a) contacting a plurality of fluid samples with predetermined distinct free iron concentration values with the composition of claim 1;

(b) detecting and quantifying said signal; and

(c) generating a calibration curve depicting said signal against said predetermined distinct free iron concentration values.

36. The method of claim 24, further comprising contacting said plurality of samples with an iron chelator so as to release said iron binding moiety following step (b); and detecting and quantifying a signal' generated following contact with said iron chelator, and wherein said calibration curve depicts said change in said signal vs said signal' against said predetermined distinct free iron concentration values.

37. A method of quantifying labile cellular iron in a biological sample, the method comprising:

(a) loading cells of the biological sample with a iron indicator composed of an iron binding moiety and a signal generating moiety, wherein an intensity of said signal generated by said signal generating moiety is related to an amount of said iron bound be said iron binding moiety;

(b) contacting loaded cells of step (a) with a cell permeant metal chelator so as to remove said iron from said iron binding moiety;

(c) recording a signal generated in (a) and in (b), wherein the mean change in signal intensity is indicative of a level of labile cell iron;

(d) quantifying said change using a calibration curve generated according to the method of claim 35 or 36.

38. The method of claim 37, wherein said iron indicator comprises calcein.

39. A method of quantifying redox active iron levels in a biological fluid, the method comprising:

(a) contacting a sample of the biological fluid with a reducing agent, to obtain redox active iron;

(b) contacting said sample with a detector molecule selected capable of measurable activation upon contact with redox active iron reaction products, wherein said measurable activation is related to an amount of said redox active iron reaction product; and

(c) quantifying said measurable activation using the composition-of-matter of claim 1, thereby quantifying redox active iron levels in the biological fluid.

40. The method of claim 39, wherein said reducing agent is ascorbic acid, dithionite, dithiothreitol and mercaptoacetic acid.

41. The method of claim 39, wherein said detector molecule is selected from the group consisting of dihydrorhodamine, dihydrorhodamine, carboxy-dihydrofluorescein and dihydroresorufin.

42. The method of claim 39, wherein said redox active iron reaction products are reactive oxygen species.

43. A method of determining a presence, absence or risk of a disorder associated with abnormal levels of free iron in a biological fluid or cells of a subject, the method comprising:

- (a) determining levels of the free iron in the biological fluid or cells of the subject according to any of the methods of claims 24-42; and
- (b) determining in the subject based on said levels a presence, absence or risk of the disorder associated with abnormal free iron levels.

44. A method of treating a subject having a disorder associated with abnormal levels of free iron in a biological fluid or cells, the method comprising:

- (a) determining levels of the free iron in the biological fluid or cells of the subject according to any of the methods of claims 24-42;
- (b) determining in the subject a presence of the disorder associated with abnormal free iron levels based on said levels obtained in (a); and
- (c) treating the subject using a medicament for the disorder associated with abnormal free iron levels.

45. The method of claim 44, wherein said medicament comprises an iron chelation therapy.

46. A method of determining efficacy of treatment of a disorder associated with abnormal levels of free iron in a biological fluid or cells;

(a) treating a subject in need thereof using a medicament for the disorder associated with abnormal free iron levels; and

(b) determining levels of said free iron in a biological fluid or cells of said subject according to any of the methods of claims 24-42, wherein a change in said levels following said treating is indicative of treatment efficacy.

47. The method of claim 46, wherein said medicament comprises iron chelation therapy, and whereas a reduction in said levels following said therapy is indicative of efficacious treatment.

48. A kit for determining a presence of a disorder associated with abnormal levels of free iron in a biological sample of a subject, the kit comprising the composition of matter of any of claims 1-21.

49. The kit of claim 48, further comprising at least one of a mobilizing agent and a compound devoid of iron and being capable of binding endogenous apo-Transferrin.

