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(54) Title: PHOTOGRAPHIC COLOR COUPLERS USED AS CYTOCHEMICAL CONTRAST MARKERS

(57) Abstract

Novel methods of detecting peroxidatively active species in biological samples, such as cells, are disclosed. The methods comprise using combinations of peroxidase substrates and photographic color couplers.
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PHOTOGRAPHIC COLOR COUPLERS USED AS CYTOCHEMICAL CONTRAST MARKERS

BACKGROUND OF THE INVENTION

All animal and plant cells are equipped with a large variety of protein enzymes which chemically catalyze the various biochemical reactions that are necessary for maintenance, growth and specialized cell functions. Among this variety of enzymes are subsets which are present in only a few, or even only one kind of cell. Thus, identifying specific enzymes is a useful method for identifying cells of a particular type. This identification is usually carried out using the techniques of enzyme-histochemistry.

Gomori, Proc. Soc. Exp. Biol. Med., 42:23 (1939) and independently Takamatsu, Trans Soc. Path. Japan, 4:277 (1939) first described enzyme-histochemistry when they realized that if an appropriate substance or combination of substances were introduced into a cell where there existed an enzyme that could produce a light-absorbing and insoluble product from that substance or those substances, all cells which contained that enzyme would become selectively colored (or dark) and could easily be distinguished from all other cells which lacked that enzyme by observing such cells with a light microscope. The introduced substances are natural or synthetic substrates for a specific enzyme which, after reaction with that enzyme, either spontaneously become light-absorbing and insoluble or become so because of reaction with other introduced reagent substances.


In addition to staining of enzymes which are naturally present in cells, the field of histochemical staining has expanded to include methods of detecting cells by using exogenously introduced labels. Enzymes, in particular peroxidases, are commonly used as
labels. When enzyme labels are used, a substrate is added which reacts with the enzyme to provide a detectable response. Whether the label is an enzyme or some other directly or indirectly detectable compound, the exogenous labels are typically used in the form of a conjugate, in which the label is linked to an antiligand that specifically binds to a ligand molecule (such as a protein) that is found in the cells of interest. The antiligand to which the label is conjugated may be a low molecular weight molecule, or a larger molecule such as an immunoglobulin.

Another, related, group of methods for identifying the presence of specific cell markers is immuno-cytochemical staining methods. Immunocytochemistry uses the high binding specificity of immunoglobulin molecules to bind selectively to unique molecular sites which are present on, or in, cells of interest. Immunoglobulins that are bound to the cells of interest may be directly conjugated to a label such as an enzyme ("direct immunocytochemistry") or, alternatively, they may be visualized indirectly ("indirect immunocytochemistry"). In the latter method, a primary antibody is bound to the cells or cell molecules of interest, and is then itself bound by a secondary antibody (e.g., an anti-primary antibody IgG) which is conjugated to a label, such as an enzyme, for which high-resolution enzyme-cytochemical methods exist. When such an antibody and enzyme are directly or indirectly bound to their target cell, the preparation can be stained by an appropriate enzyme-cytochemical method, and only the labeled cells will accumulate light-absorbing (colored) product on or in them.

The peroxidase based methods of cellular detection rely on the availability of peroxidase substrate systems. The systems presently used, however, have certain limitations. In particular, the peroxidase substrates and dyes now available provide a limited number of distinguishable colors. Thus, using the reagents presently available, it is difficult to distinguish more than one peroxidase in a cell or biological sample. In addition, as noted supra, peroxidases are often used as the enzyme label for antibodies or other binding molecules. Increased capability for distinguishing different peroxidase-labeled antibodies would increase the ability to detect antibodies and distinguish them from other antibodies with different specificities, from other cell stains, or from background.

The availability of additional, and better, peroxidase detection systems would greatly increase the convenience and utility of detection techniques in research and medicine. Therefore, a need exists for improved peroxidase substrate systems.
SUMMARY OF THE INVENTION

The invention provides a method of identifying, in a biological sample, the presence of a peroxidatively active species. In one embodiment the method entails exposing the biological sample to a reagent comprising a peroxidase substrate and a photographic color coupler, detecting a product formed by reaction between the peroxidatively active species and the reagent; and relating detection of the product to the presence of the peroxidatively active species.

In a second embodiment, the method is carried out by forming a complex comprising a ligand (e.g., antigen) in the biological sample, a receptor (e.g., antibody) capable of binding the ligand, and a peroxidatively active species conjugated to, or associated with, the receptor. The complex is exposed to a reagent comprising a peroxidase substrate and a photographic color coupler and the product formed by reaction between the peroxidatively active species and the reagent is detected and related to the presence of the specific cell type.

In a third embodiment at least two different peroxidatively active species in a single biological sample may be detected by exposing the sample to a first reagent comprising a peroxidase substrate and a first photographic color coupler under conditions wherein only a first peroxidatively active species is peroxidatively active; exposing the sample to a second reagent comprising the peroxidase substrate and a second photographic color coupler under conditions wherein only a second peroxidatively active species is peroxidatively active, wherein the product formed by reaction between the first peroxidatively active species and the first photographic color coupler can be distinguished from the product formed by reaction between the second peroxidatively active species and the second photographic color coupler; detecting the products formed by reaction between the peroxidatively active species and the reagents; and relating detection of the products to the presence of the first and second peroxidatively active species. The first and second peroxidatively active species may be present in the same cell in the sample or in different cells.

In a forth embodiment, a cell carrying both a peroxidatively active species and a non-peroxidative enzyme, may be detected by exposing the sample to a reagent comprising a photographic color coupler and a substrate for the non-peroxidative enzyme.
The substrate for the non-peroxidative enzyme is capable of conversion by the non-peroxidative enzyme to a peroxidase substrate. The product formed by reaction among the peroxidatively active species, the photographic color coupler and the peroxidase substrate is detected and its presence related to the presence of the cell carrying both the peroxidatively active species and the non-peroxidative enzyme.

In related embodiments of the invention, the biological sample is contacted with a reaction mixture comprising a peroxidase substrate, a peroxide and a photographic color coupler. The order of addition of the reagents to the sample is not critical, so long as the peroxidase substrate and the peroxide are not both present before the photographic color coupler.

These methods are particularly useful when the biological sample is blood, e.g., a red blood cell such as a fetal red blood cell.

The invention also provides kits useful for carrying out the methods.

**BRIEF DESCRIPTION OF THE DRAWINGS**

*Figure 1* shows the spectrum of individual cells as determined by Zeiss filter monochromator, microscope, and COHU camera. (a) Upper curve--DAB peroxidase on an eosinophile. (b) Lower curve--DAB pseudoperoxidase on a red blood cell (RBC).

*Figure 2* shows the spectrum of an individual eosinophile after peroxidase reaction with 3-amino-9-ethylcarbazole (AEC).

*Figure 3* shows the spectra of individual RBC, using AEC, 4-chloro-1-napthol (4CN) or 2, 4-dichloro-1-napthol (2, 4-CN).

*Figure 4* shows spectra of individual RBC using DAB, AEC or 2,4-dichloro-1-napthol as peroxidase substrate. An unstained cell spectrum is also shown. All cells have the hemoglobin background spectrum.

*Figure 5* shows spectrum of individual white blood cells (neutrophils with no hemoglobin background) showing change in spectrum from that close to DAB to that of the color coupler as coupler concentration increases.

*Figure 6* shows spectra of individual eosinophiles stained with peroxidase, using DAB plus an indicated color coupler, and also DAB without any coupler. When DAB is not included, there is no color with any coupler.
Figure 7 shows the spectra of the pseudoperoxidase reaction of hemoglobin in RBC in the presence of DAB and several yellow couplers, illustrating the spectral differences from one to another. Spectrum of an unstained RBC is included.

