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Optical Biosensors for Immunoassays: The Fluorescence Capillary-Fill Device [and Discussion]

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Optical biosensors for immunoassays: the fluorescence capillary-fill device

By R. A. Badley, R. A. L. Drake, I. A. Shanks, F.R.S., A. M. Smith and P. R. Stephenson

Unilever Research, Colworth Laboratory, Sharnbrook, Bedford MK44 1LQ, U.K.

This paper reports, for the first time, details of a novel type of optical biosensor for immunoassays, the fluorescence capillary-fill device (FCFD). This is based on a straightforward adaptation of the technology used to mass manufacture liquid-crystal display (LCD) cells to give cheap disposable immunosensors. These merely require contact by the sample to give a result in about a minute, and use certain principles of optical fibres and waveguides to avoid the need for operator attention, for physical separation methods or for washing steps.

After a very brief introductory review and classification of optical biosensors, the main features of the FCFD and its associated instrumentation are described. The optical characteristics of the FCFD are then described, followed by accounts of the immunoassay method, the measurement system used in the experiments, the fabrication of FCFD sensors and a detailed description of the design of a competitive immunoassay for human immunoglobulin G (hIgG). The experimental details and the results of a first attempt at such an assay are then presented and discussed.

It is concluded that the demonstration of this assay is a significant achievement, because the format of the FCFD, its manufacturing process and its instrumentation are completely novel. Certain problem areas have been identified and quantified; intended further work on these is outlined.

Introduction

Many new types of optical biosensor are at present under consideration or investigation (see, for example, reviews of Seitz (1984), Nylander (1985) and Andrade *et al.* (1985)). These range from optical oximeters for measuring oxygen saturation in blood (BTI Biox 1984; Schweiss 1983), through ligand-binding systems which measure serum albumin concentrations by using colour changes in dyestuffs which selectively bind such proteins (Goldfinch & Lowe 1980), to more exotic sensors that use surface plasmon resonance to measure antigen concentration (Liedberg *et al.* 1983). Many of these, however, use optical-fibre light guides simply to carry light to and from a transducing element at the measurement site. Others may make use of the inherent optical properties of such optical waveguides or of other more subtle optical effects.

To provide some focus for this paper, the following working definition of an optical biosensor will be adopted. An optical biosensor is a small device which, together with its measuring instrument, uses optical principles quantitatively to convert chemical or biochemical concentrations or activities, of interest in biology, into electrical signals. These electrical signals may be compensated, calibrated and interpreted, by using electronic signal processing in the instrument, into a format suitable for display or for driving an actuator, e.g. in a process control system. The sensor may also itself incorporate biological molecules, such as antibodies or

enzymes, to provide a transducing element that gives the desired specificity. There are many applications in which such biosensors may be useful:

(i) clinical and diagnostic medicine (use at bedside, in doctor's office or in home, e.g. for monitoring fertility);

(ii) monitoring and control of chemical, food and drug processes and fermenters;

(iii) quality control and safety of food and drugs and of raw materials for these;

(iv) veterinary medicine (e.g. animal fertility);

(v) environmental (e.g. pollution) monitoring;

(vi) farming and agriculture (soil nutrients, condition and disease organisms, animal fertility and diseases, etc.);

(vii) monitoring chemical and biological warfare;

(viii) research.

Such applications usually require the development of cheap, rapid and simple sensor systems with appropriate sensitivity and specificity. Some applications also have requirements for the inherent safety of an optical biosensor, to minimize risks from fire or explosion, or for biocompatibility if the sensor is to be used *in vivo* or in a fermenter. These requirements, taken with other considerations such as the temperature range, sterilizability and lifetime of the sensor, provide the need and justification for carrying out such challenging multidisciplinary research into optical biosensors.

It is possible to classify such sensors in two ways:

i (*a*) Direct sensors achieve their measurement without the need for additional reagents being added to the sample fluid.

(*b*) Indirect sensors require the addition of known concentrations of reagents (e.g. labelled conjugates) to the sample as an inherent feature of their operation. This usually requires that the sample volume to be tested should be accurately defined.

ii (*a*) Reversible sensors are capable of following changes in analyte concentration continuously over a suitable period.

(*b*) Irreversible sensors are only capable of one measurement or assay and must then be discarded or regenerated.

Some illustrative examples of sensors corresponding to these different categories are shown in table 1. The low value of the dissociation constant or reverse rate constant obtained in immunological reactions means that most immunosensors produced to date have been irreversible ones. Although some of these can be of the direct-measurement type (see, for example, Liedberg *et al.* 1983; Sandstrom *et al.* 1985; Sutherland *et al.* 1984) which measure the mass of antigen bound, the sensitivity of these is rather limited, and most immunosensors are of the single-assay disposable variety. Both of these observations may, however, change in the future.