50. The kit of claim 49, wherein said compound comprises gallium or cobalt.

Fluorescence metal sensors on beads (FMSB) as probes for labile iron concept and application

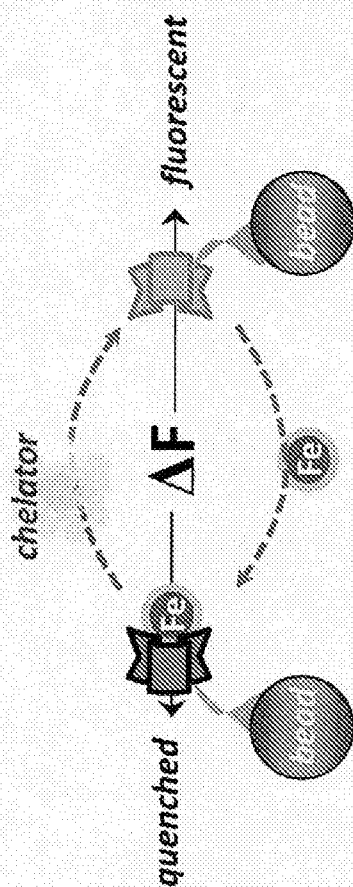


FIG. 1A

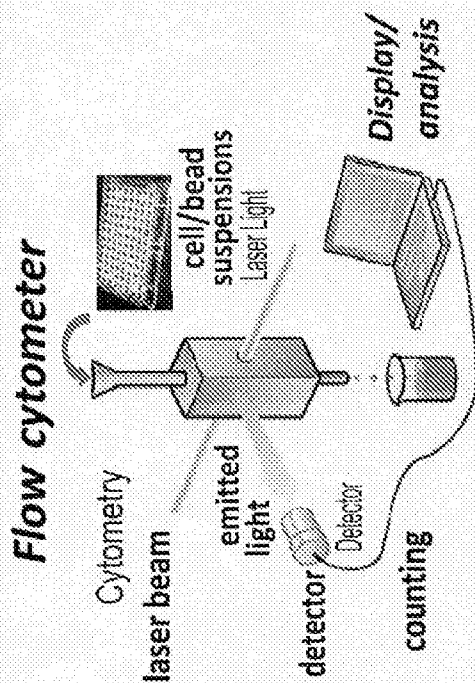


FIG. 1B

FIG. 2 Fluorescence metal sensors on beads (FMSB) as probes for labile iron
FMSB properties

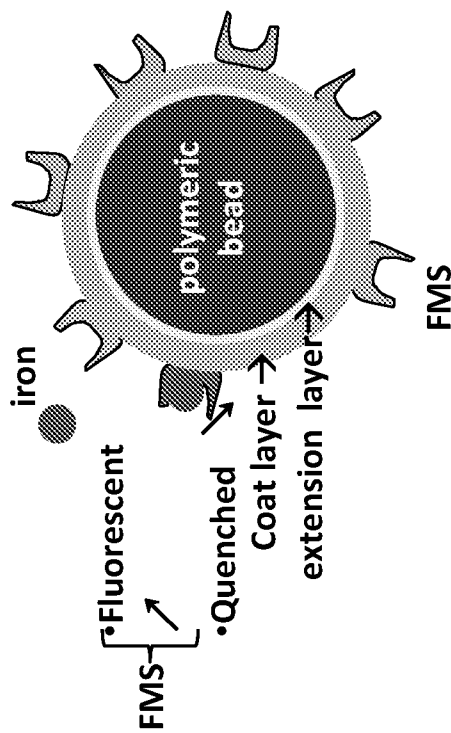
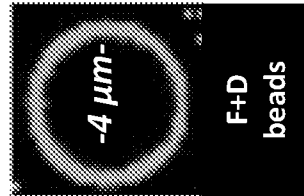


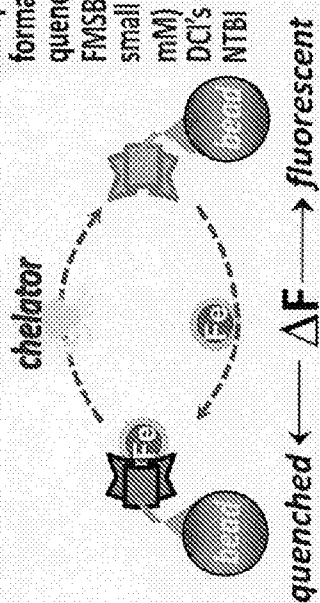
FIG. 3 Fluorescence metal sensors on beads (FMSB) as probes for labile iron
FMSB preparation



