Review Article

An overview of various labeled assays used in medical laboratory diagnosis

Immune and non-immune assays

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ABSTRACT

في هذه المراجعة تم إلقاء بعض الضوء على مختلف المقايسات المناعية الموسومة التي تعتمد على تفاعلات الضد بالمستضد Ag-Ab والتي تشمل المقياسات المناعية التألقية، و المناعية الشعاعية، و المناعية الإنزيمية (EIA, ELISA). تم تعريف هذه المقايسات الثلاثة أولاً مع وصف مختصر لمبدأ التفاعلات و التطبيقات، ثم نوقشت حسب التدرج التاريخي لتوضيح تطورها التدريجي الذي قاد في النهاية إلى تقنية آلية بشكل كامل. كما تم مناقشة المقايسات المناعية الأنزيمية (لطخة ويسترن، اللطخة البقعية، و المقايسة المناعية اللطخية المأشوبة) و ظاهرتي التلألؤ البيولوجي و اللمعان الكيميائي وذلك حسب التدرج التاريخي. ثم تم وصف المقايسات الموسومة والتي لا تحتوي على تفاعل الضد بالمستضد Ag-Ab وتحتوي على تفاعل البيوتين بالستربتافيدين، و تفاعل مسبار المستهدف DNA. بالإضافة إلى وصف بعض التقنيات اللمعانية الموسومة التي تستخدم بنفس الوقت تفاعل الضد بالمستضد Ag-Ab و تفاعل البيوتين يالستربتافيدين BS مثل مقايسة اللمعان لوسي و شكلها التجاري ألفاليزا اللتين حذفت منهما عمليات الغسيل دون التأثير على الحساسية العالية، و المجال الديناميكي الواسع.

In this review, some light is thrown on various labeled immunoassays that depend on antigen-antibody (Ag-Ab) reactions, including immunofluorescence, radioimmunoassay, and enzyme immunoassay (EIA or ELISA). Their definitions, principles, and applications are described, then they are discussed chronologically to show their stepwise development that led finally to full automation. Enzyme labeled immunoblot assays (Western blot, blot spot, and recombinant immunoblot assay), and luminescence (bioluminescence and chemiluminescence) are also discussed chronologically. Labeled assays, that do not involve Ag-Ab reaction but rather, utilizing biotin-streptavidin (BS) interaction and probe-target DNA interaction, are described, together with their applications for DNA/RNA detection and genotyping. Finally, included in the discussion were some luminescent labeled techniques that utilize the immune Ag-Ab reaction together with non-immune BS reaction, such as the luminescent oxygen channeling immunoassay, and its commercialized AlphaLISA, both eliminate the washing steps without sacrificing high sensitivity, or wide dynamic range.

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The second half of the last century witnessed the development of a vast number of techniques revolutionized both biology and medicine. It led to many Nobel prizes' laureates[1] (**Table 1**), whose discoveries have highly influenced, in particular, the area of techniques utilizing labels, namely, labeled assays. These are, in addition to the scientific reasons, that prompted the writing of this review on the immunological and non-immunological labeled assays used in medical laboratory diagnosis.[2-8] In this review, various labeled assays will be gathered and discussed to show their concepts and principles, to demonstrate their applications in various disciplines of the laboratory in medicine and biology, and to monitor their chronological development that led finally to full sophisticated automation.

I. Development of labeled immunoassays (LIAs). The LIAs possess a significant wide range of applications, in almost every field of biomedical research, and depend on the antigen-antibody (Ag-Ab) interaction, in which the choice of the molecular labels contributes to their high sensitivity.[9] When radioactive isotopes are used as a label, for example, iodine-125, the test