Figure 8 shows the spectra of one RBC and one nucleated RBC after pseudoperoxidase stain with DAB + orthoacetoacetanisidide (o-AAA) and the Astrazon Blue nuclear stain.

Figure 9 shows the spectrum of an eosinophile after peroxidase reaction with DAB and one of the three different color couplers commonly used in color photography. Each spectral trace represents measurements from a single eosinophile.

Figure 10 shows the spectrum of RBC from different slides. Each slide is exposed to DAB plus a color coupler. In each case, a pair of slides includes or excludes hexamine cobalt chloride as an aid to oxidation.

DETAILED DESCRIPTION OF THE INVENTION

1. Definitions

As used herein, a ligand-receptor pair is broadly defined and refers to two molecules that recognize and specifically bind one to the other. "Specific binding" of a receptor to a ligand in a sample is evidenced by the binding of the receptor to the ligand (or the ligand to the receptor) without concurrent binding to other components in a sample. The ligand and receptor pair are often referred to as ligand and antiligand and may be referred to as a specific binding pair member. Examples of ligand-receptor pairs include antigen-antibody, biotin-avidin, hormones-hormone receptors, nucleic acid duplexes, IgG-protein A, and the like. No particular biological function, other than specific binding, is implied by use of the terms ligand and receptor. As noted, the ligand or anti-ligand can also be referred to as a "specific binding pair member" (sbp member) refers to a molecule which is one of two different molecules, having an area on the surface or in a cavity which specifically binds to and is thereby defined as being complementary with a particular spatial and polar organization of the other molecule. The two molecules are related in the sense that their binding to each other is such that they are capable of distinguishing their binding partner from other assay constituents having similar characteristics.

"Antibody" shall mean an immunoglobulin having an area on its surface or in a cavity that specifically binds to and is thereby defined as complementary with a
particular spatial and polar organization of another molecule. The antibody can be polyclonal or monoclonal. Antibodies may include a complete immunoglobulin or fragments thereof, which immunoglobulins include the various classes and isotypes, such as IgA (IgA1 and IgA2), IgD, IgE, IgM, and IgG (IgG1, IgG2, IgG3, and IgG4) etc. Fragments thereof may include Fab, Fv and F(ab')2, Fab', and the like. It will be appreciated that the immunoglobulins include chimeric or genetically engineered species (e.g., humanized antibodies).

The term "hydrocarbyl" shall refer to an organic radical comprised of carbon chains to which hydrogen and other elements are attached. The term includes alkyl, alkenyl, alkynyl and aryl groups, groups which have a mixture of saturated and unsaturated bonds, carbocyclic rings and includes combinations of such groups. It may refer to straight chain, branched-chain, cyclic structures or combinations thereof.

"Alkyl" refers to a cyclic, branched or straight chain, alkyl group. "Lower alkyl" refers to an alkyl with one to eight carbon atoms. This term is further exemplified by such groups as methyl, ethyl, n-propyl, i-propyl, n-butyl, t-butyl, i-butyl (or 2-methylpropyl), cyclopropylmethylyl, i-amyl, n-amyl, and hexyl. "Substituted lower alkyl" refers to lower alkyl as just described including one or more functional groups such as lower alkyl, aryl, aralkyl, acyl, halogen, hydroxyl, amino, acylamino, acyloxy, alkoxy, mercapto and the like. These groups may be attached to any carbon atom of the lower alkyl moiety.

"Alkenyl" refers to a cyclic, branched or straight chain group containing a carbon-carbon double bond, i.e., an unsaturated hydrocarbon. This term is further exemplified by such groups as ethylene, propylene, butene, 2-methylpropene, pentene, hexene and the like.

"Alkynyl" refers to a branched or straight chain group containing a carbon-carbon triple bond. This term is further exemplified by such groups as acetylene, propyne, butyne, pentyne, 3-methyl-1-butyne, hexyen and the like.

The term "aryl" refers to an aromatic carbocyclic radical having one (e.g., phenyl) or more condensed rings (e.g., naphthyl), which can optionally be mono-, di-, or tri-substituted, independently, with alkyl, lower-alkyl, cycloalkyl, hydroxylower-alkyl, aminolower-alkyl, hydroxyl, thiol, amino, halo, nitro, lower-alkythio, lower-alkoxy, mono-lower-alkylamino, di-lower-alkylamino, acyl, hydroxycarbonyl, lower-
alkoxycarbonyl, hydroxysulfonyl, lower-alkoxysulfonyl, lower-alkylsulfonyl,
lower-alkylsulfanyl, trifluoromethyl, cyano, tetrazoyl, carbamoyl, lower-alkylcarbamoyl,
and di-lower-alkylcarbamoyl.

The term "optionally substituted" refers to optional mono-, di-, or
tri-substitution, independently, with substituents including hydroxylower-alkyl,
aminolower-alkyl, hydroxyl, thiol, amino, halo, nitro, lower-alkylthio, lower-alkoxy,
mono-lower-alkylamino, di-lower-alkylamino, acyl, hydroxycarbonyl,
lower-alkoxycarbonyl, hydroxysulfonyl, lower-alkoxysulfonyl, lower-alkylsulfonyl,
lower-alkylsulfanyl, trifluoromethyl, cyano, tetrazoyl, carbamoyl, lower-alkylcarbamoyl,
and di-lower-alkylcarbamoyl.

The term "lower" as used herein in connection with organic radicals or
compounds respectively defines such with up to and including 6, preferably up to and in
including 4 and more preferably one or two carbon atoms. Such groups and radicals may
be straight chain or branched.

The term "effective amount" refers to the amount required to produce the
desired effect. Thus, an effective amount of a photographic color coupler or a peroxidase
substrate refers to the amount of each which is needed to obtain sufficient staining of the
sample being analyzed.

2. Description

A) Introduction

This invention relates to the use of photographic color couplers in detection
of peroxidatively active substances. In an embodiment of the invention, the biological
sample containing one or more peroxidatively active species is exposed to a peroxidase
substrate and a photographic color coupler. The peroxidatively active species in the cell
or other biological sample reacts with the peroxidase substrate to form an intermediate
product which subsequently, but rapidly, couples to the photographic color coupler to
yield a detectable product.

For example, a peroxidase will act on a substrate in the presence of
hydrogen peroxide (or certain other electron donors), to produce an oxidized product
(along with water and oxygen). Usually the oxidized product is a reactive molecule,
which can then polymerize, or couple with other compounds. Thus, a peroxidase acting
on a phenolic compound (as a substrate) and hydrogen peroxide, \( \text{H}_2\text{O}_2 \), (as a cosubstrate) will produce a reactive quinoid intermediate. If a photographic coupler is present, the intermediate can then react with the coupler to produce a colored compound, and the appearance of the color correlated with the presence of the peroxidase or other peroxidatively active substance.

The invention can be used to detect peroxidatively active substances in biological tissue, and has particular utility in histocytochemical reactions, including immunohistochemical reactions. Using the methods disclosed herein, a peroxidase reaction product with almost any specified absorbance characteristic may be produced and used as a marker for the presence of a peroxidatively active species. In particular, the invention provides methods for viewing multiple reaction products from a single tissue preparation (e.g., on one slide). Among other uses, the invention may be used advantageously for blood cell typing.

One aspect of this invention provides novel methods of varying the color of reaction products produced in peroxidase-based histochemical staining techniques by varying the nature of a coupling agent which is used to couple with the initially produced peroxidase oxidation product. The coupling agents are drawn from the color couplers used in photographic color chemistry to couple to initially formed developer oxidation product. As described in more detail below, yellow, magenta and cyan couplers can be used to stain cells, or cell components, with different colors and produce cells having varying absorption spectra.