Fluorescence metal sensors on beads (FMSB) as probes for labile iron

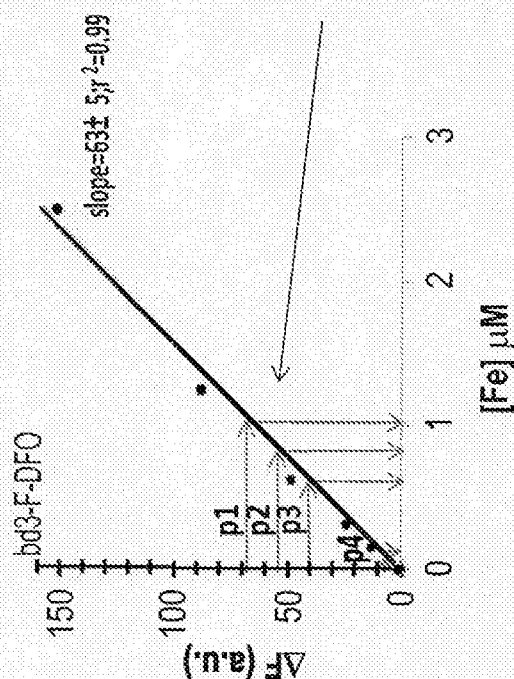
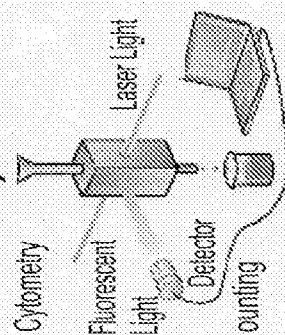
Measurement of plasma NTBI as directly chelatable iron (DCI) by flow cytometry (FC)

1. Addition of labile iron (present in a given fluid sample) to the FMSB (e.g. (F+D)B) leads to formation of FMSB-Fe with concomitant quenching of fluorescence by a factor $\square F$ FMSB-Fe, monitored in a flow cytometer. A small concentration of mobilizing agent (NTA 0.1 mM) may be optionally used to increase the DCI's assay sensitivity by revealing also cryptic NTBI



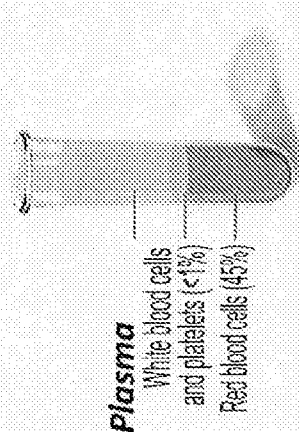
2. Addition of a strong chelator leads to removal of Fe from FMS-Fe and thereby to the regeneration of the fluorescent FMS by a factor $\square F$ FMS-Fe

Flow cytometer



3. The relationship between $\square F$ and $[Fe]$ is used to establish a calibration curve for the beads

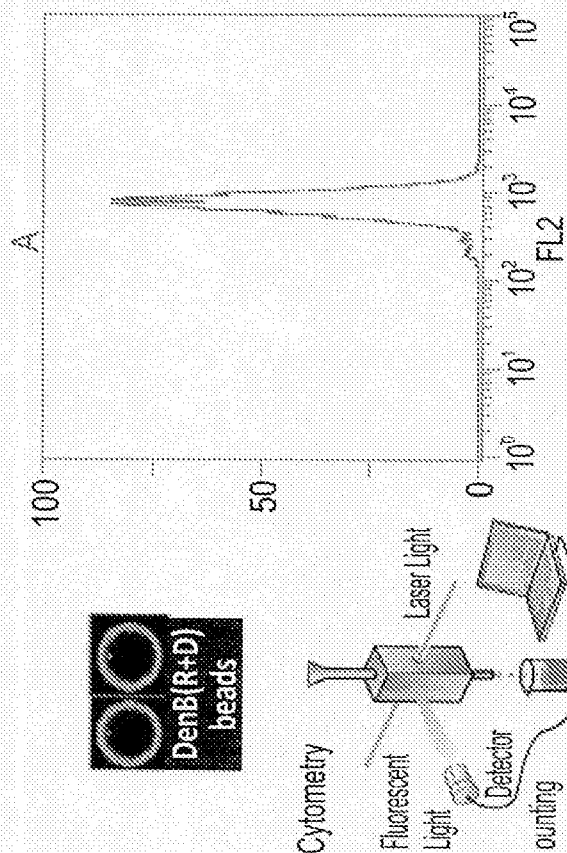
4. example: The $\square F$ obtained with plasma from four different thalassemia major patients is converted into Fe concentrations with the aid of the calibration curve



