Fluorescent Excitation Transfer Immunoassay

A GENERAL METHOD FOR DETERMINATION OF ANTIGENS*

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A general immunochemical method for the assay of haptens and proteins has been devised and applied to morphine, a morphine-albumin conjugate, and human immunoglobulin G. A fluorescein-labeled antigen and a quencher-labeled antibody are employed. By use of fluorescein and rhodamine as the fluoroscer and quencher, respectively, dipole-dipole-coupled excitation energy transfer can occur within the antigen-antibody complex. The resulting quenching of fluorescence can be inhibited by competitive binding with unlabeled antigen. Alternatively, separate antibody samples can be labeled with fluorescein and rhodamine, respectively. Unlabeled antigen causes aggregation of the separately labeled components with resultant quenching.

Using the latter method, experiments suggest that up to about 20 anti-morphine antibody binding sites will associate with morphine-albumin conjugates. When an excess of the conjugate is present the antibodies appear to assemble in clumps on the protein surface. Mathematical analysis of the quenching of fluorescein-labeled morphine by rhodamine-labeled anti-morphine gives an approximate fit to the quenching data, but the calculations are very dependent on the assumptions used.

Immunchemical methods for the assay of antigens often employ either an antibody or antigen labeled with a reporter group. In so-called heterogeneous immunoassays the labeled and unlabeled components are combined, and the distribution of the label is determined by separating the antigen-antibody complex from the unbound components. Homogeneous immunchemical techniques, by contrast, require no separation step but depend instead on some physical measurement to permit detection of the antigen-antibody interaction in solution. Homogeneous methods have been developed for the assay of haptens using spin labels (1) and enzyme labels (2) in which electron spin resonance and spectrophotometric measurements are employed. However, they are generally less satisfactory for high molecular weight substances such as proteins. Bacteriophages also serve as useful labels for homogeneous immunoassay of protein antigens (3, 4) but the utility of the method is limited by the long periods of time required to culture the bacteria for the assay of phage activity.

Fluorescence techniques appeared to us to be an attractive alternative for the direct detection of the interaction of antibodies with both haptens and proteins without the necessity of employing a separation step. Fluorescer-labeled antibodies are commonly used for antigen localization in tissues (5), but heretofore have not found broad application for the immunochemical detection of substances in solution. Among the fluorescence techniques that have received most attention is fluorescence polarization. The technique has been applied to the detection of antibodies using fluorescein-labeled antigens but is highly subject to interference from nonspecific protein-protein interactions (6-8).

Fluorescence excitation transfer has received little attention despite the observation that haptens having an intrinsic chromophore (9) or bearing a chromophoric fluorescent label (10) can quench the intrinsic fluorescence of antibody tryptophans. We report here a method employing fluorescence excitation transfer which permits rapid detection of very low concentrations of both hapten and protein antigens. This approach employs two labels. Fluorescein was employed as a donor (fluoroscer) because of its high extinction coefficient and high fluorescence quantum yield, and rhodamine, which has good spectral overlap with the fluorescein emission maximum, was employed as the acceptor (quencher).

Two variants of this method were examined. In the first the antigen (Ag) was labeled with fluorescein (F) and the antibody (Ab) was labeled with rhodamine (R). The average distance between the quencher and the fluoroscer within the antigen-antibody complex was expected to be sufficient to permit energy transfer. Thus complexation should lead to fluorescence quenching. The inclusion of unlabeled antigen would be expected to reduce the available binding sites by competitive binding and thus reduce the amount of quenching.

\[
\text{Ag} + (\text{Ag}-F) \rightarrow (\text{Ab}-R) \rightarrow (\text{Ag}) + (\text{Ab}-R) + \text{Ag}-F
\]

The second method was similar to the first except that the antigen was labeled indirectly by employing a fluorescein-la-
beled antibody. For this purpose separate portions of antibody were labeled with fluorescein and rhodamine, respectively. Provided multivalent antigen is used, admixture of the different labeled antibody fractions with antigen should reduce the fluorescence intensity by bringing the donor and acceptor within close proximity.

\[ \text{Ag + Ab-F + Ab-R} \rightarrow (\text{Ab-R})_n - \text{Ag} - (\text{Ab-F})_m \]

**MATERIALS AND METHODS**

Fluorescein isothiocyanate (FNCS) was prepared from fluorescein isothiocyanate (Isomer I, Sigma) (11, 12). Tetramethylerhodamine 5'-isothiocyanate (Me, RNCs, Isomer R) (13) was obtained from Baltimore Biologi- cal Laboratories and showed no detectable impurities by thin layer chromatography. Human immunoglobulin G (HiG) and rabbit anti-serum to HiG\(^+\) were obtained from Antibodies Inc. The latter had been purified by DEAE-cellulose chromatography and assayed by quantitative precipitin test by the manufacturer (27.8 mg/ml of protein, 6.4 mg of antibody/ml). Crystallized bovine serum albumin and bovine y-globulin, Fraction II, were obtained from Miles.

