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Analytical Survey

On the use of fluorescent labels in immunoassay

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Abstract: The principles and use of fluorescent labels in immunoassay are reviewed. Comparison is made with radio- and chemiluminescent immunoassays. Possible fluorescent labels are listed. The following types of assay are described: separation fluoroimmunoassay, immunofluorometric assay, fluorescence enhancement and fluorescence quenching assay, fluorescence polarization immunoassay, fluorescence energy transfer immunoassay, fluorescence protection immunoassay, alternative binding immunoassay, release fluoroimmunoassay and time-resolved fluorimetry and phosphorimetry.

Keywords: *Fluoroimmunoassay; immunoassay systems; chemiluminescent assays; fluorescent labels.*

Introduction

For many years clinical chemists have been exploiting the unique specificity of the antigen–antibody reaction for diagnostic purposes. Immunoassays are now available for a wide range of hormones, macromolecules and drugs. Although non-isotopic labels for immunoassay are not new, the sensitivity and reproducibility of radioactive counting have meant that radioisotopes have become the most widely used type of label in immunoassay. More recently, however, increasing interest in alternative labels has led to the development of non-isotopic immunoassay systems which offer sensitivity and precision rivalling those of radioimmunoassay. Instruments and reagents are now commercially available for enzyme immunoassay, chemiluminescent immunoassay and fluoroimmunoassay, and in some cases non-separation methodology allows complete automation of the assays — a significant feature in view of the rising demand for immunoassays.

It is likely that further development, particularly of fluoroimmunoassay, will lead to the replacement of radioimmunoassays in many applications by non-isotopic immunoassays.

Advantages and Limitations of Radioimmunoassay

Radioimmunoassay has unquestionable advantages. Detection sensitivity for radioactive labels is very high: radioactive emission occurs against a very low background, and

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this background is normally constant and independent of the sample. Radioactive counting is precise (the standard deviation is proportional to the square root of the number of counts). Radioactive decay is unaffected by the environment of the label, and therefore interferences with the counting process do not normally arise. The most commonly used radioactive labels (^{125}I, ^{57}Co) are small enough that their effect on the immunoreactivity of the labelled material can be disregarded. The technology of radioactive counting is thoroughly established, and a variety of instruments is available specifically designed to reduce the work involved in counting large numbers of samples.

There are, however, a number of disadvantages. The decay of a radioactive label limits the shelf-life of reagents containing it, not only because the activity declines with time, but also because the energetic disintegration of an atomic nucleus can fragment the labelled molecule. This "decay catastrophe" leads to progressive deterioration of the purity of the labelled material. Radioactive isotopes are regarded as a special hazard to health, and whilst the quantities used in most radioimmunoassays are too small to constitute a serious risk, there is some hazard for those producing the labelled compounds. Radioisotopes are also the subject of restrictive legislation in some countries.

A radiolabel is a highly inefficient marker: an ^{125}I label, counted for 600 s, emits radiation arising from the decay of *ca* 0.0013% of the radioactive iodine atoms present. For each count recorded, some 80,000 ^{125}I atoms are required.

For a non-isotopic label to merit serious consideration as a replacement for radioisotopes in immunoassay, it must overcome at least some of these problems without compromising the clear advantages of radiolabels in terms of sensitivity, precision and ease of performance without increasing costs.

An assortment of non-isotopic labels has been described, including bacteriophages [1], red cells [2], free radicals [3], metal atoms [4], particles [5], enzymes [6], and fluorescent [7] and chemiluminescent [8] substances. These have been used to develop immunoassays most of which are less sensitive than RIA, or less precise, or both. Of those which are suitably sensitive — those using enzymes and luminescent labels — the latter have particular advantages.