Table 1. Examples of sensors by category

	direct	indirect
reversible...	fibre-optic pH sensor (Peterson 1980)	fibre-optic glucose sensor (Schultz 1982)
irreversible...	surface plasmon resonance immunosensor (Liedberg *et al.* 1983)	fluorescence capillary-fill device immunosensor (FCFD)

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This paper concentrates on a novel, indirect, irreversible (i.e. disposable) type of immunosensor, the 'fluorescence capillary fill device' (FCFD). This can provide a simple, rapid and cheap immunosensor with sensitivity and specificity adequate for a range of uses in medical diagnostics and in industrial process applications. It is based on the same principles as optical fibres and waveguides and on the well-developed, low-cost, mass-production technology for producing liquid-crystal displays (LCDs). In a modified form it can also be considered for use in carrying out immunoassays based on chemiluminescence or enzyme-chemiluminescence detection methods. Some of the main features of the FCFD are contrasted and compared with those of a conventional 'ELISA' (enzyme-linked immunosorbent assay) in table 2.

TABLE 2. COMPARISON OF CONVENTIONAL IMMUNOASSAY AND THE FCFD IMMUNOSENSOR SYSTEM

conventional test (e.g. enzyme-linked immunosorbent assay (ELISA)	FCFD sensor
sample of blood or urine needs accurate measurement	capillary action measures sample effortlessly on contact
several reagents added in precise amounts at various stages	dissoluble reagents pre-dosed in device
waiting time often hours	test result available in minutes
washing and separation steps needed	no washing or separation
laboratory style instrumentation	low-cost, simple readout unit
suitable for trained technicians in pathology laboratory	suitable for untrained users in doctor's office or home

MAIN FEATURES OF THE FCFD

The FCFD consists of two pieces of glass, separated by a narrow gap, as shown in schematic form in figure 1. The lower plate is coated with an immobilized layer of specific antibody and acts as an optical waveguide. One of the plates has a dissoluble reagent layer of antigen (or hapten) labelled with fluorescent dye. When a sample is presented to one end of the CFD it is drawn into the gap by capillary action and dissolves the reagent. If the device is set up for a competition assay, the fluorescently labelled antigen in the reagent will compete with sample antigen for the limited number of antibody binding sites on the waveguide solid phase. Because the capillary gap is narrow (typically 100 μm) the reaction is expected to go to completion in a short time, probably less than 5 min. The needs for sample and reagent metering have been removed by precise choice of the capillary gap (and hence the volume of sample collected per unit area of waveguide), and accurate loading of antibody and reagent during the device manufacture process, respectively.

The need for a separation or washing step is avoided by using an optical phenomenon known as evanescent wave coupling. When the labelled reagent in the device is optically excited by a suitable wavelength light source, it fluoresces at a longer wavelength. Reagent molecules that remain in solution can only fluoresce into relatively large angles, measured relative to the plane of the lower glass plate waveguide, and hence emerge at large angles to the end of the guide in accordance with Snell's law of refraction. Reagent molecules that are bound to the surface of the plate will, however, emit light into all angles within the waveguide (El-Hang Lee *et al.*

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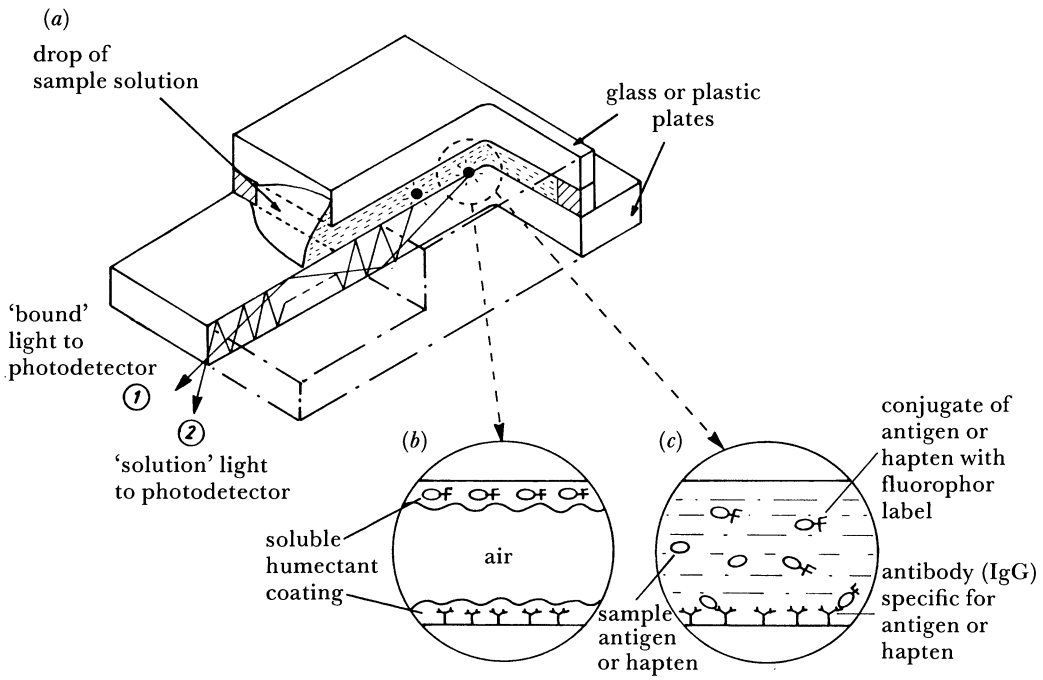

FIGURE 1. Schematic diagram of the fluorescence capillary-fill device (FCFD) illustrating a competition immunoassay for a desired antigen or hapten. (*a*) Cutaway view of device showing construction and optical waveguiding principles. (*b*) Enlarged inset of gap in the empty device showing immobilized antibody layer on lower plate and dissoluble reagent layer on upper plate. (*c*) Enlarged inset of gap in the filled device showing competitive immunological binding of the desired antigen or hapten and its fluorescently labelled counterpart (the conjugate) to the limited number of antibody molecules. Sandwich-type immunoassays are also possible in this device.