Protein determinations of purified IgG fractions were obtained assuming 66.15 and a molecular weight of 145,000 for sheep IgG. A molecular weight 106,000 for HiG\(^+\) was determined by rabbit IgG (15). Anti-morphine binding site concentrations were determined by titration with spin-labeled morphine (1, 16). Fluorescence measurements were made with a Perkin-Elmer MPF-2A spectrophuorometer equipped with Baird-Atomic B-4 and B-5 fluorometer filters. Excitation was at 470 nm. The emission maxima appeared near 520 nm. Based on the unsupported assumption that this product was pure IgG fluorescence measurements were made with a Perkin-Elmer MPF-2A spectrofluorometer equipped with Baird-Atomic B-4 and B-5 fluorometer filters. Excitation was at 470 nm. The emission maxima appeared near 520 nm. Based on the unsupported assumption that this product was pure IgG

**Morphine-Fluorescein**—A solution of 0.5 g of O\(^-\)cyanoethylmorphine (17) in 40 ml of tetrahydrofuran was added dropwise under nitrogen to a suspension of 0.5 g of lithium aluminum hydride in 26 ml of the same solvent. After heating at reflux for 1 hour with stirring, the solution was cooled and the excess hydride destroyed with 4 ml of 25% NaOH. The mixture was then filtered and the solution dried over Na\(_2\)SO\(_4\). Evaporation of the solvent and purification of the residue by thin layer chromatography gave O\(^-\)a-morphine as an oil showing characteristic splitting of the O-CH\(_3\) group in the NMRI. A solution of 100 mg of FNCS in 5 ml of acetone was added during 15 min to 100 mg of O\(^-\)a-morphine in 25 ml of 20% aqueous acetone containing 70 \(\mu\)l of triethylamine. The mixture was maintained at pH 9.5 over a period of 1 hour by adding more amine. The acetone was then partially removed in vacuum and the pH was adjusted to 5.5 by passing carbon dioxide into the solution with slow addition of water. A total of 30 mg of precipitate was obtained. The product was pure by thin layer chromatography. It showed the characteristic NMR pattern (21). The Ab-R conjugates had absorption maxima in 0.01 M phosphate, pH 7.5, at 280 nm, 369 nm, and 515 to 518 nm and 551 to 553 nm. The relative fluorescence emission labeling and with the concentration of the conjugates. A spectroscopic

**Morphine-Albumin Conjugates** (Albumin-M)—O\(^-\)Carboxyethylmorphine (18) was treated with 1 eq of isothiouronium chloride in dimethylformamide at 0\(^\circ\). This solution was added to a 0.5% solution of albumin in 0.25 M sodium bicarbonate and the mixture stirred in the cold overnight. The conjugate was purified by Sephadex G-25 (Phar- macia) gel filtration. The number of morphine molecules was estimated by comparing the absorption spectra of physical mixtures of O\(^-\)carboxyethylmorphine and albumin.

**Anti-morphine IgG** (Ab\(_m\))—Sheep were immunized with albumin-M\(_n\) and the globulin fraction of the resulting antiserum precipitated with 50% saturated ammonium sulfate. A portion of the precipitate was resuspended in 2 ml (0.2 mg/ml) of 0.01 M buffered saline, pH 7.5, and fractionated on Sephadex G-200. Further purification was achieved by treatment with albumin-immunoadsorbent prepared from 2% CNBr-activated Sepharose 4B (Pharmacia) and 30 mg of albumin. The resulting IgG solution contained 50% of specific anti-morphine antibodies (Ab\(_m\)) estimated by spin-immunoassay (1, 16) and was homogeneous by cellulose acetate electrophoresis (Tris-barbital buffer, pH 8.5, \(\eta = 0.1\)).