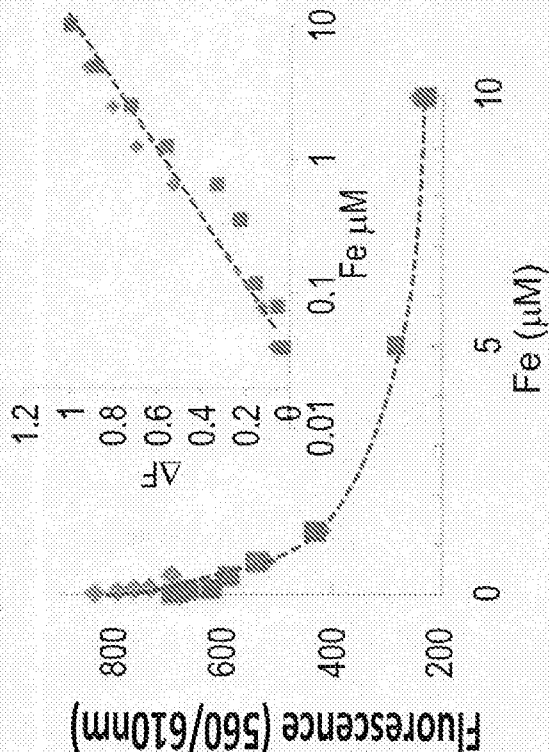
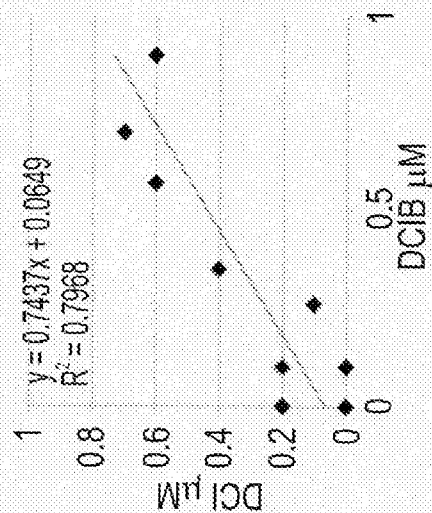
Cabantchik & Breuer

FIG. 4A

Rhodamine+DFO coated beads DenB(R+D) used for quantitative assessment of DCI by flow cytometry



Fluorescence intensity dispersity FL2 (560-610 nm) of Rhodamine+DFO coated beads DenB(R+D) analyzed by flow cytometry



Titration of DenB(R+D) with the indicated concentrations of Fe as analyzed by flow cytometry (tested 1 month apart). The relative change in fluorescence ΔF produced by the indicated concentration of Fe is plotted in the inset (semilog scale)

Comparison of NTBI values of 10 thalassemia patients obtained by the plate reader method of DCI (based on FI-DFO) versus those obtained by the flow cytometric method based on DenB(R+D)

FIG. 4B

Fluorescence metal sensors on beads (FMSB) as probes for labile cell iron
(LCI) by flow cytometry (FC)