B) The Biological Sample

The biological sample may be a biological fluid, tissue, cell, or preparative fraction from a tissue or cell, including, but not limited to, whole blood, serum, plasma, blood cells such as leukocytes, nasal secretions, sputum, urine, saliva, transdermal exudates, pharyngeal exudates, bronchoalveolar lavage, tracheal aspirations, cerebrospinal fluid, synovial fluid, fluid from joints, vitreous fluid, vaginal or urethral secretions, or the like. Disaggregated cellular tissues such as, for example, hair follicles, skin, synovial tissue, tissue biopsies and nail scrapings are also considered as biological samples. Preferred samples include blood and tissue sections.
Tissues may be processed by microtome section or by touch preparation. If tissue sections are used, there may be optional pretreatment by freezing, fixation by conventional fixing agents and/or embedding (e.g., in paraffin). It is known that peroxidase enzymes survive the majority of such pretreatments. When fixation is carried out on a biological sample containing a ligand to which a directly or indirectly labeled receptor is intended to bind, the fixation conditions will be selected so as to not destroy the ability of the ligand and receptor (e.g., antibody and antigen) to bind.

Preparative fractions from a tissue or cell may be prepared by well known methods that may include steps such as cell lysis, centrifugation, filtration, precipitation, chromatography, electrophoresis, and the like.

The sample may be localized on a microscope slide or be present on an electrophoretic strip, chromatographic matrix, or other such separation medium. In general, any technique which localizes peroxidatively active species into a discrete area, especially within a cell, or in any of the intracellular organelles, can be used in this invention.

C. Peroxidatively Active Species

As used herein, the term "peroxidatively active species" or "peroxidatively active substance" refers to those substances which are capable of catalyzing the oxidation of another substance using hydrogen peroxide or another peroxide as an obligate cosubstrate. The term "peroxidatively active cell" refers to a cell containing a peroxidatively active species. Peroxidase is a particularly useful peroxidative species.

Peroxidase is usually found as a conjugated protein containing iron porphyrin, and is present in horseradishes (horseradish peroxidase), potatoes, sap in fig trees, turnips (vegetable peroxidase), cow's milk (lactoperoxidase) and white blood cells (verdoperoxidase). It is also present in microorganisms and can be obtained by extraction or fermentation. Such peroxidases include lactoperoxidase, myeloperoxidase and microperoxidase. Frequently, the peroxidatively active species which serves as cellular marker in this detection method is a naturally occurring peroxidase isozyme. (An isoenzyme is one of a group of enzymes which all have the same biological catalyst activity. Isoenzymes can be distinguished one from another by physical or chemical
conditions that change their activity. An inhibitor of one isoenzyme may not inhibit another isoenzyme with the same catalytic activity.)

In addition, cells bearing peroxidatively active species other than peroxidase enzymes may be detected. In particular, cells carrying pseudoperoxidases, e.g., hemoglobin and the like can be detected by using the methods disclosed herein. Thus, as used herein, the term "peroxidatively active species" is not limited to species considered enzymes by conventional definition. For example, cytochromes, hemin, forms of hemoglobin, alkaline hematin, methemoglobin, myoglobin, oxyhemoglobin, and the like may, along with natural and synthetic peroxidases, be peroxidatively active species. In one embodiment, hemoglobin present in fetal nucleated red blood cells is detected.

It will be recognized unless special steps are taken, such as for example, adding reagents which inhibit specific peroxidases, most peroxidase containing cells may be detected by this method. In particular, granulocytes (e.g., neutrophils and eosinophils) can be advantageously detected by the methods disclosed herein in the presence of other cells (e.g., lymphocytes and monocytes). When detection is accomplished by adding an exogenous sources of a peroxidatively active species (e.g., in an immunoperoxidase based detection system), a catalytic amount of the peroxidative species can be used, as is known in the art.

D) Peroxidase Substrates

Generally, any peroxidase substrate which reacts with a peroxidatively active species to form an intermediate capable of coupling with a photographic color coupler can be used in the methods disclosed herein. Peroxidative activity has generally been detected by using oxidizable organic compounds, usually hydroxy- or amine-substituted aryl compounds. The oxidation is effected by action of hydrogen peroxide (or other peroxide) on the enzyme to form a free radical which then reacts with the substituted aryl. The substituted aryl free radical formed by this reaction polymerizes forming detectable colored precipitates. Representative substrates are disclosed in the references listed in the Worthington Manual of Enzymes, following sections on peroxidase and lactoperoxidase, pages 66-73; and Practice and Theory of Enzyme Immunoassays, P. Tijsen (Elsevier, 1985). Preferred substrates are p-phenylene diamines (e.g., N,N-dimethylparaphenylenediamine), dianimobenzidines (e.g., 3,3'-diaminobenzidine),
aminoantipyrines and aminocarbazoles (e.g., 3-amino-9-ethylcarbazole). Other substrates include o-tolidine, o-dianisidine and 4-N,N-dimethyl-p-naphthalenediamine.

Since a peroxide, typically hydrogen peroxide, is an obligate cosubstrate for the peroxidatively active species, hydrogen peroxide or a source of hydrogen peroxide is typically part of the reagent mixture. Suitable sources of hydrogen peroxide are oxidases such as for example, various enzymes such as glucose oxidase, uricase, cholesterol oxidase, glycerol oxidase, glycerin-3-phosphate oxidase, sarcosine oxidase, pyruvate oxidase, D-(or L-)amino acid oxidase, L-gulono-gamma-lactone oxidase, L- (or D-)hydroxyacid oxidase, pyridoxine oxidase, hexose oxidase, o-aminophenol oxidase, amine oxidase (containing pyridoxal, or containing flavin), xanthine oxidase, alcohol oxidase, ethanolamine oxidase, choline oxidase, acyl CoA oxidase, sulphite oxidase, ascorbate oxidase, etc. in conjunction with their respective substrates. If the hydrogen peroxide source is an enzyme substrate pair such as, but not limited to, those described above, then supplying a peroxidatively active species (e.g., peroxidase) allows the detection of the specific hydrogen peroxide producing enzyme. See, U.S. Patent No. 3,925,018. It will be appreciated that sources of peroxide other than hydrogen peroxide (e.g. perborate) may be used. An advantage of perborate is that it may be maintained in a powdered form.

E) Photographic Color Couplers

The term "photographic color coupler" refers to those compounds used in color photography to couple with a "developer oxidation product" (DOP) to produce a colored product. An extensive collection of such compounds are known to those skilled in the photography art and all such color couplers, including those subsequently identified are contemplated as within the scope of the present invention. A description of the more commonly used color couplers can be found in "The Theory of the Photographic Process," by J.F. Hamilton, G.C. Higgins and J.E. Starr, particularly at Chapter 12 (Macmillan, New York, 1977). Couplers are of several major classes. Those that contain open chain active methylene groups generally form yellow to orange dyes and are called yellow couplers; those that contain the active methylene group in a heterocyclic ring generally form magenta dyes and are called magenta couplers; and those that contain an active
methine group (e.g., the para position of a phenol or naphthol) generally form blue to cyan dyes and are termed cyan couplers.