is called radioimmunoassay (RIA), and the amount of the resulted radioactivity is detected by a γ counter (a liquid scintillation counter). When fluorescent dyes are used as a label, for example, rhodamine, the test is called immunofluorescence (IF), and the resultant shiny greenish-yellowish color is seen under a fluorescent microscope. However, when enzymes are used as a label, for example, horseradish peroxidase (HRP), the test is called enzyme immunoassay (EIA), or enzyme linked immunosorbent assay (ELISA), which requires the addition of a specific substrate to give a color that can be seen by the naked eye, or can be read by an ELISA reader (a simple spectrophotometer).[10-12] The LIAs involving Ab-Ag reactions differ in the way, in which they detect Ag (by immobilized specific Abs), or Ab by using an immobilized specific Ag (on a glass slide, plastic surface, bead, or nitrocellulose [NC] strips). When using immobilized Abs to capture the specific soluble Ag from a patient's serum, another specific labeled Ab is needed. This technique is called sandwich LIA. While the use of immobilized Ag to capture the specific Abs from a patient's serum, different specific labeled Abs (anti human immunoglobulins) are needed, thus, the technique is called indirect LIA.[10,11,13] Tissue antigens can be detected by using a tissue section fixed on a slide, and adding labeled (by fluorescent dyes) specific Abs, this technique is known as direct IA, or direct IF.[11,14,15]

1. Fluorescent IAs. There were many landmarks throughout the development of LIAs used in biological research and clinical diagnosis. Heidelberger et al in 1933,[16] attached a salt of benzidine to egg albumin through a diazo linkage, which was then used in colored quantitative studies of the precipitation reaction, for which a spectrophotometric method was employed. Marrack in 1934,[17] showed that dyes could be introduced into Ab molecules without altering their immunological specificity. In 1941, Coons et al[18] at Harvard Medical School were the first to label Ab molecules with fluorescent dyes. Coons' group later developed an improved optical filter system, a procedure for the preparation of isocyanate, and a technique for the conjugation of Ab globulin with fluorescent dye.[19] This fluorescent dye-LIAs, termed IF, made a major revolution in immunology and cell biology that permitted the detection of both Abs and Ags, and found wide applications in diagnostic medicine and research.[11,15,20] Coons was awarded many prizes, the Lasker medal in 1959, the Erlich Prize in 1961, and the Behring Prize in 1966.[20] The most commonly used fluorochromes in IF technology are fluorescein isothiocyanate (FITC) and rhodamine, both of which absorb light (ultraviolet [UV]) that is not visible to the human eye, and emit light that is visible.[11,14] The IF procedure in public health laboratories was first utilized in the diagnosis of influenza,[21] and was subsequently applied to many other microbial diseases.[22] The IF has also been used to identify the anatomic distribution of an Ag and Ag-Ab complexes within tissues,[11,12,15,20] or autoAbs in sera. Some good examples of which are anti-double-stranded DNA (using *Crithidia luciliae*

Table 1 - Nobel Prize laureates with their discoveries that have highly influenced the area of labeled assays, during the second half of the last century.

Year	Laureates	Country	Field	Contribution(s)	Ref
1972	Gerald M. Edelman Rodney R. Porter	USA UK	Physiology or Medicine	For their discoveries concerning the chemical structure of antibodies	2
1977	Rosalyn Yalow	USA	Physiology or Medicine	For the development of radioimmunoassays of peptide hormones	3
1980	Paul Berg	USA	Chemistry	For his fundamental studies of the biochemistry of nucleic acids, with particular regard to recombinant-DNA	4
1980	Paul Berg Walter Gilbert Frederick Sanger	USA USA UK	Chemistry	For their contributions concerning the determination of base sequences in nucleic acids	4
1984	Niels K. Jerne Georges J. F. Köhler César Milstein	Basel, Switzerland Basel, Switzerland UK	Physiology or Medicine	For theories concerning the specificity in development and control of the immune system and the discovery of the principle for production of monoclonal antibodies	5
1993	Kary B. Mullis	USA	Chemistry	Contributions to the developments of methods within DNA-based chemistry, and for his invention of the polymerase chain reaction method	6
2008	Osamu Shimomura Martin Chalfie Roger Y. Tsien	USA USA USA	Chemistry	For the discovery and development of the green fluorescent protein that started in the 1960's	7

