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[54] **FLUORESCENCE QUENCHING WITH IMMUNOLOGICAL PAIRS IN IMMUNOASSAYS**

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[*] Notice: The portion of the term of this patent subsequent to Dec. 7, 1993, has been disclaimed.

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Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 731,255, Oct. 12, 1976, which is a continuation-in-part of Ser. No. 591,386, Jun. 30, 1975, Pat. No. 3,996,345, which is a continuation-in-part of Ser. No. 497,167, Aug. 12, 1974, abandoned.

[51] **Int. Cl.**[2] **G01N 33/16**

[52] **U.S. Cl.** .... **424/8;** 23/230 B; 23/915; 424/12

[58] **Field of Search** .... 23/230 B; 424/8, 12; 195/103.5 R, 103.5 A

[56] **References Cited**

U.S. PATENT DOCUMENTS

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3,996,345 12/1976 Ullman 424/12

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[57] **ABSTRACT**

Immunoassays are provided employing antibodies and a fluorescer-quencher (F-Q) chromophoric pair, wherein one or both of the chromophoric pair are bonded to antibodies. Depending on the particular ligand of interest, or whether antibodies are to be assayed, various reagent combinations can be employed, where the amount of quenching is directly related to the amount of ligand or antibody present in the assay medium.

In carrying out the assay for ligands, the unknown and antiody specific for the ligand of interest to which is bound one of the F-Q pair, are combined in an aqueous buffered medium. Depending on the protocol, different assay reagents are employed in the aqueous buffered medium: (1) ligand analog bonded to the other of the F-Q pair; (2) antiobodies specific for the ligand to which is bound the other of the F-Q pair or; finally, (3) a combination of a plurality of ligands bonded together through linking groups to a hub molecule, usually a polymer, in combination with antibody bound to the other of the F-Q pair. The fluorescar is electronically excited, for example by irradiation with light at a wavelength, absorbed by the fluorescing molecule and the amount of emitted light determined. By employing appropriate standards, the presence and amount of the ligand can be determined.

The technique for the determination of antibodies in a competitive mode is substantially the same except that ligand is added as a reagent.

26 Claims, No Drawings

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FLUORESCENCE QUENCHING WITH IMMUNOLOGICAL PAIRS IN IMMUNOASSAYS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application Ser. No. 731,255, filed Oct. 12, 1976, which is a continuation-in-part of application Ser. No. 591,386, filed June 30, 1975, now U.S. Pat. No. 3,996,345 which is a continuation-in-part of application Ser. No. 497,167, filed Aug. 12, 1974, now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

There is a continuing need for rapid sensitive methods for determining minute amounts of organic compounds. A number of techniques have been developed toward this end. Among the commercially available techniques are radioimmunoassay, spin-labeled immunoassay, for which reagents are sold under the trademark FRAT ®, homogeneous enzyme immunoassay, for which reagents are sold under the trademark EMIT ®, and hemagglutination (HI). These techniques are effective for determining amounts of materials in the range of 10^{-6} to 10^{-10} M or less.

These techniques all involve the ability of a receptor molecule, usually an antibody, to be able to recognize a specific spatial and polar organization of a molecule. Except for hemagglutination, the techniques depend upon providing a reagent which can compete with the molecule being assayed for the receptor. By being able to distinguish between the reagent which is bound to receptor and reagent which is unbound, one can determine the amount of the compound of interest which is present.

These techniques can also be employed for the determination of antibodies by adding known amounts of ligand or known amounts of reagent (labeled ligand) or both.

In developing immunoassays for ligands, one is limited by the availability and properties of an appropriate receptor. However, as for the other reagents and the technique of measurement, there are a number of different considerations which make for a more accurate, convenient or commercially desirable assay. First, it is desirable that there be a minimum number of measurements of the various reagents, as well as transfers of the various reagents. Secondly, the equipment for measuring should be reasonably economical, so as to be accessible to a broad range of users. Thirdly, the reagents employed should be relatively stable, so as to be capable of storage and shipment. Fourthly, the method should not be subject to significant interference from other materials which may be adventiously present in the sample to be assayed. Other considerations are ease of training of technicans, absence of health hazards, sensitivity, reproducibility, and applicability to a wide variety of ligands.