Typical yellow couplers are the $\beta$-ketocarboxamides R-C(O)-CH$_2$-C(O)-NH-R$^1$, wherein R and R$^1$ are optionally substituted hydrocarbyl groups. Yellow couplers include alkanoylacetanilides R$^2$-C(O)-CH$_2$-C(O)-NH-Ar (I), benzoylacetanilides Ar-C(O)-CH$_2$-C(O)-NH-Ar (II) and pivaloylacetanilides t-Bu-C(O)-CH$_2$-C(O)-NH-Ar (III), where Ar represents an optionally substituted aryl. See, e.g., U.S. Patent Nos. 2,186,849; 2,875,057; 3,265,506; 3,770,446; 3,778,277; and GB 808,276. Yellow couplers also include $\beta$-ketoanilides substituted at the $\alpha$-position with a leaving group (i.e., a 2-equivalent coupler). 2-equivalent couplers require two ions of Ag$^+$ to form two atoms of Ag for their oxidation. Such couplers are represented by R$^3$-C(O)-CH(Z)-C(O)-NH-Ar, where R$^3$ represents an optionally substituted hydrocarbyl group, Ar an optionally substituted aryl and Z is a leaving group (also termed a "coupling off group" in color photography). Coupling off groups include, halo, phthalimido, succinimido, 5,5-dimethylhydantoinyl and various other 5-membered heterocycles, acyloxy, sulfonyloxy, aryloxy, urethane, imido, pyridone, pyridazine and the like. See, U.S. Patent Nos. 2,278,658; 3,849,140; 3,277,155. Another family of yellow couplers, unusual in not containing an active methylene group, are the substituted indazolones and the benzisoxazolones (see e.g., GB 875,470 and GB 778,089). Other examples include pivaloylacetanilides (see Hamilton et al., supra).

Magenta couplers contain a heterocyclic active methylene structure, such as the 5-pyrazolones (U.S. Patent No. 1,969,479) and may be pyrazolines, pyrazolones, pyrazolobenzimidazoles, or pyrazolotriazoles. Particular magenta couplers are the 1-aryl-5-pyrazolones (G. Brown et al., J. Am. Chem. Soc., 73:919 (1951). Many 5-pyrazolones contain a nitrogen containing substituent at the 3-position including carbonamido, sulfonamido, alkylamino, arylamino, heterocyclicamino, guanadino, ureido and the like. The 1-phenyl-5-pyrazolones can be substituted by up to three halogens in the phenyl ring. Other variations include the presence of a heterocyclic ring at the 1-position as in the benzimidazoyl pyrazolones. As with the yellow couplers, the magenta couplers can carry leaving groups at the coupling position giving 2-equivalent pyrazolone couplers. Related
magenta couplers include pyrazolo[2,3-a]benzimidazoles, pyrazolo-(3,2-c)-5-triazoles and others (W. Pelz, "Farbkuppler" in Mitteilungen aus den Forschung-laboratorien der Afga Leverkusen-München, Vol III pp. 111-175 (Springer-Verlag, Berlin 1961). In one embodiment, the magenta coupler is a compound having a structure of Formula

![Chemical Structure]

where R is independently hydrocarbonyl, n is an integer from 0-4 and X and Y are -C(O)-, -S-, -O- or -NH-.

Cyan couplers are typically phenols or naphthols which may carry electron donating (e.g., alkyl, alkoxy, alkylamino) or electron withdrawing substituents (e.g., halo, cyano). Frequently, cyan couplers carry substituents, such as carbonamido, ureido, carbamyl, heterocyclic groups and the like, at the 2-position. In the naphthol family, cyan couplers include the substituted 1-naphthols, particularly derivatives of 1-hydroxynaphthoic acids, such as, for example, the 1-hydroxy-2-naphthamides. Various coupling-off groups, such as halo, carboxy, sulfo, alkoxy, hydroxymethylene and alkylidine, may also be found attached to the 4-position of cyan couplers to give 2-equivalent couplers.

Photographic color couplers may also be functionalized with a combination of hydrophilic and hydrophobic groups to promote water solubility and prevent excessive diffusion. Alternatively, the couplers may also be attached to polymer chains, such as polycrylamides and polycrylates. Other exemplary compounds useful as couplers include those described in U.S. Pat. No. 4,978,612 and p-amino-N-dialylanalines such as those described by Bent et al. (1951) "Chemical Constitution, Electrochemical Photographic and Allergenic Properties of p-Amino-N-Dialylanilines" (1951; communication no. 1385 of Kodak Research Laboratories.

Generally, the photographic color coupler is present in a concentration of up to about 10−3 molar, and preferably from about 5 x 10−5 to about 5 x 10−4 molar. When added exogenously, the peroxidatively active species is present in an amount sufficient to react with the coupler. The amounts of the composition components (e.g., buffer, surfactant, peroxidative substance, etc.) are within the skill of a worker in the art.
F) Reaction Conditions

Substantially any buffer can be used in the practice of this invention as long
as the pH is maintained at a level conducive to dye formation as well as to the reactions
required for a given assay. Generally, the pH is maintained within the range of from about
4 to about 11, typically between about 6 and about 9, but a specific pH will depend on the
particular analyte being assayed and the reagents used therein. Useful buffers for various
assays include carbonates, borates, phosphates, malates, maleates, glutarates, the tris
materials, e.g., tris(hydroxymethyl)aminomethane, and others known in the art. It will
sometimes be advantageous to add a salt such as cobalt, nickel or the equivalent, to the
reaction to increase the absorbance peak of the color coupler.

The wavelength of peak absorption will also change, depending on the
proportion of peroxidase substrate to photographic color coupler. For example, the color
of DAB substrate reaction without any color coupler is orange-brown, with a wide peak
that centers at about 500 nm. When o-acetoacetaniside (a) is added as a color coupler the
absorption peak moves toward 400 nm as the relative concentration of a is increased, up to
a ratio of between about 1:10 or 1:20 (DAB:a), at which point the effect is saturated. For
example, at a ratio of 1:5 DAB:a, the peak absorption is in the range of 440-450 nm.
Thus, it is possible to adjust an optimum wavelength to match the sensitivity of a sensor
(e.g. a video camera) or to avoid overlap with a second color dye (e.g., a nuclear stain).

It will be appreciated that the method by which the biological sample is
exposed to, or contacted with, the reagents will vary with the type of sample. For
example, when the biological sample is a tissue section or blood preparation on a
microscope slide, the reagents can be overlaid so as to cover the cells or tissue sections or,
alternatively, the slide can be immersed into a reagent solution. It will be apparent to one
of skill that the particular method of contacting the peroxidatively active species in the cell
with the peroxidase substrate, peroxide, and color coupler is not critical to carrying out
the claimed method. As used herein, the terms "exposed to" and "contacted with" have
the same meaning.

Although it will generally be convenient to contact the biological sample
with a reagent comprising a peroxidase substrate, a peroxide and a photographic color
coupler, it will be recognized that these reagents may be added separately. The order of
addition of the reagents to the sample is not critical, so long as the peroxidase substrate
and the peroxide are not both present before the photographic color coupler is added. As used herein, the term "reagent" is used to describe the case in which the peroxidase substrate, a peroxide and a photographic color coupler are combined before they are contacted with, or exposed to, the biological sample. The term "reaction mixture" refers to the combination in the biological sample of the compounds in the mixture, whether added as a reagent (i.e., premixed) or added separately or in subcombinations. It will be appreciated that the terms are generally interchangeable, and embodiments described herein in which the term "reagent" is used can also be carried out by adding the components (e.g., peroxidase substrate, peroxide and photographic color coupler) separately.

G. Detection Methods

The presence of a peroxidatively active species in a biological sample such as a cell can determined by several methods, including, but not limited to, microscopic slide techniques, flow cytometry (including both transmission and fluorescence spectroscopy (e.g., for a fluorescent product)), and direct visual observation. It will be appreciated that in detecting the presence of a peroxidatively active species in a sample, the practitioner of the present method will be able to (1) discover the presence of the species (as when it is discovered that there is a cell containing fetal hemoglobin in a sample of maternal blood) or (2) differentiate the peroxidatively active species from a background matrix (e.g., a matrix used in electrophoresis) or from other, e.g., nonstained or nonlabeled, cells in the sample, or both (1) and (2).