**RESULTS**

Binding of fluorescein-labeled morphine to antimorphine y-globulin (Ab\(_m\)) was demonstrated by addition of morphine-fluorescein to approximately equimolar mixtures of antibody binding sites and spin-labeled morphine (1). Increases in the
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| Dye/protein added | Per cent protein recovered a | Binding sites/protein recovered b | Dye/protein recovered c |  
|-------------------|-----------------------------|----------------------------------|------------------------|
| mol/mol           | mol/mol                     | mol/mol                           |                        |
| 0.0               | 1.00                        | 1.15                             |                        |
| 1.18              | 0.85                        | 0.8                              | 1.07                   |
| 2.36              | 0.90                        | 1.4                              | 1.02                   |
| 4.71              | 0.90                        | 2.4                              | 0.85                   |
| 8.28              | 0.99                        | 4.7                              | 0.75                   |
| 14.2              | 0.81                        | 8.1                              | 0.56                   |
| 21.3              | 0.76                        | 11.6                             | 0.46                   |
| 28.4              | 0.75                        | 14.5                             | 0.32                   |

a Binding sites were determined by spin-immunoassay (1, 16).
b The fluorescence of solutions of 2 nM morphine-fluorescein in 0.2 M borate, pH 8.0, containing 0.5 mg/ml albumin and 20 nM antibody binding sites are recorded relative to the fluorescence of the same solutions in the absence of antibody. Solutions were incubated for 40 min at room temperature prior to measurement.
c Binding sites per M, 145,000 in the unlabeled Abm preparation.

ESR signal were observed due to competition of morphine-fluorescein and the spin label for binding sites. Approximately 80% as much morphine-fluorescein as morphine was required to produce equivalent signals. Apparently the fluorescein-labeled drug, like codeine (O\textsuperscript{2}-methylmorphine), binds more strongly than morphine to the antibody because the O\textsuperscript{2}-methylene group serves as an added structural feature in common with the original immunogen (1).

Addition of increasing amounts of rhodamine-labeled antimorphine (Abm-R) to a fixed concentration of morphine-fluorescein produced decreases in the fluorescence intensity which approached a minimum value with excess antibody (Fig. 1). The minimum intensities were directly related to the average number of rhodamines bound to antibody molecules. Interestingly, with unlabeled antibody a slight increase in the fluorescence intensity was observed which reached a maximum near an equimolar ratio of Abm-R to morphine-fluorescein. Possibly this phenomenon is due to a change in the polarity of the medium near bound morphine-fluorescein molecules that is caused by a conformational change when the second antibody binding site becomes occupied. After correction for this fluorescence enhancement, a maximum of about 72% quenching was observed with an excess of Abm-R. The specificity of binding of morphine-fluorescein and the absence of nonspecific quenching effects was demonstrable by the observation that excess codeine blocked quenching by antibody. The effects of both codeine and morphine on the fluorescence intensity of a mixture of morphine fluorescein and Abm-R\textsubscript{2} are shown in Fig. 2.

Similar results were obtained in an assay for HlgG using HlgG labeled with fluorescein (HlgG-F) and rabbit anti-HlgG antibodies labeled with rhodamine (Abh-lgG-R). Addition of excess Abh-lgG-R\textsubscript{12} to HlgG-F\textsubscript{8} produced a maximum of 88% quenching (Fig. 3). Similar quenching was obtained with 6 fluoresceins bound per HlgG but with 12 or more fluoresceins per HlgG the quenching was reduced. The specificity of the quenching effect was demonstrated by nearly complete reversal of the quenching with added HlgG (Fig. 2). As little as 0.1 nM HlgG in an assay mixture produced a 15% increase in fluorescence intensity.

**FIG. 1.** Effect of anti-morphine antibody (Abm-R) on the fluorescence intensity of 2.0 nM M-F in 0.2 M borate, pH 8. M-F, morphine-fluorescein.

**FIG. 2.** Changes in fluorescence intensity upon combining, (a) codeine (O) or morphine (●) with 3.2 nM (1.6 nM) Abm-R\textsubscript{2} and 0.46 nM morphine-fluorescein; (b) HlgG with 7.6 nM Abh-lgG-R\textsubscript{2} and 1.1 nM HlgG-F\textsubscript{4} (●); (c) codeine with 0.08 nM Abh-lgG-F\textsubscript{8}, 3.5 nM Abm-R\textsubscript{4}, and 0.31 nM albumin-M\textsubscript{fg} (●). Intensities are given relative to that of the fluorescent component alone.