CALG beads (CALGB) for calibration of LCI

FIG. 5A

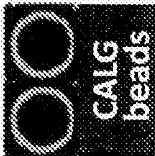


FIG. 5B

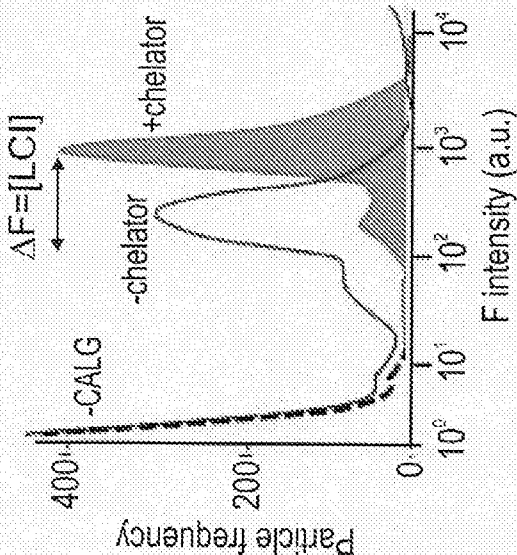
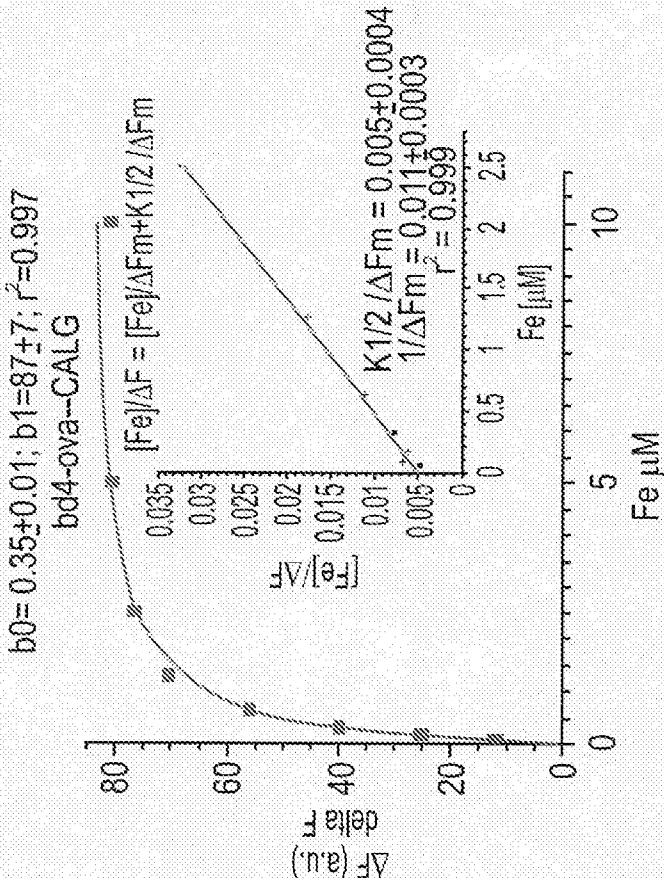