Chemiluminescent and Fluorescent Immunoassays

These are closely related techniques. Both use a label which emits visible light when raised to an excited electronic state. Some substances, such as luminol, 3-aminophthalhydrazide (Fig. 1), have long been known to emit light when oxidised in alkaline solution in the presence of certain catalysts. Hydrogen peroxide is commonly used as the oxidant, and haem as the catalyst. Chemiluminescent labels have been used in the development of immunoassays which are amongst the most sensitive non-isotopic immunoassays available, capable of measuring such analytes as TSH in plasma [9]. The light from the chemiluminescent reactions used in immunoassay is usually emitted as a flash and precise timing of reagent addition is required, with rapid and reproducible mixing, if the

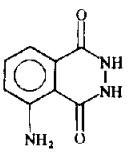

Figure 1
Structural formula of luminol.

precision of a chemiluminescent immunoassay is to be acceptable. The efficiency of light generation is low; generally, less than 1% of the molecules which do react do so with the emission of a photon [10]. The emission of a chemiluminescent label is a one-off phenomenon; measurements cannot be repeated, and the ultimate sensitivity of a chemiluminescent immunoassay will be limited by the extent of labelling. In contrast, fluorescent labels can be repeatedly excited in a reproducible manner. Measurements can be repeated, and a single fluorescent label can, in principle, emit many photons. Given a sufficiently stable fluorophore, emission intensity can be increased by increasing the intensity of excitation. With the advent of techniques such as time-resolved fluorimetry [11], it is to be expected that fluorescent labels will eventually be the most useful for very sensitive non-isotopic immunoassays. In the meantime, most types of fluorescent immunoassay (q.v.) are also amenable to the use of a chemiluminescent label with no fundamental change in assay protocol.

Interferences in FIA

As in any form of immunoassay, substances which interfere with the antigen–antibody reaction will degrade the performance of a fluorescent immunoassay. The interferences which specifically affect FIA are those which relate to the measurement of fluorescence intensity. The most important of these are scattered light, endogenous sample fluorescence, non-specific binding effects and inner filter effects.

Scattering of the exciting light occurs from inhomogeneities in the sample and sample holder; it is especially a problem in homogeneous assays where proteins, and possibly lipid micelles, are present in relatively high concentrations. Labels with a small Stokes shift (the difference between the wavelengths of the excitation and emission maxima), such as fluorescein, are particularly susceptible to interference by scattered light.

The fluorescence of serum components can be a source of marked interference in homogeneous FIA. Fluorescein measurement is particularly prone to interference from bilirubin, which has both absorption and emission maxima close to those of fluorescein. Whole serum, when excited at wavelengths around 300 nm, shows a broad emission peak with a maximum at about 400 nm, prompting efforts to find fluorophores which emit at long wavelengths.

Many fluorescent-labelled haptens, in particular, bind non-specifically to albumin. This can cause errors in some types of FIA, notably fluorescence polarization assay (where the binding greatly increases the tumbling time) and enhancement FIA (where spurious enhancement may occur).

The presence of coloured material, for example haemoglobin or bilirubin, can cause a reduction in the measured fluorescence intensity by absorbing the exciting, or more usually the emitted radiation.

Fluorescent labels

Fluorescein isothiocyanate (Fig. 2a) (FITC) was used by Coons [12] in his fluorescent antibody technique, and has subsequently become the most widely used label in fluorescent immunoassay. It has a high quantum yield (around 0.3 for the protein-bound dye at neutral pH) [13] and it is readily available, reasonably soluble in water, and cheap. Its disadvantages lie in a small Stokes shift, and the fact that its absorption and emission maxima overlap with those of bilirubin.

Rhodamine B isothiocyanate (Fig. 2b) (RBITC) has an emission maximum at a longer wavelength than fluorescein, but a similarly small Stokes shift. Its relative instability, and