**FIG. 3.** Quenching of 5 nM HlgG-F\textsubscript{8} (●), 5 nM HlgG-F\textsubscript{2} (O), 5 nM HlgG-F\textsubscript{5} (●), and 5 nM HlgG-F\textsubscript{10} (●) by Abh-lgG-R\textsubscript{2} in 0.05 M phosphate, pH 8, containing 0.5 mg/ml of albumin. Antibody binding site concentrations are estimated assuming two sites/M, 140,000, and no loss of specific activity during labeling.

It was found that excitation transfer could also be employed for an assay in which antigen was labeled indirectly through an antibody. Since antibodies to morphine were available, morphine-albumin conjugates were used as model protein anti-
The change in the fluorescence efficiency upon labeling the protein model with Ab₄-F₄ was studied first. Addition of albumin-Mₐ to a solution of Ab₄-F₄ 2.4 nM in binding sites, resulted in slight quenching of the fluorescence up to a maximum of 7% at a concentration of 1.0 nM albumin-Mₐ (Fig. 4). Additional albumin-Mₐ produced less quenching.

The Ab₄-F₄ concentration was then varied while holding the albumin-Mₐ concentration constant at 2.2 nM. Fluorescence measurements were made both without inclusion of unlabeled antibody and with the addition of an amount of unlabeled antibody sufficient to maintain a total binding site concentration of 40 nM. In both cases the reduction in fluorescence produced by albumin-Mₐ became more pronounced with higher concentrations of Ab₄-F₄. However, in the presence of unlabeled antibody there was a smaller reduction in fluorescence which was particularly evident at low concentrations of Ab₄-F₄ (Fig. 5A). By contrast, if Ab₄-R₁₆ was used in place of the unlabeled antibody, the reduction in fluorescence efficiency caused by albumin-Mₐ was greatly enhanced. Optimal quenching was reached when the Ab₄-F₄/Ab₄-R₁₆ ratio was < 1/5.

Based on this result 1/5.4 mixtures of Ab₄-F₄ and Ab₄-R₁₆ were incubated with increasing amounts of the protein model, albumin-Mₐ. The fluorescence intensities decreased to as low as 50% of their original values and then recovered as the albumin-Mₐ concentrations were further increased. The positions of the fluorescence minima were affected by the total concentration of antibody. With lower antibody concentrations the minima appeared at lower concentrations of albumin-Mₐ and the total quenching was reduced (Fig. 4). The degree of quenching was also affected by the number of haptenic sites (morphines) on the protein model. Thus the total quenching at the fluorescence minimum was reduced on decreasing the number of morphines per albumin molecule (Fig. 5B).

The quenching of the fluorescence of Ab₄-F by Ab₄-R and albumin-Mₐ could be used not only as a sensitive assay (0.1 nM) for albumin-Mₐ (Fig. 4) but also as an assay for codeine. Thus when codeine was included in a mixture of Ab₄-F₄, Ab₄-R₁₆, and albumin-Mₐ, the quenching was reversed (Fig. 2). This assay permitted the detection of as little as 1 nM codeine.

An assay for HlgG could be constructed in a similar manner. Separate portions of affinity-chromatographed antibodies against HlgG were labeled with rhodamine and fluorescein, respectively. Using a 1:7 molar ratio of Ab₄HlgG-F₄₈ to Ab₄HlgG-R₁₆, addition of HlgG produced a maximum of 40% quenching. The minimum detectable HlgG concentration was below 100 pm (Fig. 4).

**DISCUSSION**

**Direct Labeling Method**—The above observations suggest relatively efficient energy transfer between fluoroscer-labeled antigens and quencher-labeled antibody within the antigen-antibody complex. Efficient energy transfer by a dipole-dipole resonance transfer mechanism is expected when the absorption maximum of the acceptor strongly overlaps the emission maximum of the fluoroscer. The effect of the degree of rhodamine labeling on the efficiency of quenching morphine-fluorescein fluorescence by excess labeled antibody (Fig. 1) can be compared to that predicted by this mechanism. For this purpose we assume a mathematical model in which morphine-fluorescein binds to Ab-R at the end of one of two cylindrical Fab fragments attached to a cylindrical Fc fragment with the dimensions shown in the diagram (23). In the presence of excess antibody all of the morphine-fluorescein is expected to be bound.