USA - United States of America, UK - United Kingdom

organisms as substrate),[23] or anti-nuclear antibodies (ANA) (by using human epidermoid cancer cells [HEp-2], or other substrates). Although ANA can also be detected by ELISA, IF ANA is still the "gold standard" in ANA testing, because it exhibits distinct fluorescent patterns, which are associated with a specific disease, or a subset of collagen/vascular disorders, such as the nucleolar pattern that is associated with scleroderma; the centromere pattern, which is associated with calcinosis, Raynaud's phenomenon, esophagus disease, sclerodactyly, telangiectasias (CREST) variant of scleroderma, the rim pattern that indicates for systemic lupus erythematosus and the speckled pattern ,which is seen in mixed connective tissue diseases (MCTD).[24-26] In 1959, Singer[27] developed methods for the detection and localization of Ag or Ab-Ag complexes by electron microscopy. He conjugated ferritin to Ab molecules, rendering them detectable in the electron microscope, thus, permitting their detection where Ag have been located. Colloidal gold labeled Abs are also being widely used as an electron-dense immunolabel by electron microscopists.[12]

The introduction of fluorescence into flow cytometry (FC) was carried out by Wolfgang Göhde from the University of Münster in 1968.[28,29] The FC is a fluorescent technique that detects (using special apparatus called flow cytometer) fluorescence on individual cells in suspension, which had been incubated with fluorescent-labeled monoclonal Abs, or other probes. This allows counting the number of cells that express a fluorescence by passing the cells, one at a time, through a spectrofluorometer with a laser-generated incident beam.[11,15] The first fluorescent FC device (ICP 11) was called pulse cytophotometry.[28,29] Then, according to Goehde,[30] was changed to FC at the 5th American Engineering Foundation Conference on Automated Cytology in Pensacola, Florida, USA. The FCs can also analyze cell sizes, and their internal complexity by measuring the forward and side light-scattering properties of cells. For example, due to their cytoplasmic granules, neutrophils cause greater side scatter than lymphocytes, but monocytes cause greater forward scatter due to their size. Additionally, modern FCs can simultaneously detect many different molecules on a cell, because they can detect 3, or more different colored fluorescent signals.[31] The introduction of fluorescence into cell sorting was carried out in Herzenberg's laboratory.[32] Therefore, fluorescence-stained cells can also be separated from unstained cells by applying an electric charge to the stained cells during passage through the FC, and deflecting them into a collection tube. This technique was called fluorescence activated cell sorting (FACS)[20] for which Becton Dickinson developed the first FACS instrument in 1974.[33,34] It is worth mentioning, that such purification of cells with a particular phenotype can also be achieved by using Abs (attached to magnetic beads) that will bind specifically to certain cells, and the bound cells can then be pulled out of suspension by using a strong magnet.[31]

Advanced improvements in IF, included the development of computer-aided confocal fluorescent microscopy for ultra thin optical sections, the 2 photon microscopy, which prevents out-of-focus light from forming. In addition to a time-lapse video microscopy, in which sensitive digital video cameras record the movement of fluorescently labeled molecules in cell membranes.[31,35,36]

2. The RIA. In 1960, RIA was first reported by 2 groups. In New York, Yalow and Berson[37] from the Veterans Administration Hospital reported an RIA for the measurement of endogenous plasma insulin. While in London, Ekins[38] from Middlesex Hospital reported "saturation analysis" for measuring thyroxine in human plasma. Not only Ag labeling but also Abs labeling were reported when in 1968, Miles and Hales[39] published their first results of an "immuno-radiometric" technique with radioactive labeled Abs, rather than labeled Ag for measuring insulin in human plasma. In 1977, Rosalyn Yalow was a Nobel Prize laureate in Physiology or Medicine "for the development of radioimmunoassay of peptide hormones"[3] (**Table 1**). The rapid and increasing utilization of RIA led to the development of special facilities for safe work usage. At the beginning, iodine-131 (ß and γ radiation) was used since no other alternative was available at that time.[37-39] However, the potential health hazard of radioactivity was greatly reduced by the use of iodine-125, which has a weak γ radiation.[40]