The subject invention is predicted on the phenomenon of energy transfer between two chromophores. When a fluorescing chromophor is irradiated with light absorbed by the chromophore, the fluorescing chromophore can dissipate the energy of the absorbed light by emitting light of longer wavelength, that is, fluorescing. If another chromophore is within less than 100 Å of the fluorescer and absorbs light at the wavelength of emission, there is a probability, depending upon other factors, that the fluorescer will transfer to the other chromophore the energy which would otherwise have been emitted as light, in effect, quenching the fluorescer.

2. Description of the Prior Art

U.S. Pat. No. 3,709,868 is exemplary of a radioimmunoassay. U.S. Pat. No. 3,690,834 is exemplary of a spin immunoassay. U.S. Pat. Nos. 3,654,090 and 3,817,837 are exemplary of enzyme immunoassays. Articles of interest include an article by Ludwig Brand and James R. Gohlke, entitled, Fluorescence Probes for Structure, *Annual Review of Biochemistry,* 41, 843–868 (1972); and Stryer, *Science,* 1962, 526 (1968). Also of interest is co-pending application Ser. No. 402,693, filed Oct. 2, 1973 U.S. Pat. No. 3,998,943.

SUMMARY OF THE INVENTION

A method is provided for determining the presence or amount of an organic compound to which a receptor, usually antibody, is available or can be prepared or for the determination of antibodies. The organic compound will be hereinafter referred to as a ligand.

In carrying out the assay, two chromophores are employed which are a fluorescer-quencher pair. The amount of fluorescer within quenching distance of quencher is affected by the amount of ligand present in the assay medium.

One chromophore is introduced into the assay medium covalently bonded to a receptor composition which specifically binds to the ligand. The second chromophore can be introduced into the assay medium in different ways: (1) covalently bonded to a receptor composition which is the same or different from the receptor composition conjugated to the first chromophore, but in both instances specifically binds to the ligand, and in the presence or absence of polyligand; or covalently bonded to ligand analog, where the ligand analog can compete with ligand for the receptor composition. The choice of modes of introduction will depend to a significant degree on the number of independent epitopic or haptenic sites present in the ligand.

Where the ligand has only one independent epitopic site (monoepitopic), usually one chromophore will be covalently bonded to a receptor for ligand, and the other chromophore will be provided as covalently bonded to a ligand analog or a combination of poly(ligand analog) and the chromophore covalently bonded to receptor for ligand.

Where the ligand has a plurality of independent epitopic sites (polyepitopic), the modes indicated above may be used in addition to the following modes. In one mode, the two chromophores are individually bonded to receptor for ligand. In another mode, receptor for ligand is obtained from different species and one chromophore is bonded to receptor for the ligand-receptor from one species and the other chromophore bonded to receptor for ligand-receptor from the other species. The latter method expands the versalitity of the subject assay in allowing for common reagents for a wide variety of assays, simplifies purification procedures, and allows for the determination of the presence of assemblages, as distinct from the component parts.

Where the determination of an antibody is involved, the same techniques can be employed as for the determination of ligands, with the additional variation of adding ligand to the medium when no ligand analog or polyligand is employed.

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The various materials are brought together in an aqueous buffered medium, incubated and the fluorescer is electronically excited into an electronically excited state capable of emitting light. By determining the amount of emitted light, after incubation for a predetermined time interval or after the system has approached equilibrium, and comparing the results obtained with one or more known standards, the presence or amount of ligand or antibody can be determined.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Definitions

Ligand—an organic molecule or assemblage, normally greater than 100 molecular weight and having at least one functionality, normally polar, for which a receptor is either naturally available or can be prepared.

Ligand analog—a mono- or polyvalent radical a substantial proportion of which has the same spatial and polar organization as the ligand to define one or more determinant or epitopic sites capable fo competing with the ligand for the binding sites of a receptor, and differs from the ligand in the absence of an atom or functional group at the site of binding to another molecule or in having a linking group which has been introduced in place of one or more atoms originally present in the ligand. The ligand analog precursor is the compound employed for conjugating ligand or ligand analog to another molecule, e.g., chromophore.