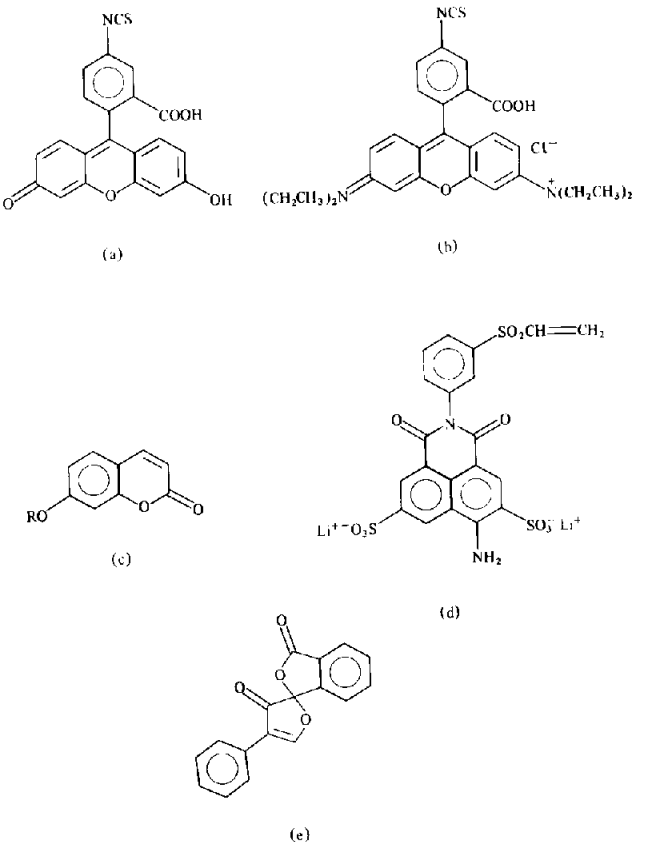

Figure 2
Structural formulae of (a) FITC; (b) RBITC; (c) umbelliferones; (d) Lucifer yellow VS; (e) fluorescamine.

the tendency of rhodamine-labelled proteins to come out of solution, have limited its application in immunoassay. Rhodamine B also illustrates a general point to be borne in mind in connection with the use of new labels: fluorescent labels are frequently described as being free from any health hazard, in contrast to radioactive labels. However, at least one manufacturer's catalogue entry [14], listing rhodamine B as a suspected carcinogen, emphasises the need for continued caution.

Umbelliferones (Fig. 2c) have high quantum yield and a moderately large Stokes shift, and have been used in immunoassay [15]. An emission maximum at around 400 nm means that sensitivity is limited by the high background in this region.

Lucifer yellow VS (Fig. 2d) has a large Stokes shift (110 nm) and an emission maximum at 540 nm. It is more convenient in use than fluorescein, and its use has been described in assays for albumin in plasma [16, 17]. Its recent commercial availability may lead to its use becoming more widespread.

Phycobiliproteins are red-fluorescent proteins isolated from bacteria. Their use in immunoassays has been described [18], and they have the advantage of an emission maximum well into the red (575–650 nm), where the fluorescence of serum is relatively slight.

Fluorescamine (Fig. 2e) has the special advantage that it becomes fluorescent only after reaction with an amino group, so that labelled proteins can be prepared without the need for separation of unbound dye. The use of fluorescamine in immunoassay has been described [19], but unfortunately the emission maximum of labelled protein is at a wavelength (475–490 nm) where serum background is high.

Fluorescent Immunoassay — Variations on the Theme

Separation fluoroimmunoassay

Separation fluoroimmunoassay is analogous to conventional separation radioimmunoassay. Serum is mixed with analyte labelled with a fluorescent marker, and a limiting quantity of antibody added. After immunoreaction, the free and antibody-bound labels are separated by one of the established techniques, and the fluorescence of either fraction is measured. The separation step can serve as a useful means of removing interfering substances: the bound fraction is precipitated, the supernatant removed, and the precipitate redissolved for measurement.

Immunofluorometric assay

Immunofluorometric assay (IFMA) is analogous to immunoradiometric assay. Typically, serum is added to an excess of antibody bound to a solid phase; this may be one of a variety of particulate materials, or the antibody may be adsorbed to the wall of the assay tube. Fluorophore-labelled antibodies, added either simultaneously or after an incubation period, bind to the antigen and are thereby bound to the solid phase. Removal of unbound labelled antibody leaves the bound fraction to be measured, after elution if necessary.

IFMA has particularly good sensitivity: the separation step allows the effective removal of material which would interfere in the fluorescence measurement, and the most sensitive fluorescent immunoassays described to date use this approach. IFMA shares with immunoradiometric assay the advantages of antibody excess: highly purified antigen is not required; incubation times are reduced; and by the use of a two-site assay, specificity can be enhanced.