In one embodiment, a biological sample, e.g., an anticoagulated blood sample, is deposited on a microscope slide, and if necessary, dried and fixed with a conventional fixing agent (formalin, glutaraldehyde and the like). Optionally, the slide is pretreated with one or more inhibitors and then exposed, or contacted with, a reagent comprising a peroxidase substrate and a color coupler. By using different couplers one can obtain different colors of reaction product. Excess reagents are washed off, the cells of interest are placed in an immersion medium and the slides examined under a microscope.

When flow cytometry is used, the cell sample is introduced into a fluid stream flowing in a conduit or analysis channel in the flow cytometer. See, "Practical Flow Cytometry," by H. Shapiro (Alan Liss, 1985). This preferably comprises
establishing a flowing stream of a flowing fluid sheath stream in the conduit or analysis channel and thereafter introducing the said sample into the flowing fluid sheath stream. Such sheath streams are usually of fluids having a refractive index substantially identical to that of the cell sample suspending medium. One such flow cytometer which uses a sheath stream carrier fluid is used in the Technicon Hemalog D and H-6000 systems, which handle all routine hematology tests. Detailed information on the Hemalog D, H-6000, H-1, H-2 and H-3 systems is available from Technicon Instruments Corporation (now Bayer, Inc.), Tarrytown, N.Y.

Detection may be either qualitative (e.g., noting the presence of absence of a cell containing a peroxidatively active species), semi-quantitative or quantitative. It will be recognized that appropriate controls will be done as necessary to subtract background signal formation or establish standard curves for positive signals. In addition, automated methods for cell screening are well known and may be applied used in conjunction with the present invention. For example, in one embodiment, an automated microscope is used. In this embodiment, the microscope is equipped with a motorized stage and a computer based image analysis system (including algorithms for automated focusing and cell detection). Examples of automated microscopes that include motorized stages include the LSC microscope (CompuCyte Corp., Cambridge MA).

H) Staining With Multiple Color Couplers

Multicolor staining using different color couplers allows the detection of different cell types from a single sample. This methodology generally involves performing sequential reactions which take advantage of the specific activity or inactivity of the various cellular peroxidases under different conditions and/or when exposed to different reagents. For example, at pH 3 only eosinophil peroxidases are reactive with 4-chloro-1-naphthol, whereas neutrophil peroxidase is inactive. Therefore, if the pH 3 reaction with 4-chloro-1-naphthol is followed by a second reaction at pH 5 incorporating an eosinophil peroxidase inhibitor, a magenta coupler and diaminobenzidine, one obtains blue-black eosinophils (from the 4-chloro-1-naphthol reaction) and magenta neutrophils. Monocytes, whose peroxidase activity increases with increasing pH can be detected along with neutrophils or detected in a separate third reaction with a yellow coupler. Similarly,
by increasing the hydrogen peroxide concentration and using a yellow coupler, the pseudoperoxidase activity of hemoglobin in red blood cells can be detected.

Fixation conditions can also differentially affect the activity of peroxidatively active species. For example, peroxidases are stable to aldehyde (e.g., glutaraldehyde) fixation, while pseudoperoxidase activity tends to decrease with aldehyde. Reactions in which acetonitrile (about 85% in water) plus about 0.5% glutaraldehyde or glyoxal are present result in a strong staining of neutrophils and eosinophiles but relatively little staining in red blood cells (which contain pseudoperoxidase). The difference is augmented when low concentrations (i.e., less than about 0.03%) of hydrogen peroxide are used. On the other hand, use of alcoholic fixatives (e.g., ethanol or methanol) and high concentrations of hydrogen peroxide tends to destroy true peroxidase activity, so that only staining due to pseudoperoxidases takes place. Thus, it is possible to stain for hemoglobin without staining neutrophils or eosinophils.

In one embodiment, when more than one color coupler is used, a cell of interest can be identified by containing a combination (i.e., two or more) of peroxidases or antigens that distinguish it from other cells in the sample. Alternatively, in a sample with at least two cell types, cells can be distinguished from each other when each cell type contains a peroxidase or antigen not found in the other cell type (e.g., when the two cells have different isoenzyme contents). Thus, one of skill will recognize that when, e.g., two different coupler couplers are used, a cell may be characteristically labeled with neither color, one color, or both colors and thus distinguished from other cells with a different labeling pattern.

When cells are detected by exposure of a sample to an exogenous peroxidatively active species, linked directly or indirectly to a receptor (or sbp member), several methods are available to form a detectable complex between the peroxidatively active species and the cell. A particular class of enzyme-labeled specific binding techniques for forming such complexes is the "immunoperoxidase" method, which includes five basic protocols. Among the following methods, those which bind larger numbers of peroxidase molecules for each molecule of primary antibody bound increase sensitivity by "enzyme amplification". First, in the "direct conjugate" protocol, a peroxidase-antibody conjugate binds directly to the cell. Second, in the "indirect conjugate" protocol a primary antibody binds the cell and is, in turn, bound by a
peroxidase-secondary antibody (anti-primary antibody) conjugate. Third, the "labeled antigen" protocol is essentially a sandwich technique in which primary antibody binds to both the cell and to an analogous antigen which has been conjugated with peroxidase. Fourth, in the "enzyme bridge" protocol, primary and secondary antibodies are bound as described above and the secondary antibody is bound by a third antibody which has been conjugated with peroxidase. Fifth, the "peroxidase-anti-peroxidase" protocol is as described for the enzyme bridge protocol with the addition that the peroxidase-tertiary antibody is followed by an anti-peroxidase antibody and excess peroxidase. In each protocol, hydrogen peroxide, a peroxidase substrate and a photographic color coupler are introduced to exhibit the extent of available or localized peroxidase by enzyme-cytochemical staining and, thus, tissue specific antigen. See, generally, Falini, et al., Arch. Pathol. Lab Med., 107:105 (1983).

It will be appreciated that the target of the primary antibody may be any number of antigens chosen to differentiate the cell of interest from other cells. Examples of such antigens include fetal hemoglobin, transferrin receptor, and cell surface markers (e.g. tumor markers).

I) Inhibitors

In one aspect of the invention, the sample is pretreated with differential inhibitors of specific peroxidases. This enables one to detect multiple isoenzymes in a single slide preparation. For example, cells containing myeloperoxidases and hemoglobin related pseudoperoxidases can be observed within a single slide. Alternately, specific inhibitors for eosinophil and neutrophil myeloperoxidase can be used. Each peroxidase can be selectively inhibited completely, allowing the other to retain full activity.

Representative inhibitors include resorcinol, 4-hydroxyresorcinol, phloroglucinol, pyragallol, 3-hydroxycatechol and hydroquinone (A. Saunders, J. Histochem. Cytochem., 25(8):1001-1012 (1977); L. Ornstein et al., Blood Cells, 2:557-558 (1976)). Polyhydroxybenzenes typically inhibit one or more of the peroxidase isozymes, allowing pseudoperoxidase containing cells to be selectively detected. Similarly, simple alkyl alcohols also inhibit peroxidases but not the pseudo peroxidase hemoglobin. Thus, as noted above, inhibition can be achieved by varying the fixation or reaction conditions.
J) Detection of Nonperoxidase Enzymes

In another aspect of the invention, the peroxidase substrate or the color coupler may be used in a protected form which is a substrate for another cellular enzyme, in which case both the peroxidase and this other enzyme are required to be locally present for reaction product to be formed. By way of example and not limitation, a phenolic hydroxyl of a peroxidase substrate such as 4-chloro-1-naphthol may be protected as an ester or a phosphate, in which case the presence of both the specific esterase (or phosphatase) and the peroxidase will be necessary. In another example, an oxidase substrate capable of forming hydrogen peroxide in the presence of the oxidase is used instead of hydrogen peroxide. With such a system, color is formed only when the oxidase and peroxidase are both present in the cell. Representative oxidases are listed supra.