Assemblage—a combination of organic molecules bound together by other than covalent bonds, generally having molecular weights exceeding 600, usually exceeding 1,000 and may be 1,000,000 or more, for which receptor is either naturally available or can be prepared; an illustrative assemblage is an antigen and antibody) or, a molecule prepared from two discrete entities, normally joined together by weak bonds, such as polar bonds or disulfide bonds, which under the conditions of the system are capable of being in equilibrium with the individual entities.

Chromophore—a fluorescer or quencher molecule; in the subject invention, the fluorescer and quencher are interrelated. The fluorescer molecule is a chromophore which is able to be electronically excited to an electronically excited state from which it returns to the ground state by the emission of light, for example, the chromophore is able to absorb light at one wavelength and emit light at a longer wavelength. Various means for transferring energy to the fluorescer to electronically excite the fluorescer to an excited state capable of fluorescence may be employed, the particular mode being primarily one of convenience. The quencher molecule is capable of inhibiting fluorescence, when within a short distance, usually less than about 100 Å, of the fluorescer molecule, by accepting the energy which would otherwise be emitted as fluorescent light. As far as the molecule or composition to which the chromophores are joined, in most instances, the fluorescer and quencher will be interchangeable, although there will frequently be some preference. Therefore, for purposes of generality, the two molecules will be referred to as chromophores, and individually referred to as Ch_1 and Ch_2.

Ligand analog-chromophore (ligand analog-$(Ch_2)_x$)—ligand analog is covalently bound to one or more fluorescent molecules or quencher molecules. With small ligands, those below about 10,000 molecular weights, usually below about 2,000 molecular weight, the ligand analog will usually be joined to fewer than 10 chromophores, usually from 1 to 10 chromophores, not more than about 1 chromophore per 1,000 molecular weight. With a large ligand, at least 2,000 molecular weight, usually at least about 10,000 molecular weight, a plurality of chromophores may be covalently bound to ligand analog. The number of chromophores present will be limited by the number which may be introduced without masking too many epitopic sites of the ligand and the desire to have a sufficient number of chromophores to insure a substantial amount of quenching when receptor-Ch_1 is bound to the ligand analog-$(Ch_2)_x$.

Poly(ligand analog)-poly(chromophore)[poly(ligand analog)-poly(Ch_2)]—ligand analog and chromophore are bonded to a high molecular weight (as compared to the ligand analog and chromophore) water soluble polyfunctionalized hub or nucleus molecule, to provide a plurality of ligand analog groups and chromophore groups spaced on the surface of the molecule, so that when receptor-Ch_1 is bound to ligand analog, some Ch_1 groups will be present within quenching distance of Ch_2 groups.

Poly(ligand analog)—ligand analog groups are bonded to a high molecular weight (as compared to ligand analog) water soluble polyfunctionalized hub or nucleus molecule, so that there are a sufficient number of ligand analogs per unit area for quenching to occur when the poly(ligand analog) is saturated with receptor-Ch_1 and receptor-Ch_2 in appropriate proportions.

Receptor-chromophore (receptor-Ch_1 and receptor-Ch_2)—a receptor is a molecule which is capable of distinguishing an epitopic site and binding to such site. Usually receptors will have binding constants in excess of 10^4, frequently, in excess of 10^6. For the most part, receptors are antibodies, although enzymes, nucleic acids, and certain globulins, may also act as receptors. In the subject invention, for the most part, the receptors will be antibodies to which one or more, usually at least two or more, chromophore groups will be bound.

Receptor composition—receptor composition is a homogeneous or heterogeneous composition capable of specific non-covalent binding to ligand and ligand analog and includes anti-ligand (a composition which specifically recognizes the ligand) and a combination of anti-ligand and anti(anti-ligand) (a composition which specifically recognizes the anti-ligand).