A development which is significant for all types of immunoassay, including fluorescent immunoassay, is the increasing availability of monoclonal antibodies [20]. Whereas the antisera in most immunoassays to date are mixtures of antibodies differing widely in their affinity and specificity, a monoclonal antibody preparation is derived from a single antibody-producing cell. It is a preparation of antibodies directed against a single antigenic determinant, and with a single affinity constant. Polyclonal antisera contain antibodies with a non-uniform distribution of affinities: a few have very high affinity; many more have low affinity. Consequently, most monoclonal antibodies have low affinity. This low affinity makes most monoclonal antibody preparations unsuitable for limited-antibody procedures. They are, however, excellently suited to antibody excess methods, and in particular to two-site assays, since it is possible to select antibodies to different antigenic determinants on a single molecule. Such assays have lower non-specific binding than the equivalent assays using polyclonal antisera. Furthermore, the clones from which the antibodies are obtained are immortal: the supply of a particular antibody can continue indefinitely, and variations in assay performance resulting from batch to batch variations in antibody characteristics can be eliminated.

Fluorescence Enhancement and Fluorescence Quenching Assay

In some instances, the fluorescence of an antigen–fluorophore conjugate is enhanced or quenched on binding to the antibody. Such changes in fluorescence have been exploited as the basis for homogeneous immunoassays. Fluorescein-labelled thyroxine, for example, has an anomalously low quantum yield because of the presence of four iodine atoms in the molecule. In the free state the molecule is flexible enough for interaction to take place between the iodine atoms and the label, causing quenching of the fluorescence. Binding to *anti*-T4 antibody restricts the relative motion of the two parts of the molecule, and results in a three- to four-fold increase in fluorescence.

The sensitivity of a T4 assay based on this effect [21] is not sufficient for routine use. However, direct quenching fluoroimmunoassays for gentamicin have been described [22], and a direct quenching assay for serum cortisol has been developed [23] which uses an initial solvent extraction to remove interfering substances. These enhancement and quenching assays have not become widely used, because the effects are relatively uncommon, generally unpredictable and usually variable from batch to batch of antibody.

Fluorescence Polarization Immunoassay

Molecules in solution are in constant random thermal motion (Brownian motion), and rotate with a period which depends on their size and shape. A large molecule such as an antibody, with a large moment of inertia, has a tumbling time of, typically, tens to hundreds of nanoseconds. By contrast a small molecule, for example a drug, has a tumbling time measured in hundreds of picoseconds or in nanoseconds. The fluorescent emission of fluorescein has a lifetime of 4.5 ns. If, then, a fluorescein-labelled hapten, free in solution, is illuminated with plane-polarized light, the rotation of the excited state molecules is fast enough that the emitted radiation has largely random polarization. When the same hapten is bound to its antibody, the tumbling time of the complex is not significantly different from that of the antibody alone. An individual antibody–excited state–hapten complex moves only slightly during the lifetime of the excited state, and the emitted radiation retains much of the polarization of the exciting light. The proportion of label bound to antibody can be determined by measuring the degree of polarization of its fluorescence.

Fluorescence polarization immunoassay has found application in the monitoring of drug therapy. Its use is restricted to such applications since its sensitivity is relatively low, and its range of measurement limited. It is not suitable for the measurement of analytes of high molecular weight.

Fluorescence Energy Transfer Immunoassay (FETI)

FETI makes use of two labels. One of these is a fluorophore attached to the antigen, and the other is a quencher attached to the antibody. The quencher may or may not be fluorescent, but should have an absorption spectrum which overlaps as closely as possible with the emission spectrum of the fluorescer. Energy transfer can take place between the excited state of the fluorescer and the ground state of the quencher by a process of dipole–dipole interaction, and the extent of this energy transfer is dependent on, amongst other factors, the degree of overlap of the two spectra and the sixth power of the

distance between the two molecules. In practice this sixth-power relationship between separation distance and energy transfer means that, for a suitable donor–acceptor pair, energy is transferred efficiently within a distance of, typically, about 10 nm, but very inefficiently at greater separations. In energy transfer immunoassay there is little quenching of free labelled antigen, but binding to antibody brings the fluorescer into close proximity to one or more quencher molecules and results in quenching of as much as 90% of the label fluorescence. If the quencher is itself a fluorescent compound, the transferred energy may be re-emitted at the emission maximum of the quencher, and in principle it is possible to measure either free-label fluorescence or bound-label fluorescence in a homogeneous assay [25].

FETI may also be carried out as an antibody excess technique if the fluorescent label and the quencher are attached to two populations of antibodies in a two-site assay: this approach is not applicable to haptens.