In allergies, radiolabeled allergens were used in 1967, to detect the specific immunoglobulin E (IgE) Abs, either on immunodiffusion plates (by radioimmunoelectrophoresis), or in the fluid phase.[41] Although radioimmunoelectrophoresis could be considered as a reliable technique for demonstrating the presence of IgE antibodies,[42] it proved very difficult to establish a quantitative technique for measuring IgE binding activity with the allergen.[43] In 1967, Wide et al[44] introduced the radioallergosorbent technique (RAST) to detect the amount of IgE that reacts specifically with suspected allergens that are covalently coupled to an immunosorbent paper disk. The RIA for total IgE in serum was also developed, which was a direct modification of the assays used routinely for measuring hormone levels,[45,46] either by double Ab inhibition RIA, the technique which was developed in 1971 by Gleich

et al,[47] by radioimmunosorbent technique (RIST), which is a solid phase competitive technique, and was the first commercially available assay for IgE levels,[48] or by non-competitive RIST, which does not depend on inhibition, and is often called paper RIST or PRIST.[49]

3. Enzyme LIAs. Some drawbacks were encountered in radioisotope labels, including the radiation hazards of reagents, regular requirements for disposal of the radioactive materials, and the short shelf-life of the labeled reagents. These drawbacks of radioisotopes hampered their applications, and prompted the development of other nonisotopic labeling systems.[50] In 1966, methods utilizing enzyme conjugated Abs were reported by Nakane and Pierce,[51] and by Avrameas and Uriel.[52] In California, Nakane and Pierce,[51] proposed that enzymes can be used instead of fluorochromes to prepare enzyme labeled Abs for immunohistological applications, in which after the Ab-Ag reaction takes place, the enzyme label reacts with a substrate to yield an intensely colored product that can be analyzed with an ordinary light microscope, or by making the product electron dense for electron microscopy.[51] In France, Avrameas and Uriel[52] published the coupling of Ags or Abs with enzymes. In immunohistochemical stains, when peroxidase enzyme (such as HRP) labeled Abs are used, the technique is termed as immunoperoxidase.[51] Whereas, the peroxidase-antiperoxidase (PAP) technique of Sternberger[53,54] is another immunohistochemical method that employs specific unlabeled rabbit Abs (primary Abs), which bind specifically to the Ag being sought. Afterward, a peroxidase-rabbit antiperoxidase complex (PAP complex) is added together with sheep antirabbit Ig Abs (secondary Ab) that link the specific rabbit Abs into the rabbit antiperoxidase Abs, then a color is developed by adding the substrate.[20,54] In this manner, PAP is capable of detecting very small quantities of Ag in tissues.[55] Since PAP complex represents a soluble Ab-Ag complex (but not a labeled Ab), the PAP method was described as an unlabeled Ab enzyme method of immunohistochemistry,[53] in which the same PAP complex may be used for dozens of different unlabeled antibody specificities.[20] At the same time in 1966, an immunosorbent technique was reported, in which Abs were immobilized by coupling them to cellulose and Sephadex beads,[56] or to tubes (Ab-coated tubes).[57] However, ELISA and EIA were independently reported in 1971, both of which utilized an enzyme rather than radioisotopes as the reporter label, but they differ in their assay design. In 1971, an alkaline phosphatase dependent ELISA, for the measurement of IgG in rabbit serum, was reported by Engvall and Perlmann[58] at Stockholm University in Sweden. At the same time, an HRP dependent EIA, for the measurement of human chorionic gonadotropin (HCG) concentrations in urine. This was reported by van Weemen and Schuurs in the Netherland.[59] The ELISA had then been applied in a wide range of different fields, and commercial EIA/ELISA kits are being developed that utilizes 96-well microtiter plates coated with either an Ag or an Ab, in addition to the manufacturing of automated ELISA instruments.[60]