From the equations developed by Förster (24, 25), the rate of energy transfer between two point dipoles at a distance r is

\[
\chi = \frac{1}{\tau} \left( \frac{\kappa^2 \theta G J}{n' \tau^*} \right) = \frac{1}{\tau} \left( \frac{R_G}{r} \right)^6 \text{sec}^{-1}
\]

where \( J \) is the spectral overlap integral, \( \kappa^* \) is the dipole-dipole
the expression

\[ \text{t} \text{ation, which had previously been employed by Gennis and} \]
\[ \text{Cantor, the rhodamines were assumed to be localized at one} \]
\[ \text{point on the surface, whereupon the summation is simplified to} \]
\[ \text{which rhodamine can be distributed. In the second approxima-} \]
\[ \text{tion. For this purpose it was assumed that each fragment} \]
\[ \text{was labeled to an extent that was in proportion to its surface} \]
\[ \text{area. Following the treatment of Gennis and Cantor the relative} \]
\[ \text{fluorescence efficiency of morphine-fluorescein bound to anti-} \]
\[ \text{bodies labeled randomly with rhodamines was calculated} \]
\[ \text{assuming a Poisson distribution of rhodamines on the antibody} \]
\[ \text{molecules.} \]

\[ (5) \quad P(N) = \mu^N e^{-\mu} / N! \]

In this expression \( P(N) \) is the probability of finding an antibody molecule with \( N \) rhodamines in a mixture having an average of \( \mu \) rhodamines per antibody molecule. The average fluorescence efficiency of morphine-fluorescein relative to that in the absence of quencher is then

\[ (6) \quad \phi_O = \bar{E} = \sum_{N=0}^{m} P(N) \phi(N) \]

where \( m \) was arbitrarily taken as a large enough value (\( \mu + 10 \)) to avoid significantly affecting the numerical result. Our measurements give \( \bar{E} \) expressed as the ratio of the fluorescence efficiency of morphine-fluorescein in the presence of excess Ab-R, \( \phi_O \) to that measured with unlabeled antibody \( \phi_A \).

Evaluation of Equations 2 and 4 requires a value for \( R_s \). Energy transfer from fluorescein to rhodamine B in glycerol has been studied by Kawski et al. (27) who reported \( R_s = 56 \) Å based on both a theoretical study and experimental data. In their calculations, random, but rigid orientations of the acceptor and donor were assumed. Since this is likely to also be true when the acceptor and donor are attached to a protein, their measured value for \( R_s \) can be taken without correction of the orientation factor \( \kappa^2 \). However, a correction is required for the different fluorescence quantum yield of morphine-fluorescein under the present experimental conditions. This was found to be 61% of the fluorescence quantum yield of fluorescein under the conditions used by Kawski et al. (27). Moreover the spectral overlap of the morphine-fluorescein emission and Ab-R absorption was 79% of the Kawski value. Thus we estimate the expected \( R_s \) under our conditions as 56 (0.61)^1/6 (0.79)^1/2 = 50 Å.

In Fig. 6 the calculated fluorescence efficiencies using \( R_s = 50 \) Å are compared with the experimental fluorescence intensities of morphine-fluorescein in the presence of excess antibody. The model in which the rhodamines are assumed to be uniformly distributed over the antibody surface is seen to lead to the prediction of unrealistically efficient quenching. In this model every antibody molecule bearing a given number of rhodamines contributes the same degree of quenching. Statistical variations in the distribution of rhodamines on different molecules are ignored. The effect is to overestimate the quenching contribution, particularly of lightly labeled antibody molecules, by failing to properly account for molecules with all of their rhodamines distributed far from the binding site.

By contrast, the Gennis and Cantor (26) model, in which all the rhodamines are localized on each antibody molecule at a single randomly selected site, gave a rough correlation with the observed quenching. However, this correlation was obtained only when a third of the rhodamines were assumed to be associated solely with the binding Fab fragment. Quenching by
rhodamines ignored, -.

binding Fab fragment and quenching contributions from the remaining rhodamines assumed to be distributed uniformly over the antibody surface, --; rhodamines on each molecule assumed to be localized at molecule) relative to the efficiency when p = 0. Calculated efficiencies: in the presence of a lo-fold excess of Ab,-R (μ rhodamines/antibody albumin-MS, was present in excess (see below and Fig. 4 for titration method), the only function of the unlabeled antibody evidence favoring the self-quenching hypothesis. Since the observation that the inclusion of unlabeled antibody reduced self-quenching of fluorescein upon bringing several AbM-F bulky fluorescein groups.