Fluorescence Protection Immunoassay (Indirect Quenching Immunoassay)

In a fluorescence protection immunoassay, the fluorescence of the label is quenched by the binding of antibodies raised against the fluorophore [26, 27]. These antibodies are prevented from binding if the labelled antigen has already been bound to antigen-specific antibodies, since the bulk of these sterically hinders the necessary close approach of the antifluorophore antibodies (see Fig. 3).

In a further refinement of this type of assay, the antifluorophore antibody is labelled with a quencher, as in energy transfer immunoassay [28]: energy transfer to the quencher increases the efficiency with which unhindered fluorophore is quenched.

Alternative Binding Immunoassay

Fluorescence protection immunoassay is not applicable to haptens: binding of antifluorophore antibody is not prevented, and the labelled hapten serves simply to bind together the two antibodies. Alternative binding assay [15] has been developed to overcome this deficiency. Antibodies to hapten and to fluorophore are combined in a complex. Fluorophore-labelled hapten can bind to this complex in one of two ways: the hapten moiety may bind to an anti-hapten site, or the fluorophore may bind to an anti-fluorophore site, in which case its fluorescence will be quenched. The presence of

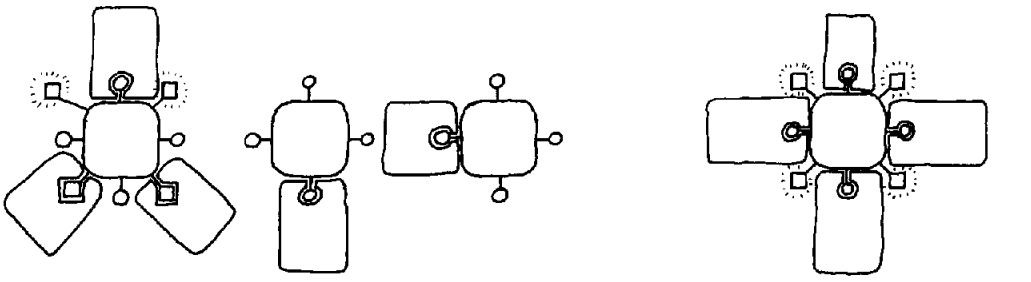

Figure 3
Illustration of fluorescence protection immunoassay.

unlabelled hapten, competing for the anti-hapten sites, increases the proportion of labelled hapten binding to the antifluorophore sites and decreases the fluorescence intensity of the assay mixture.

Release Fluoroimmunoassay

Also known as substrate-labelled fluoroimmunoassay (SLFIA), release fluoroimmunoassay may also be considered as a variant of enzyme immunoassay. The antigen is labelled with a non-fluorescent fluorophore-precursor, for example umbelliferyl-β-galactoside. In the free state, the labelled antigen can be split by a suitable enzyme — in this example, β-galactosidase — with the release of a highly fluorescent umbelliferone. The presence of antibody bound to the antigen sterically hinders the approach of the enzyme and prevents the development of fluorescence. Assays for several therapeutic drugs have been described using this approach [29, 30].

Time-resolved Fluorimetry and Phosphorimetry

Fluorescence occurs when the excited state of a fluorophore decays by a radiative transition to the ground state. The excited state, like a radioactive nucleus, decays in a random fashion with a characteristic lifetime. The fluorescence lifetime of a fluorophore is defined as the time taken for the emission to fall to $1/e$ of its initial value after the excitation has stopped. Most of the fluorophores used in fluorescent immunoassay have lifetimes of the order of 1–10 ns [31], and the scattered light and endogenous fluorescence from serum has similar lifetimes. In the assay of substances which exhibit long-lived fluorescence or phosphorescence, background rejection can be greatly increased by the use of time-resolved detection: the sample is illuminated by a short pulse from a flashlamp or laser source. During the first 100–200 ns, the scattered light and interfering fluorescence decay rapidly, and the long-lived emission can then be measured against a very low background. The sequence is repeated and the fluorescence intensity is integrated over a number of pulses. Figure 4 illustrates this principle.