FIG. 5C



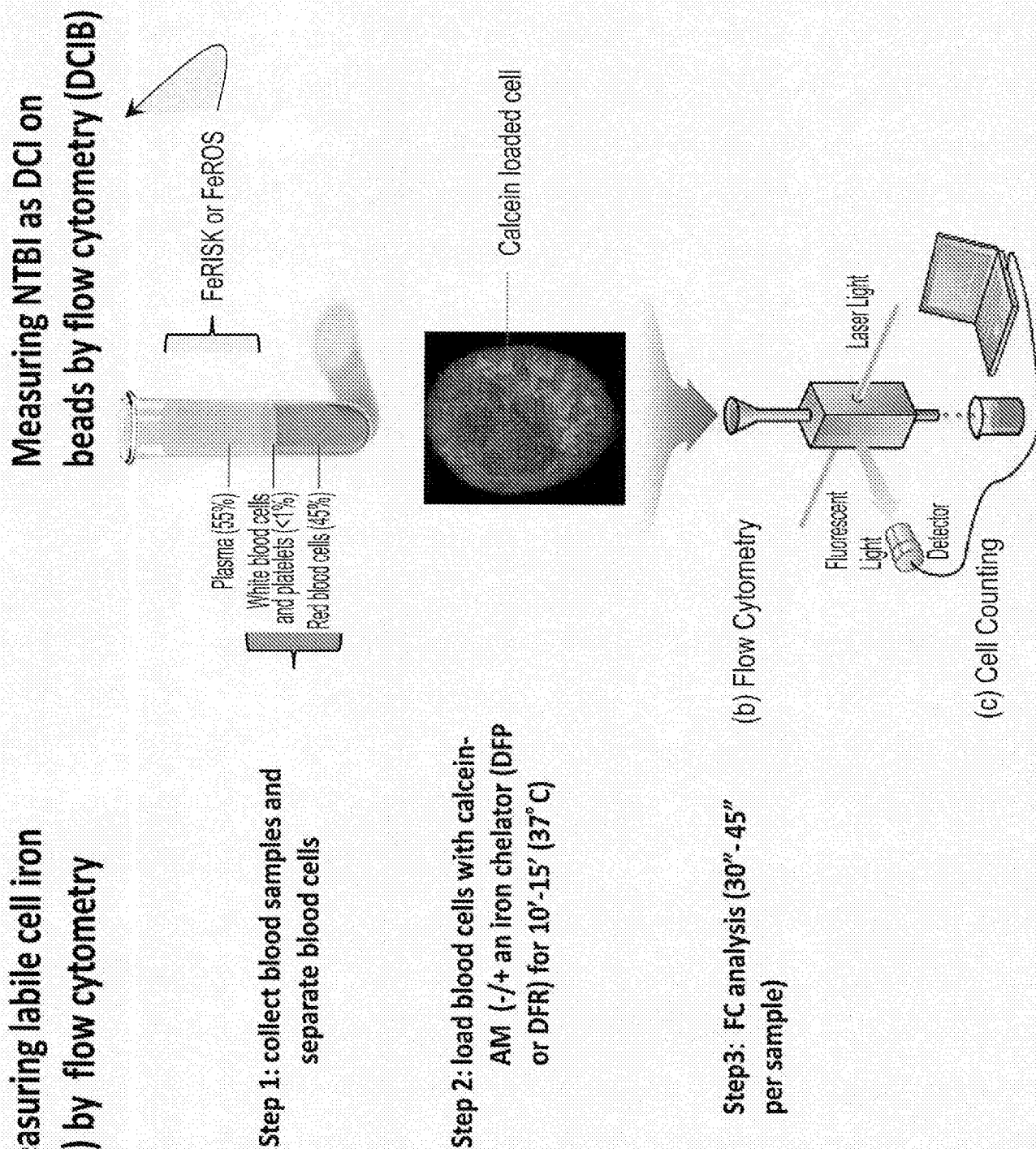


FIG. 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2012/050119

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/84 G01N33/90
ADD.

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)
G01N

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)

EPO-Internal, WPI Data, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BREUER WILLIAM ET AL: "Iron acquired from transferrin by K562 cells is delivered into a cytoplasmic pool of chelatable iron(II)", JOURNAL OF BIOLOGICAL CHEMISTRY, THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC, US, vol. 270, no. 41, 13 October 1995 (1995-10-13), pages 24209-24215, XP002211549, ISSN: 0021-9258, DOI: 10.1074/JBC.270.33.19330 page 24209 - page 24210 -----	1-50
A	WO 00/36422 A1 (YISSUM RES DEV CO [IL]; CABANTCHIK ZVI YOAV [IL]; BREUER WILLIAM [IL];) 22 June 2000 (2000-06-22) the whole document ----- -/-	1-50



Further documents are listed in the continuation of Box C.



See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search

11 June 2012

Date of mailing of the international search report

04/07/2012

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2012/050119

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BREUER WILLIAM ET AL: "A fluorescence-based one-step assay for serum non-transferrin-bound iron", ANALYTICAL BIOCHEMISTRY, ACADEMIC PRESS INC, NEW YORK, vol. 299, no. 2, 15 December 2001 (2001-12-15), pages 194-202, XP002211522, ISSN: 0003-2697, DOI: 10.1006/ABIO.2001.5378 abstract	1-50
A	----- WO 2004/040252 A2 (YISSUM RES DEV CO [IL]; CABANTCHIK IOAV ZVI [IL]; BREUER WILLIAM [IL];) 13 May 2004 (2004-05-13) the whole document -----	1-50

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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		WO 2004040252 A2 13-05-2004	