The quenching of fluorescence on binding HIgG-F by Ab,-R has not been studied quantitatively. It is however noteworthy that the degree of quenching was greater than the highest quenching of Dmphine-fluorescein effected by Ab,-R (cf. Figs. 1 and 3). Very probably this is due to the ability of two or more antibody binding sites to come in close proximity to a single fluorescein label on HIgG. Interestingly, increasing the number of fluoresceins on the antigen (HIgG) reduced the efficiency of quenching (Fig. 3). This effect may be due to a reduction in the number of intact haptenic sites due to the antigen surface. The introduction of unlabeled antibody would decrease the concentration of fluorescein in the clumps and thus decrease the self-quenching phenomenon. The surface aggregation of the antibody could be a consequence of specific or nonspecific binding interactions between antibody molecules.

The source of this self-quenching is not well defined. It is well known from fluorescence polarization measurements that energy can be transferred between like molecules through a dipole-dipole mechanism (28). However this process need not decrease the emission quantum yield. Emission inefficiency could arise from migration of excitation to a few unusually situated fluoresceins whose environment provides a mechanism for rapid nonradiative deactivation. The higher the density of Ab,F on the albumin-M10 surface the greater would be the probability of energy transfer.

When Ab,-R was used in place of unlabeled antibody the quenching was markedly increased due to energy transfer to rhodamine within the complex (Fig. 5A). The biphasic response observed with increasing concentrations of albumin-M10 (Fig. 4) was not unexpected. With antibody in excess both the strongly fluorescent free Ab,F and the weakly fluorescent bound Ab,F contribute to the total emission. On adding albumin-M10 the fraction of unbound antibody is decreased and there is a corresponding reduction in the fluorescence intensity. With albumin-M10 in excess the antibody becomes predominantly bound. However, some of the haptenic sites on the antibody surface are no longer complexed and the average distance between antibody molecules on the surface is thereby increased. This leads to a reduction in the efficiency of energy transfer and an increase in the fluorescence intensity of bound antibody. A similar phenomenon explains the decrease in fluorescence intensity upon removal of antibody from albumin-M10 by competitive binding with a-dodecim (Fig. 2).

It follows that for antibody concentrations well above the antibody-antigen dissociation constant, the fluorescence minima should be deepest and occur at the equivalence point. With decreasing concentrations the minima should be increasingly shallow due to incomplete binding, and they should appear at antigen concentrations that are higher than the stoichiometric equivalent. In point of fact, at the two highest antibody concentrations the positions of the minima were proportional to the concentration and corresponded to about 20 binding sites per albumin-M10 (Fig. 4). At the lowest antibody concentration the minimum became shallower and corresponded to only about 10 binding sites per albumin-M10. It is of interest that a similar number, 18, of anti-NPPh binding sites has been found to associate with an albumin-dinitrophenyl conjugate (29).

Unfortunately a simple analysis of the quenching expected by energy transfer between groups on adjacent antibodies is not possible. Assuming that the antibodies bind through both binding sites to a single albumin-M10, the Fc fragments would extend radially away from the antigen and some of the Fc surfaces might be expected to be quite distant from their nearest neighbors. Maximal quenching of significantly less than 100% is thus not surprising. The sensitivity of the quenching to distance between bound antibodies is apparent from Fig. 5B in which the fluorescence minima are shown for albumin-M conjugates bearing different numbers of morphine.
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CONCLUSION

The two types of fluorescence excitation transfer assays described here provide significant advantages over heterogeneous immunoassays for antigens in the 100 pm range and above. The most important of these may be the elimination of the frequently troublesome separation step required for heterogeneous immunoassays. Additional advantages include the stability of the reagents, high sensitivity and speed of the measurement.

One disadvantage of the indirect labeling method is that antibodies of a relatively high degree of purity are required so as to avoid excessive fluorescence background due to inactive fluorescer-labeled proteins. The direct labeling method avoids this problem since the antibody only bears the quencher, and the fluorescer is attached to the antigen. The latter method may therefore be more generally useful despite the fact that it is a competitive rather than direct assay and should theoretically be somewhat less sensitive. However, even here, too much dilution of the antibody with inactive labeled protein must be avoided because of the danger of excessive trivial quenching through direct reabsorption of fluorescence emission by high quencher concentrations.

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