4. Enzyme labeled immunoblotting assays. In 1975, Southern[61] at the University of Oxford, transferred DNA fragments from agarose, after an electrophoretic separation, onto nitrocellulose. This technique was accordingly known as Southern blotting.[61] Soon after in 1977, Alwine et al[62] described the transfer of separated RNA strands from an agarose gel to a suitable solid support, a technique which was called the Northern blotting technique. These nucleic acid blotting techniques had led the laboratory of George Stark at Stanford to develop an immunoblotting technique, which was called the Western blot assay by Burnette in 1981.[63] This was used in 1985 for monitoring infectivity of anti-human T cell lymphotropic virus-III/lymphadenopathy-associated virus (HTLV/LAV [HIV]) positive blood.[64] Western blot can be used, both for the detection of Ag by using known specific Abs, and for detecting Abs by using specific Ags. These proteins (the Ags) are separated by sodium dodecyl sulphate polyacrylamide (SDSPA) gel electrophoresis, then transferred to an absorbent sheet of material, such as a NC, or polyvinylidene diflouride membrane. The serum containing the studied Abs is added, and incubated to allow the studied Abs bind to the specific proteins, next after washing, a solution containing an enzyme labeled secondary Ab (peroxidase-labeled goat anti-human Ig) is added, followed by washing, and incubation in substrate to yield colored bands.[64]

According to Brown,[65] a dot blot (or slot blot) technique was introduced in 1982, that represents a simplification of the Southern blot, Northern blot, or Western blot methods in which the biomolecules to be detected are not first separated by electrophoresis, but instead, immobilized directly on a membrane as dots by a suitable method such as, vacuum filtration method,[66] which was then followed by detection, either by nucleotide probes (for a Southern blot and Northern blot), or by Abs (for a Western blot).[65] Terms such as dot blot, slot blot, dot spot, dot IA, dot immunoblot, immunodot, or Ag dot test are essentially identical techniques, in which a variety of methods had been used to transfer protein patterns to membranes.[65] However, an extension to immunoblot assays had led to the development of recombinant immunoblot assays (RIBA). This could also be considered as a

Western blot assay, in which recombinant and synthetic proteins are immobilized directly as bands, on solid support (NC strip) without prior size separation by gel electrophoresis.[67,68]

5. Homogeneous enzyme IAs. The previously described enzyme IAs are termed "heterogeneous" enzyme IAs, as the free and bound enzymes are physically separated prior to the measurement of the enzyme activity.[69] In "homogeneous" enzyme IAs, the activities of the free and bound enzyme are different, and the total activity is determined without separation.[70] In these latter assays, an immune reaction brings 2 enzymes into proximity on a surface, such as, multienzyme complexes systems, so that (at such surface) one enzyme produces a product that serves as a chromogenic substrate (for the second enzyme) that reacts with the second enzyme prior to escape into bulk solution.[71] The first product can thus be "channeled" directly to the second enzyme. Hence, the method is called a "homogeneous" enzyme-channeling IA.[72]

II. Development of labeled non-IAs. 1. Labeled probes. Enzyme labeled assays, which are not IAs, have also been widely used for the detection and genotyping of DNA/RNA (amplified by the polymerase chain reaction [PCR]) in a manner not involving IAs (Ab-Ag reaction), but rather using biotin-streptavidin (BS) interaction due to the extremely high affinity between biotin and streptavidin (association constant 10^{15}/M), and since biotin moieties can be incorporated within an oligonucleotide on any place, and in any number.[73,74] It was in 1964 when streptavidin from the *Actinobacterium Streptomyces avidinii* was shown to be a biotin-binding protein that binds 4 molecules of biotin.[75] In the 1980's, reports appeared for the synthesis of biotin-labeled analogs of TTP that can be enzymatically incorporated into DNA, and of UTP for RNA.[76,77] The resultant DNA probes were hybridized to cytological samples for the viral visualization (parvovirus, polyomavirus, herpes simplex virus, adenovirus, and retrovirus genetic material) in infected cell cultures, and in paraffin-embedded autopsy tissues.[77] However in 1983, a rapid and sensitive colorimetric procedure was described for visualizing biotin-labeled DNA probes after hybridization, to DNA or RNA immobilized on NC filters (bio-blots).[78]