K) Kits

The peroxidase substrates, photographic color couplers and/or peroxidatively active species can be provided as part of a diagnostic test kit. The kit components can be supplied as lyophilized reagents in individual packets having predetermined amounts. Alternatively, they can be provided in bottled or otherwise packaged solutions sufficient in size for one or more assays. Other reagents or non-reactive addenda, such as selective peroxidase inhibitors, fixing agents and the like, can also be supplied in the kit along with suitable assay utensils or containers for performing the assay, if desired. For pseudoperoxidase detection, kits may specifically comprise alcoholic solutions of peroxidase inhibitors and a concentrated solution of peroxide in packaged combination. Usually kits will include instructions describing the methods disclosed herein.

EXAMPLES

METHODS

A. Spectral Measurements

All spectra in following examples were performed on the system here described.
A Zeiss continuous running filter monochromator, Catalog No. 47 43 10 of 1967 Zeiss catalog, was set up with adapter-support for standard GFL Microscope, Catalog No. 47-43-16. The adapter was slightly modified to fit snugly onto an Olympus BX60 microscope light source at the image plane. The adapter was fitted with a 2.5 mm slit, which is approximately equivalent to 4 nm band width on the continuous running monochromator.

A 40X objective on the microscope is an infinite focus lens. The image is projected onto a COHU 4915 single color (black & white) camera. The color response of the camera was tested and found to agree with the manufacturer’s specifications. A didymium filter was used to confirm the spectral orientation of the filter monochromator, because the scale can be read in two ways, yet only one is correct.

The camera response is digitized such that each pixel has a range of 0 to 255. After a choice of a convenient dark level, the full light response in the 500 nm region is approximately 240 arbitrary digitized units.

When cells are measured, they are enclosed in a constant sized digital aperture. The same size aperture over a clean background region is taken as 100% transmission. The reading over cells is transformed to optical density (O.D.) with a standard equation.

**B. Stock Reagents**

1) **Buffer**

50 mm Tris-HCl pH 7.6
3.45 g Trishydroxymethyl aminomethane
400 mL Deionized Water

Adjust pH to 7.6 with about 18.5 mL 1N HCl.
Adjust total volume to 500 mL.

2) **DAB (diaminobenzidine) Solution**

A 10mg/ml solution is prepared by dissolving three (3) tablets of DAKO Diaminobenzidine (Catalog # 53000) in 3 mL of TRIS pH 7.6 buffer. Filter and dispense into smaller volumes for storage and use. Store in freezer.
3) **Coupler Solutions**

Dissolve 1 g of coupler (for example, o-acetoacetanilsidol) in 2 mL of N,N-dimethylformamide. Store at room temperature.

4) **Inhibitors of Peroxidase**

In separate containers, and also in combination where indicated, dissolve 1 g of Resorcinol or 1 g of Catechol in 1 mL of N,N-dimethylformamide. Store at room temperature.

5) **Hydrogen Peroxide**: 3% Commercial Stock Solution

6) **Imidazole Solution**: Dissolve 0.068 g of Imidazole in 10 mL of deionized water. Store at room temperature.

7) **Working Solution**

400 µL DAB solution
490 µL 50 mM TRIS buffer
80 µL Coupler
10 µL Imidazole or TRIS buffer
20 µL Inhibitor or TRIS buffer
1.67 µL Hydrogen Peroxide (added immediately before use).

C. **General Slide Preparation:**

1) **Method 1**

Blood films are prepared by the "pull" method, using 2 µL of whole blood or cell suspensions of previously enriched samples. The thin films are permitted to dry completely in air.

Slides with blood films are immersed for two (2) minutes in acetonitrile containing 15% deionized water and 0.25% glutaraldehyde.

Slides so fixed in glutaraldehyde are washed in several changes of deionized water and may be used while wet, or may be dried and used later.
2) **Method 2**

Blood films are made as in Method 1. Fixation is by immersion in 100% Methanol or in 80% Ethanol for at least two (2) minutes.

3) **Method 3**

Slides prepared as in Method 2 are further treated by the Kleihouer-Betke extraction of adult hemoglobin, which leaves fetal hemoglobin intact, and therefore, provides specificity for fetal hemoglobin.

The extraction is for one (1) minute in each of two fresh solutions of 0.050 M citrate-phosphate Buffer at pH 3.4, and a third solution of pH 5.5 to neutralize and stop extraction.

Slide is dried before further use.

**D. General Sample Exposure Method**

The slide is pretreated with inhibitors, if necessary, by immersion into solutions containing the appropriate inhibitor(s). If this is done, excess solution is washed away with water or solvent.

The slide is exposed to the working solution containing coupler reagent, enzyme substrate and peroxide. Changing the coupler in this step enables the production of reaction products of different colors. The excess is washed away and the slide is dried. If desired, the cells are counterstained. The cells of interest are covered in an immersion medium and a coverslip is placed over the same area to match the refractive index of the cells and give a clearer optical image. The cells are examined on a microscope as described above, either visually or by automation.

The samples may also be prepared for examination by flow cytometry, by preparing the cells in suspension. If desired, the cells can be fixed in suspension to maintain their structural integrity and retain enzyme activity in the location within the cell where it originated. The working solutions can be added directly to the suspension. Excess reagent may be removed by centrifugation and resuspension. Alternatively, dilution may be sufficient to reduce the reagent concentration to a level where they do not interfere with subsequent steps.
By adding different color couplers, at varying pH, either in the presence of, or subsequent to the addition of specific peroxidase inhibitors, several different colors of peroxidase containing cells can be separately identified. Using the H-1 hematology system (Technicon Instruments (now Bayer, Inc.) , Tarrytown, NY), it will be possible to automate these reactions.

E. General Counterstain:

Following any example with enzyme staining with or without coupler, the final procedure is to stain nuclei in the slide with a low concentration 0.05% of Astrazon Blue in pH 4.5 mM Buffer. Excess of blue dye is eliminated by two rapid dips in fresh deionized water.

EXAMPLE 1

STAINING CELLS WITH DIAMINOBNENZIDINE AND YELLOW COUPLERS

Fixation was carried out according to Method 1. Working Solution without the coupler and inhibitor was used. Spectrum was taken as described above. This spectrum is from an eosinophile, which is highly reactive to the peroxidase stain using DAB. The result is shown as Curve 1 in Figure 6.

The procedure was repeated but with the addition of one of the seven couplers in separate "subexperiments", as indicated in Table 1. All color couplers were obtained from Aldrich Chemical Co. (Milwaukee, WI).

```
<table>
<thead>
<tr>
<th>Number</th>
<th>Aldrich Cat #</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12,365-5</td>
<td>2-Benzyl acetoacetanilide</td>
</tr>
<tr>
<td>2</td>
<td>A813-2</td>
<td>Acetoacetanilide</td>
</tr>
<tr>
<td>3</td>
<td>A875-9</td>
<td>o-Acetoacetanisidide</td>
</tr>
<tr>
<td>4</td>
<td>15,562-4</td>
<td>2'-(2-chlorobenzoyl)-2,4'-dichloroacetanilide</td>
</tr>
<tr>
<td>5</td>
<td>32,929-0</td>
<td>2-chloro N,N-dimethylacetoacetamide</td>
</tr>
<tr>
<td>6</td>
<td>13,365-5</td>
<td>4'-chloroacetoacetanilide</td>
</tr>
<tr>
<td>7</td>
<td>26,238-2</td>
<td>4,4 dimethyl-3-oxopentone nitrile (pivaloyl acetonitrile)</td>
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</tbody>
</table>
```
The results are shown in Runs 8-14 of Table 2. Figure 6 shows spectra of white blood cells stained with these yellow couplers and Figure 7 shows spectra of red blood cells stained by this technique.

Table 2.