GENERAL STATEMENT OF THE INVENTION

The method is predicated on the employment of two chromophores which form a fluorescer-quencher pair. One of the chromophores is covalently bonded to a composition (receptor) which specifically recognizes or binds to a ligand. The other chromophore is covalently bonded to ligand analog or receptor. When the two chromophore containing compositions are introduced into the assay medium, the amount of ligand present in the assay solution will affect the amount of quencher within quenching distance of fluorescer. The assay may be carried out competitively, where ligand analog competes with ligand for receptor, ligand analog being present as poly(ligand analog) or covalently bonded to chromophore. The assay may also be carried out non-competitively with ligands having a plurality of epitopic sites, where receptor having each of the chromophores binds to ligand. The assay may be carried out for

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antibodies, where unlabeled antibody to be assayed competes with labeled antibody for binding sites.

Compositions

Depending upon the particular protocol employed and the ligand of interest, one or more of the following reagent compositions will be employed in the assay medium: ligand analog-chromophore, poly(ligand analog)-poly(chromophore), poly(ligand analog), one or two receptors and one or two receptor-chromophores. The first composition to be considered will be the ligand analog-chromophore.

Ligand Analog-Chromophore and Poly(Ligand Analog)-Poly(Chromophore)

The ligand analog-chromophore may be subdivided into two groups. The first group is where the ligand analog-chromophore has a single ligand analog and a single chromophore joined together by a relatively short linking group. In these instances, the ligand analog for the most part will be haptenic, rather than antigenic, and generally be less than about 10,000 molecular weight, more usually less than about 6,000 molecular weight, and frequently in the range of about 125 to 1,000 molecular weight, excluding the linking group employed for linking to the chromophore. For the most part, the ligand analog will differ from the ligand in having a particular functionality replaced by a bond, a hydrogen replaced by a bond, or a short carbon chain replaced by a bond (by bond, it is intended to include multiple bonds, as well as single bonds) to join to the linking group for linking to the chromophore. The various haptenic or low molecular weight ligands will be discussed subsequently.

The linking group will normally have not more than about 10 atoms in the chain between the ligand and the chromophore, more usually having either a bond or from about 1 to 6 atoms in the chain. The atoms for the most part will be carbon, oxygen, nitrogen and sulfur, particularly carbon, oxygen, and nitrogen.

The functionalities involved in the linking group will normally be non-oxo carbonyl (including imino and thionocarbonyl) oxy, amino (particularly tertiary amino or quaternary) or combinations thereof, e.g. amido, carbamyl, and amidino.

The two chromophores, either fluorescer or quencher, will normally have either an amino or alcohol function for reacting with a non-oxo carbonyl function (including the nitrogen and sulfur analogs thereof) or have a non-oxo carbonyl function, which can be reacted with an amine or alcohol functionality.

Where the ligand is of at least 2,000 molecular weight, a plurality of chromophore groups may be bound to the ligand. Usually, there will be at least one chromophore group per 20,000 molecular weight, more usually at least one chromophore group per 10,000 molecular weight and not more than one chromophore group per 1,000 molecular weight, more usually not more than one chromophore group per 2,000 molecular weight. The considerations concerning the number of chromophores conjugated to the ligand have been previously enumerated. The linking groups will be as previously described. Usually, the ligand will be an antigenic polypeptide or protein having a plurality of amino groups. Active halogen or non-oxo carbonyl (including nitrogen and sulfur analogs) can be used for conjugation to form a covalent bond or amides, amidines, thionoamides, ureas, guanidines and thioureas.

Alternatively, the ligand and chromophore (Ch_1 or Ch_2) may be linked to a hub molecule (poly(ligand analog)-poly(chromophore). The hub molecule or nucleus molecule can be employed with advantage for a variety of reasons. The nucleus molecule will generally be a polymeric molecule of relatively high molecular weight, normally in excess of 20,000 molecular weight, frequently 60,000 molecular weight, and may be 10 million or higher. The nucleus molecule will normally be water soluble or dispersible in an aqueous medium to provide a stable dispersion, where the dispersible material does not interfere with the absorption or irradiation of light. The nucleus molecule may be a naturally occurring material, a modified naturally occurring material, or synthetic. Included among nucleus molecules are polypeptides, proteins, polysaccharides, synthetic polymers, and the like. The nature of the hub molecule may be widely varied, so long as it is sufficiently functionalized to permit the introduction of the ligand and the chromophore molecules.