Moreover, the ability of streptavidin to bind biotinylated DNA was specifically used by a particular kind of reverse hybridization assay called line probe assay (LiPA), in which the biotinylated DNA is chemically denatured, and the single strands are specifically hybridized (captured) to some immobilized (as parallel lines on NC strip) oligonucleotide probes. After the unhybridized DNA is washed away, alkaline phosphatase labeled streptavidin (conjugate) is bound to the biotinylated hybridized DNA, after which a substrate forms a purple/brown precipitate when it reacts with the streptavidin-alkaline phosphatase complex, thus resulting in a banding pattern on the strip. The reaction is stopped by a washing step, and the reactivity pattern is recorded.[75] The LiPA allowed an easy and fast determination of multiple genetic variations, such as human leukocyte antigen (HLA) typing,[79] hepatitis C virus (HCV) genotyping,[80] hepatitis B virus (HBV) genotyping,[81] HIV reverse transcriptase drug resistance,[82] and for the detection of drug-resistant *Mycobacterium tuberculosis*.[83,84] Additionally, labeled sequence specific probes had been used widely in real time PCR for the detection and quantitation of the amplicon production during PCR.[85] Real time PCR thermo-cyclers, in addition to the nonspecific fluorescent dyes (such as SYBR Green I), utilize dual labelled sequence specific probes. These labeled probes possess a reporter fluorophore (such as FAM, TET, TAMRA or ROX) attached to the 5', and a non-fluorescent quencher dye (such as, DABCYL) attached to 3' ends of the probe. The non-fluorescent quencher dye suppresses the reporter fluorescence when the probes are not hybridized to the target site. Separation of the reporter dye from the quencher leads to the excitation of the reporter dye. This can be achieved by different ways such as the TaqMan system,[86] Molecular Beacons,[87] and Wavelength-Shifting Molecular Beacons.[88]

2. Labeled primers. Not only labeled probes, as mentioned above, but also labeled primers have been used in PCR molecular biology. Biotin labeled-PCR primers allowed the HLA typing to be completed in 2 steps with high accuracy, in which the resulted biotin labeled amplicons are then cached by unlabeled, sequence specific oligonucleotide probes that are immobilized separately on a membrane, followed by washing and the addition of a streptavidin-HRP, and a chromogenic substrate that yields a color when positive.[89]

III. Development of luminescent labeled techniques utilizing both the immune and the non-immune BS reaction. Luminescent techniques have also been used as labeled assays. Bioluminescence occurs when chemical reactions, within living organisms, gives off light,[8] such as the emission of a green light by certain luminous coelenterates, for example, jellyfish *Aequorea*,[8,90] *Renilla reniformis*,[91] *Obelia geniculata,* and *Aequorea forskalea*.[92] In 2008, Osamu Shimomura, Martin Chalfie, and Roger Y. Tsien were the Nobel prize's laureates in chemistry "for the discovery and development of the green fluorescent protein" (GFP)[7] (**Table 1**). According to Shimomura, Harvey in 1921 was probably the first person who studied the luminescent substance of jellyfish *Aequorea*.[90]

However, in the early 1960's, Osamu Shimomura isolated a bioluminescent protein that gave off a blue light, which was absorbed by a second jellyfish protein that was later called GFP. This protein can process blue, or UV light to green (its fluorescence) without the need for any accompanying factors, or additives.[8] The GFP can also attach itself to individual proteins, thus it could be used as a label allowing scientists to examine cellular processes under a microscope, including illuminating growing cancer tumors, and pinpointing the development of Alzheimer's disease in the brain.[93] Certain synthetic chemiluminescent compounds (such as luminol, isoluminol, and acridinium ester derivatives) can be covalently coupled to Ags and Abs. This labeling enabled development of IAs of comparable, or better sensitivity than those involving radioactive labels.[94] These chemiluminescent labels are stable-apparently indefinitely - on storage, and can be detected within a few seconds of initiation of the chemiluminescence.[95]