<table>
<thead>
<tr>
<th>Run No.</th>
<th>Coupler</th>
<th>DAB</th>
<th>N,N-dimethyl p-phenylene-diamine</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>---</td>
<td>---</td>
<td>No color in RBC or in eosinophiles</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>---</td>
<td>---</td>
<td>No color in RBC or in eosinophiles</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>---</td>
<td>---</td>
<td>No color in RBC or in eosinophiles</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>---</td>
<td>---</td>
<td>No color in RBC or in eosinophiles</td>
</tr>
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<td>5</td>
<td>---</td>
<td>---</td>
<td>No color in RBC or in eosinophiles</td>
</tr>
<tr>
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<td>6</td>
<td>---</td>
<td>---</td>
<td>No color in RBC or in eosinophiles</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>---</td>
<td>---</td>
<td>No color in RBC or in eosinophiles</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>+</td>
<td></td>
<td>Yellowish-Brown</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>+</td>
<td></td>
<td>Brown</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>+</td>
<td></td>
<td>Yellowish-Brown</td>
</tr>
<tr>
<td>11</td>
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<td>+</td>
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<td>Yellowish-Brown</td>
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<tr>
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<td>7</td>
<td>+</td>
<td></td>
<td>Brown</td>
</tr>
</tbody>
</table>
In comparison, DAB is brown in eosinophiles & orange brown in RBC, when used without a coupler.

**EXAMPLE 2**

**CELL STAINING WITH VARYING COUPLER CONCENTRATION**

The procedure of Example 1 was followed using diaminobenzidine and o-acetoacutanisidide (o-AAA). Preparation and staining were as in Experiment 1, except the concentration of the coupler, o-acetoacutanisidide was changed to represent a 1:1 and a 1:10 parts of DAB:Coupler in two otherwise similarly treated slides.

The results shown in Figure 5 indicate a more efficient narrowing of the spectrum with a higher concentration of color coupler. This indicates that coupling is a less efficient reaction than the autopolymerization of oxidized DAB and that autopolymerization can be prevented by using a higher concentration of color coupler.
EXAMPLE 3

VARIATION OF PEROXIDASE SUBSTRATE

Using 1:1 substrate:coupler and either DAB or N, N-dimethyl paraphenylenediamine, the series of couplers was tested as indicated in Tables 1 and 2.

Yellow color reaction with yellow photographic couplers was demonstrated more efficiently with DAB than the DOP.

EXAMPLE 4

AUGMENTING THE RBC SPECTRUM WITH YELLOW COUPLERS

Slides were prepared and fixed by Method 2. In the standard Working Solution, without inhibitors, several couplers in concentration ratios of 1:20 (DAB:Coupler) were evaluated and spectra were taken.

The results showed that each coupler adds absorbance (O.D.) to that of red blood cells examined without the pseudoperoxidase reaction (see Figure 7). In this case, the pure spectrum of the reaction product is not observed because the Soret band of hemoglobin is always present in the RBC.

EXAMPLE 5

CELL STAINING USING A COUNTERSTAIN

Slide preparation was by Method 2 using o-acetoacetanisidine (o-AAA) as coupler with diaminobenzidine substrate at 1:20 DAB:coupler ratio. In addition, the counterstain Astrazon Blue was added before inspection.

The results are shown in Figure 8 and indicate very good spectral separation of stained nucleus and the augmented color of hemoglobin with pseudoperoxidase. This good separation is advantageous for automated scanning of slides for cells containing both hemoglobin and a nucleus.
EXAMPLE 6

CELL STAINING WITH MAGENTA AND CYAN COUPLERS

This experiment shows that the known photographic couplers produce the expected colors when applied to histochemistry.

Slide preparation and fixing was by Method 1. DAB was used as peroxidase substrate. In three separate slides, the following couplers were used at a 1:20 DAB:Coupler ratio: o-AAA (yellow), 3-amino-1-(2,4,6-trichlorophenyl)-2-pyrazolin-5-one (3-APY, Aldrich) (magenta), and 6-amino-2,4-dichloro-3-methylphenol hydrochloride (6-AP, Aldrich) (cyan). The magenta and cyan couplers were first separately dissolved in DMSO and added at a 10X moles per mole of DAB ratio into the standard protocol. Standard spectra are taken on an eosinophile in each slide.

The results are shown in Figure 9 and provide spectra which correspond to yellow, magenta, and cyan colored eosinophiles. Visual examination also provides a yellow, magenta, and cyan colored set of eosinophiles in the corresponding slide.

EXAMPLE 7

EFFECT OF COBALT

Slide preparation and fixation were according to Method 2. The experiment consisted of 6 slides in 3 pairs. In each pair, a mole for mole equivalence of coupler and hexamine cobalt chloride is provided in one slide but not in the other.

The three yellow couplers used were benzoyl acetoacetonilide, o-acetoacetonisidide, and 2-chloro-N,N-dimethylacetoacetamide. Spectra of stained RBCs were taken from each slide.

The results are shown in Figure 10. With one exception, there was no quantitative or qualitative effect of cobalt addition. In the case of 2-chloro-N,N-dimethylacetoacetamide the addition of cobalt increased the absorbance peak, without changing the spectrum. In this case, the absorbance was lower without cobalt than in the presence of cobalt. Cobalt compounds are an aid to oxidation in both photodeveloping and histochemistry. In photographic color developing cobalt is used to convert Leuco dyes to dyes by oxidation after coupling is achieved. Though not being bound by one particular
theory, this result seems to indicate that cobalt augmentation can be used when a secondary post-coupling oxidation step is advantageous.

EXAMPLE 8

SELECTIVE CELL STAINING WITH DIFFERENTIAL INHIBITORS

Sample preparation and fixation were according to Method 1. Four slides were prepared:

Slide 1: Contains no Inhibitor
Slide 2: Contains Resorcinol
Slide 3: Contains Catechol
Slide 4: Contains resorcinol and Catechol in standard solution.

The peroxidase stain was performed and slides were visually examined. This experiment was done with both DAB only or with DAB:o-AAA at a 1:20 ratio. Results on the four slides are as follows:

Slide 1. Both neutrophils and eosinophiles contain reaction product.
Slide 2. Neutrophils have reaction product but eosinophiles have minimal or no staining.
Slide 3. Neutrophils are barely stained, but eosinophiles have the same amount of product as Slide 1.
Slide 4. Neutrophils and eosinophiles are unstained.

This experiment shows that different leukocytes can be selectively visualized by using differential peroxidase isozyme inhibitors using photographic color couplers or by previously established methods.

EXAMPLE 9

MULTICOLOR CELL STAINING

Sample treatment was as in EXAMPLE 1, except as follows:

Step 1: A peroxidase reaction is performed at pH 3.0, using 4-chloro-1-naphthol as reducing agent and no DAB. This results in dark, blue-black, reaction product in eosinophiles only.

Step 2: After washing the slide in water a second peroxidase reaction is performed with the standard solution containing DAB, o-AAA, and resorcinol.
The result is a slide with blue-black granules in eosinophiles and yellow granules in neutrophils showing that multiple colors for specific cell types may be developed in the same slide.

***

The foregoing invention has been described in some detail by way of illustration and example, for purposes of clarity and understanding. It will be obvious to one of skill in the art that changes and modifications may be practiced within the scope of the appended claims. Therefore, it is to be understood that the above description is intended to be illustrative and not restrictive. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the following appended claims, along with the full scope of equivalents to which such claims are entitled.

All patents, patent applications and publications cited in this application are hereby incorporated by reference in their entirety for all purposes to the same extent as if each individual patent, patent application or publication were so individually denoted.
WHAT IS CLAIMED IS:

1. A method of detecting the presence of a peroxidatively active species in a biological sample, comprising:
   (a) contacting the biological sample with a reaction mixture comprising a peroxidase substrate, a peroxide, and a photographic color coupler; and
   (b) detecting a product formed by reaction between the peroxidatively active species and the reaction mixture;
      whereby the presence of a peroxidatively active species is detected.