Several substances have been suggested for use as labels in time-resolved fluoroimmunoassay. Derivatives of pyrene (Fig. 5a) have been prepared for coupling to proteins [32], but the fluorescence lifetime of about 100 ns is short enough to make background rejection relatively difficult. Erythrosine (Fig. 5b) has been used in a

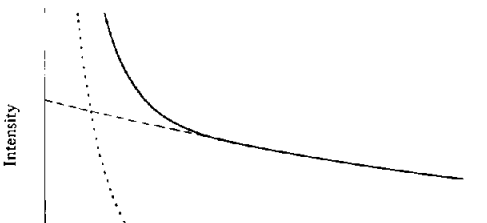

Figure 4
Time-resolved fluorimetry. A label with a fluorescence lifetime of 500 μs is present in 1000-fold excess of a fluorophore with a 200-fold smaller quantum yield, and a fluorescence lifetime of 20 μs. A pulse of illumination is supplied, and the emission monitored after the flash. The graph shows the decay of the label (– – – –), background (· · · ·), and combined (———) fluorescence. Initially the fluorescence of the short-lived fluorophore far exceeds that of the label, but after about 150 μs the background has decayed to an insignificant proportion of the total observed fluorescence. By monitoring in the period after 150 μs, the fluorescence of the label can be measured against a very low background.

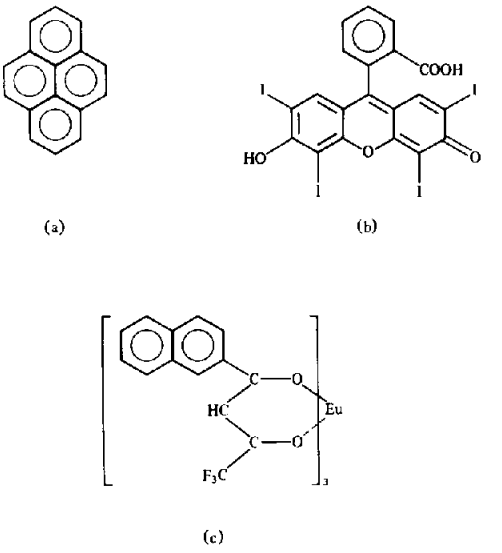

Figure 5
Structural formulae of (a) pyrene; (b) erythrosine; (c) europium tris-naphthyltrifluoroacetonate.

phosphoroimmunoassay technique [33]; it emits at 690 nm with a lifetime of several milliseconds, but has a quantum yield of only about 0.002. Perhaps the most promising approach has been the use of certain compounds of the rare earth elements. Certain diketonate complexes of europium and terbium, carrying aromatic substituents, are intensely fluorescent (e.g. Fig. 5c). The emission from these compounds is at wavelengths characteristic of the metals, and has lifetimes of 0.1–1 ms. Excitation occurs by intramolecular energy transfer from the aromatic substituents and quantum yields can be high. As little as 10^{-14}M europium can be measured as its naphthoyltrifluoroacetone complex in benzene, even without the use of time-resolved detection [34].

Europium and terbium can be attached to protein using bifunctional derivatives of ethylenediamine–tetraacetic acid [35, 36], and some energy transfer occurs to the metal ion from the phenyl ring of the chelating agent. An immunoassay for human IgG has recently been described using terbium so bound [37], but the detection limit for the phenylethylenediamine–tetraacetate (ϕ-EDTA) complex is much higher than for the diketonates. In the most sensitive time-resolved fluoroimmunoassays so far described [38–40], the analyte is labelled with the europium chelate of ϕ-EDTA. After immunoreaction and separation of the free fraction, the europium is eluted from the antibody-bound fraction and measured as its naphthoyltrifluoroacetone chelate.

Clearly it would be desirable to use a highly fluorescent lanthanide chelate directly as the label in time-resolved fluoroimmunoassay, reducing the complexity of the assay without prejudicing its sensitivity. Mixed *o*-phenanthroline–lanthanide–diketonate complexes have been described [40], and derivatives have been synthesized for labelling cell surfaces [42]. Water soluble chelates of this type can be prepared by using bathophenanthroline disulphonic acid instead of *o*-phenanthroline, and may be attached to protein using a sulphonamide linkage. Unfortunately, these compounds dissociate in dilute aqueous solution ($<10^{-5}$M) with loss of fluorescence; phosphate ion also drastically reduces the fluorescence. Once problems of this kind have been overcome, time-resolved fluoroimmunoassay promises to be a robust, and simple technique, with sensitivity and precision equalling or exceeding those of RIA.

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