Some luminescent labeled techniques utilize at the same time, both the immune Ab-Ag reaction, together with the non-immune BS reaction. An example is the luminescent oxygen channeling immunoassay (LOCI).[96] This technique uses 2 different polystyrene particles (beads) (at very low concentrations so that particle pairs are formed rather than large aggregates), and photochemically triggered chemiluminescence. One bead (donor) contains a photosensitizer that produces singlet oxygen when exposed to light. The second bead (accepter) contains an olefin (chemiluminescent), that upon reaction with singlet oxygen, produces emission.[97] When the donor and the acceptor beads are brought into close proximity by an immune reaction (complex formation), laser irradiation of the photosensitizer donor beads at 680 nm generates a flow of singlet oxygen, triggering a cascade of chemical events in nearby acceptor beads, which results in a chemiluminescent emission at 615 nm. While in the absence of the immune reaction, the acceptor and donor beads are not in close proximity, and no chemiluminescent signal is detected.[98] However, the LOCI was described as a homogeneous IA method capable of rapid quantitative determination in serum, or in lysed blood of a wide range of analytes (of high and very low concentrations, and of large and small molecules) such as 1.25 micro U/L of thyrotropin (TSH) which is 3-fold, and 5 ng/L of hepatitis B surface antigen (HBsAg), which is 20-fold lower than those of the best commercially available assays.[97] Recently in 2007, the LOCI was developed for the determination of insulin in human plasma.[99] This was described as a homogeneous, sensitive, and rapid bead-based sandwich IA of 2 steps: starting with incubating the sample with a mixture of biotinylated anti-insulin Ab, and donor beads covalently coated with anti-insulin Ab. Thus, insulin is sandwiched between the 2 types of the anti-insulin antibodies; this was followed by an incubation with accepter beads covalently coated with streptavidin to allow BS reaction to take place, and generation of light (from the accepter beads), which was then quantitated.[99]

The LOCI was commercialized as amplified luminescent proximity homogeneous assay (AlphaLISA®) or AlphaScreen,™ which is a homogeneous alternative to conventional ELISA,[97] that eliminates the tedious wash steps without sacrificing high sensitivity, or wide dynamic range.[100] The AlphaLISA can be set up as sandwich (described above), or as competitive AlphaLISA IAs, in which a biotinylated analyte bound to streptavidin donor beads is used with an Ab conjugated to AlphaLISA acceptor beads.[98] The AlphaLISA is ideal for measuring analytes in a wide variety of samples including cell culture supernatants, crude cell lysates, serum, and plasma.[101,102] This technology is suitable for detecting integral membrane proteins,[98,103] and for different HLA molecules.[104] The AlphaLISA has an expanding line of application kits for research on cancer, inflammation, and other disease areas.[100,105,106] Automation of AlphaLISA had recently been described for insulin detection by Perkin Elmer Inc.[107]

The GFP helped in the in-vivo imaging. Chishima et al[108] were the first to use GFP to visualize cancer cells in vivo. But Yang et al[109,110] were the first to use GFP for whole-body imaging. These achievements, together with the development of the intravital microscopy,[111] high resolution intravital video microscopy,[112] the confocal microscope,[113] and the visualization by the intravital imaging[114] have allowed new insights into microscopic details, and processes in real time. Future developments are expected to expand the use of *in-vivo* GFP imaging and real-time observations.[115,116] In addition to GFP, fluorescent proteins of many different colors are now available. Red fluorescent protein (RFT) was isolated from the *Discosoma sp*, and converted to a series of modified proteins with new colors from yellow-orange to red-orange.[117] It is however, expected that other additional colored proteins will be isolated from various organisms, and modified to produce more colors, which will enable simultaneous imaging of multiple cellular events *in vivo*.[115]

In spite of the developments in the various labeled assays, a test for the simultaneous measurement of a number of analytes in a single sample (microarray technology, or multiplexed analysis) is still a demand of the future. A new developed microbead-based FC system (Multiplex Bead Array Assay, or Luminex) has been used to detect at the same time, 25 different cytokines in a small volume sample of human biological material.[118] Microarray technology has also been reported for

allergens,[119] for the ToRCH-infectious antigens,[120] for detecting autoantibody profiles (of an autoimmune patient) by using up to 266 autoantigens.[121,122] Although encouraging results were obtained indicating that these technologies are promising, "they are still in their infancy," and we are still awaiting for broader applications, improvements, and processes of strict clinical and analytical evaluations.[122] By using various colored FP and the improved microarray technology, expanding and improvement of the simultaneous imaging, and the real-time observations are the obvious hope for the near future.

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