2. The method of Claim 1, wherein the biological sample is blood.

3. The method of Claim 2, wherein the peroxidatively active species is localized in a red blood cell.

4. The method of Claim 3, wherein the cell is a fetal red blood cell.

5. The method of Claim 1, wherein the peroxidatively active species is a peroxidase enzyme, a myoglobin or a hemoglobin.

6. The method of Claim 1, wherein the photographic color coupler is a yellow coupler, a magenta coupler or a cyan coupler.

7. The method of Claim 6, wherein the color coupler is a yellow coupler selected from the group consisting of benzoylacetanilides and pivaloylacetanilides.
8. The method of Claim 6, wherein the coupler is a magenta coupler selected from the group consisting of pyrazolines, pyrazolones, pyrazolobenzimidazoles, pyrazolotriazoles and a compound having a structure of Formula

\[(\text{R})_n\]

wherein:

- R is independently hydrocarbyl;
- n is an integer from 0-4; and
- X and Y are independently selected from the group consisting of -C(O)-, -S-, -O- and -NH-.

9. The method of Claim 6, wherein the coupler is a cyan coupler selected from the group consisting of phenols and naphthols.

10. The method of Claim 1 wherein the peroxidase substrate is a diaminobenzidine or a paraphenylenediamine.

11. The method of Claim 1, wherein the product is fluorescent.

12. The method of Claim 1, further comprising exposing the sample to a nuclear stain.

13. The method of Claim 12, wherein the nuclear stain is Astrazon Blue.

14. The method of Claim 1, wherein the reaction mixture further comprises a cobalt or nickel salt.
15. The method of Claim 1, further comprising exposing the sample to an inhibitor of a peroxidase isoenzyme prior to step (a).

16. The method of Claim 15 wherein the inhibitor is selected from the group consisting of: resorcinol, 4-hydroxyresorcinol, phloroglucinol, pyragallol, 3-hydroxycatechol and hydroquinone.

17. The method of Claim 1 wherein the product is detected using slide microscopy, flow cytometry, or direct visual examination.

18. The method of Claim 1 wherein the biological sample comprises a preparative fraction from a cell.

19. The method of claim 18 wherein the preparative fraction is electrophoretically separated from other cell components.

20. A method of detecting a cell of a specific type in a biological sample, said method comprising:
   (a) forming a complex comprising a ligand in the biological sample comprising at least one cell, a receptor capable of binding the ligand, and a peroxidatively active species conjugated to, or associated with, the receptor;
   (b) contacting the complex with a reaction mixture comprising a peroxidase substrate, a peroxide, and a photographic color coupler; and
   (c) detecting a product formed by reaction between the peroxidatively active species and the reaction mixture; and
   (d) relating detection of the product to the presence of the specific cell type.

21. The method of Claim 20, wherein the receptor is an antibody.

22. The method of Claim 21, wherein the biological sample is blood.
23. The method of Claim 20, wherein the cell is a fetal red blood cell.

24. The method of Claim 23, wherein the ligand is a fetal hemoglobin or transferrin receptor.

25. The method of Claim 20, wherein the photographic color coupler is a yellow coupler, a magenta coupler or a cyan coupler.

26. The method of Claim 25, wherein the color coupler is a yellow coupler selected from the group consisting of benzoylacetanilides and pivaloylacetanilides.

27. The method of Claim 25, wherein the coupler is a magenta coupler selected from the group consisting of pyrazolines, pyrazolones, pyrazolobenzimidazoles, pyrazolotriazoles and a compound having the structure

\[
(R)_n \quad \begin{array}{c}
\text{X} \\
\text{Y}
\end{array}
\]

wherein:

- R is independently hydrocarbyl;
- n is an integer from 0-4; and
- X and Y are independently selected from the group consisting of -C(O)-, -S-, -O- and -NH-.

28. The method of Claim 25, wherein the coupler is a cyan coupler selected from the group consisting of phenols and naphthols.
29. The method of Claim 20, wherein the peroxidatively active species is a peroxidase enzyme.

30. The method of Claim 20 wherein the peroxidase substrate is a diaminobenzidine or a paraphenylenediamine.

31. The method of Claim 20, wherein the product is fluorescent.

32. The method of Claim 20, further comprising exposing the cell to a nuclear stain.

33. The method of Claim 32, wherein the nuclear stain is Astrazon Blue.

34. The method of Claim 20 wherein the product is detected using slide microscopy, flow cytometry, or direct visual examination.

35. A method of detecting at least two different peroxidatively active species in a single biological sample, comprising:
   (a) contacting the sample with a first reaction mixture comprising a peroxidase substrate and a first photographic color coupler under conditions wherein only a first peroxidatively active species is peroxidatively active;
   (b) contacting the sample with a second reaction mixture comprising a second peroxidase substrate and a second photographic color coupler under conditions wherein only a second peroxidatively active species is peroxidatively active, wherein the product formed by reaction between the first peroxidatively active species and the first reaction mixture can be distinguished from the product formed by reaction between the second peroxidatively active species and the second reaction mixture;
   (c) detecting the products formed by reaction between the peroxidatively active species and the reaction mixtures; and
   (d) relating detection of the products to the presence of the first and second peroxidatively active species.
36. The method of claim 35 wherein the biological sample comprises more than one cell, and the first and second peroxidatively active species are present in different cells.

37. The method of Claim 35 wherein the product is detected using slide microscopy, flow cytometry, or direct visual examination.

38. The method of Claim 35, wherein the conditions for rendering only a selected cell type peroxidatively active comprise exposure of the cells to a peroxidase inhibitor.

39. The method of Claim 35, wherein the first peroxidase substrate is not the same as the second peroxidase substrate.

40. A method of detecting, in a biological sample, the presence of a cell carrying both a peroxidatively active species and a non-peroxidative enzyme, comprising:
   (a) contacting the sample with a reaction mixture comprising a photographic color coupler and a substrate for the non-peroxidative enzyme, which substrate is capable of conversion by the non-peroxidative enzyme to a peroxidase substrate;
   (b) detecting a product formed by reaction between the peroxidatively active species and the reaction mixture;
   whereby the presence of a cell carrying both the peroxidatively active species and the non-peroxidative enzyme is detected.

41. The method of claim 40 wherein the nonperoxidase enzyme is glucose oxidase or a phosphatase.

42. The method of claim 41 wherein the nonperoxidase enzyme is a phosphatase, the substrate is a naphthal phosphate ester, and the photographic color coupler is naphthal produced by the action of the nonperoxidase enzyme on the substrate.
43. A kit comprising in packaged combination, one or more photographic color couplers, a peroxidase substrate and instructions for their use in cytochemical staining.

44. The kit of claim 43 further comprising a peroxidase enzyme or a nonperoxidase enzyme.

45. The kit of claim 43 further comprising one or more selective peroxidase inhibitors.
### INTERNATIONAL SEARCH REPORT

**International application No.**
PCT/US97/21515

#### A. CLASSIFICATION OF SUBJECT MATTER

- IPC(6) : G01N 33/53; C12Q 1/28
- US CL : 435/ 7.24, 7.25, 28, 975

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)
  - U.S. : 435/ 7.24, 7.25, 28, 975

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)
- APS, DIALOG, MEDLINE, EMBASE, SCISEARCH

#### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>US 4,778,757 A (TESHIMA et al) 18 October 1988, entire document.</td>
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[X] Further documents are listed in the continuation of Box C.[

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**Date of the actual completion of the international search**

26 JANUARY 1998

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Commissioner of Patents and Trademarks

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