Among proteins which can find use are albumins, globulins, proteoglycans, and the like; among polysaccharides are amylose, cellulose, agarose, dextrans, or the like, either as obtained or partially degraded; among synthetic polymers, polyvinylalcohol, acrylates, copolymers thereof or the like may be employed.

Normally, there will be not less than about one conjugate (ligand analog or chromophore) molecule per 50,000 molecular weight, more usually not less than about one conjugate molecule per 25,000 molecular weight, and usually not more than about one conjugate molecule per 1,000 molecular weight, more usually not more than about one conjugate molecule per 2,000 molecular weight.

The ratio of chromphore molecules to ligand will generally be from about 0.05–20:1, more usually from about 0.5–20:1, preferably from about 1–10:1, and more preferably from about 2–8:1.

Where the chromophore is the fluorescer molecule for the purposes of this invention, generally there will be at least about 0.5–20, more usually from about 1–10, and preferably from about 2–7 fluorescing molecules per ligand molecule. Where the chromophore is the quencher molecule, the number of quencher molecules per ligand will generally be from about 0.5–20, more usually from about 1–20, and preferably from about 2–15 per ligand molecule.

The conjugates to the hub molecule will have the same type of linking group as was employed for joining the chromophore to the ligand. The particular choice of functionality will depend upon the available functional groups on the nucleus molecule.

Receptor-Chromophore

Since in most instances the receptor is antibody, the present description will refer to antibody as exemplary of receptor. Antibodies have a number of active amino groups which can be used for covalently conjugating the chromophore to the antibody. Conveniently, the chromophore can have a non-oxo carbonyl functionality (including the nitrogen and sulfur analogs thereof) or active α-halocarbonyl functionality. Illustrative functionalities for linking the chromophore to the antibody include acyl halides, mixed anhydrides, imidate alkyl esters, isothiocyanate, chlorobromo- or iodoacetyl, and the like.

The conditions for conjugation employ moderate temperatures 0° to 40° C., in aqueous media at moderate pH. Conjugation of chromophores to protein is known in the art. The, et al., Immunology, 18, 865 (1970); Cebra, et al., J. Immunol., 95, 230 (1965); Goldman, Fluo-

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rescent Antibody Methods, Academic Press, New York (1968).

The number of chromophore groups which are conjugated to the antibody may be varied over a relatively broad range, depending on the chromophore involved. There will be at least one chromophore group per antibody, and usually on the average, from about 2 to 30, more usually from about 3 to 25 chromophore groups per antibody. Where the chromophore is the fluorescer, the average number of chromophore groups per antibody will be from about 1 to 20, usually 2 to 15 and more usually 2 to 10. Where the chromophore is the quencher, the average number of chromophore groups per antibody will be from about 2 to 30, usually 3 to 25, and more usually 5 to 25.

It should also be noted that when antibodies are prepared for a ligand having a plurality of epitopic sites, the receptor composition is not homogeneous. That is, the receptor will have antibodies which recognize different epitopic sites. In referring to receptor, it is intended to include all the antibodies which are capable of specifically binding to any of the epitopic sites of the ligand.

Poly(Ligand Analog)

The poly(ligand analog) differs from the ligand analog-chromophore and poly(ligand analog)-poly(-chromophore) in that no chromophore is present, only ligand analog. The same types of nucleus molecules and the same degree of conjugation apply for the poly(ligand analog) as for the poly(ligand analog)-poly(-chromophore). However, the ligand analog may be present in much higher ratio than the hub nucleus can accomodate receptor. Therefore, while a minimum number of ligand analog groups are essential, the maximum number is one of expedience. The significant factor is that receptor molecules when bound to poly(ligand analog) can come sufficiently close to allow the chromophores to come within quenching distance.

In choosing a nucleus molecule, a number of considerations will bear on the choice. While it is not essential that the nucleus molecule be water soluble, in most instances, it will be desirable. In any event, the nucleus molecule or composition will be capable of stable dispersion in an aqueous medium. Secondly, the nucleus molecule should not absorb light at the emission wavelength of the fluorescer to cause significant quenching. Thirdly, the nucleus molecule should not fluoresce at the emission wavelengths of the fluorescer when irradiated with the exciting light. Therefore, any significant absorption by the nucleus molecule should be below about 520 nm, preferably below about 450 nm.

The nucleus molecule should be highly functionalized, preferably with amino or hydroxyl groups, although other reactive functionalities are also useful, e.g. carboxy. Fourthly, the nucleus molecule should be stable under conditions of storage and use. Fifthly, the nucleus molecule should be inert to functionalities present in the chromophore and ligand, other than the functionality for linking. Finally, the nucleus molecule should not interfere with the immunoassay, for example, by having naturally occurring receptors which may be present in physiological fluids which are studied.

While any size of molecule may be employed, very large molecules or cells will create practical problems. For example, a very large molecule passing through the light beam of the fluorometer could provide a sudden increase in the peak height. Therefore, the signal obtained would have to be averaged over a reasonable period of time. Large molecules will also result in increased scatter, but the scatter could be compensated for by an appropriate optical system. Preferably, for the most part, molecules will be employed which are less than about 10 million molecular weight, more preferably from about 30,000 to 1,000,000 molecular weight.

Chromophore

Since antibodies are normally present in the assay medium, and proteins absorb light of wavelengths up to about 310 nm, the fluorescer will have substantial absorption higher than 310 nm, normally higher than 350 nm, and preferably higher than about 400 nm. The choice of fluorescer will also be governed by the particular ligand of interest. The fluorescer should absorb light at a higher wavelength than the ligand or ligand analog of interest. A high extinction co-efficient is desirable, greatly in excess of 10, preferably in excess of 10^3, and particularly preferred in excess of 10^4. A good quantum yield should be available in the aqueous medium for the fluorescer. As a matter of convenience, the absorption peak of the fluorescer should not vary significantly with variation in the ligand.

A number of different fluorescers are described in the articles previously noted; namely, Stryer, supra, and Brand, et al., supra.

One group of fluorescers having a number of the desirable properties described previously are the xanthene dyes, which include the fluoresceins derived from 3,6-dihydroxy-9-phenyl-xanthhydrol and rosamines and rhodamines, derived from 3,6-diamino-9-phenylxanthhydrol. The rhodamines and fluoresceins have a 9-o-carboxyphenyl group, and are derivatives of 9-o-carboxyphenylxanthhydrol.

These compounds are commercially available with substituents on the phenyl group which can be used as the site for bonding or as the bonding functionality. For example, amino and isothiocyanate substituted fluorescein compounds are available.

Another group of fluorescent compounds are the naphthylamines, having an amino group in the alpha or beta position, usually alpha position. Included among the naphthylamino compounds are 1-dimethylaminonaphthyl-5-sulfonate, 1-anilino-8-naphthalene sulfonate and 2-p-toluidinyl-6-naphthalene sulfonate.

Other dyes include 3-phenyl-7-isocyanatocoumarin, acridines, such as 9-isothiocyanatoacridine and acridine orange; N-(p-(2-benzoxazolyl)phenyl)maleimide; benzoxadiazoles, such as 4chloro-7-nitrobenzo-2-oxa-1,3-diazole and 7-(p-methoxybenzylamino)-4-nitrobenzo-2-oxa-1,3-diazole; stilbenes, such as 4-dimethylamino-4'-isothiocyanatostilbene and 4-dimethylamino-4'-maleimidostilbene; N,N'-dioctadecyloxacarbocyanine p-toluenesulfonate; pyrenes, such as 8-hydroxy-1,3,6-pyrenetrisulfonic acid, and 1-pyrenebutyric acid, merocyanine 540, rose bengal, 2,4-diphenyl-3(2H)-furanone, as well as other readily available fluorescing molecules. These dyes, either have active functionalities or such funtionalities may be readily introduced.

Similar considerations involved with the fluorescer molecule are applicable to the quenching molecule, except that a good fluorescent quantum yield is not required where fluorescence of the fluorescer is being measured. An additional consideration for the quenching molecule is that it has its absorption at an emission wavelength of the fluorescer. Good overlap of the fluorescer emission and quencher absorption is desirable.

It should be noted that both the absorption and emission characteristics of the dye may vary from being free

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