1979). Hence, by measuring the intensity of the fluorescent light emerging at smaller angles to the axis of the guide, it is possible to assess the quantity of reagent bound to the surface. Measurement of the signal at larger angles can give a normalization of the total fluorescence in the device and help to compensate for background, instrumental and other effects.

The type of instrument that could be used in conjunction with the FCFD is shown schematically in figure 2. It consists of a low-cost flash-lamp, such as the type used in photographic flash guns, which illuminates the flat face of the CFD with optically filtered light (blue for a fluorescein-labelled reagent). Fluorescent light is collected and guided in the lower plate and is detected by two photodiodes, which are located behind cylindrical lenses and which collect, respectively, the light from smaller angles (approximate range of $\pm 45°$ either side of the normal to the end of the guide) corresponding to surface-bound fluorophor, and the light from larger angles. The nature of the required functionality of the instrument and the low cost of components should make low-cost–high-volume manufacture a relatively straightforward operation.

Work to date has concentrated on using fluorescent labels and a competition assay methodology within the CFD. Other assay types (e.g. sandwich, residual, etc.) are also possible and in some cases will be more appropriate. These possibilities will be investigated as the research programme develops.

The similarity of the CFD to an LCD means that many aspects of the LCD production process can be 'borrowed'. The CFDs are made in large sheets (7 in† square at present, but larger in

† 1 in = 2.54 cm.

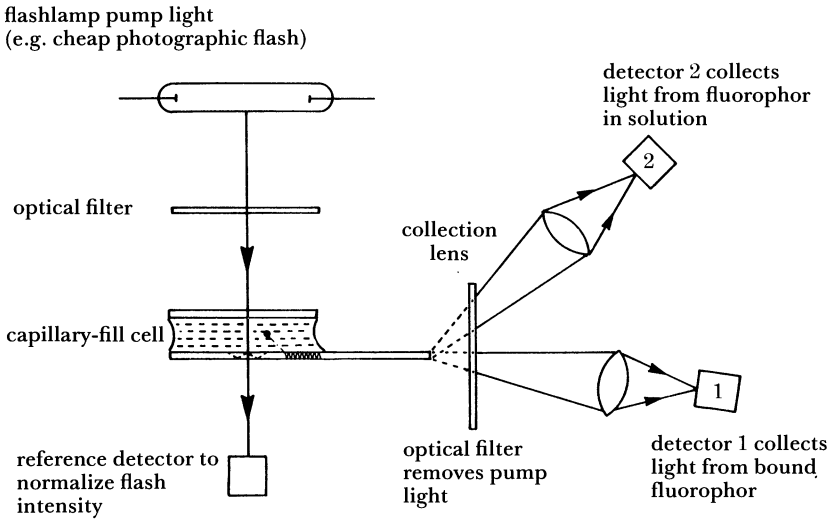

FIGURE 2. Schematic diagram of a flash photometer for the FCFD. An electronic unit processes the output of the detectors to give a ratio signal, which may be converted into a measure of antigen or hapten concentration by using a pre-stored calibration curve.

production) and then broken into individual devices (18 at present but up to about 500 in production) at the end of the production process. This technique has the potential for producing large batches of cells with uniform properties as long as the coating methods used for the plates are sufficiently well controlled.

OPTICAL CHARACTERISTICS OF FCFDS

The CFD plate structure can be viewed as a composite optical waveguide. The light that eventually emerges from the end of the lower glass plate of the CFD has quite different optical paths depending on whether it originates from a fluorescent molecule that is free in solution or a molecule bound close to the surface of the plate (figure 3*a*). Each of these cases will be considered separately, and the ways in which the optical phenomena can be used effectively will then be discussed.

(*a*) *Solution fluorescence*

Figure 3 (*b*) illustrates schematically the paths followed by light rays emitted from fluorescent molecules in solution. As a result of refraction at the sample (i.e. water) – glass boundary, all rays of light associated with solution fluorescence end up propagating at relatively large angles to the plane of the lower plate and are further refracted away from the plane when they emerge from the flat perpendicular end-face of the plate. The limiting minimum angle, α, at which solution fluorescence emerges is given by:

$$\alpha = \arcsin \sqrt{(n_1^2 - n_2^2)},$$

where n_1 and n_2 are the refractive indices of the lower plate and the sample respectively. For glass plates and a water-like sample the minimum angle is about 47°.

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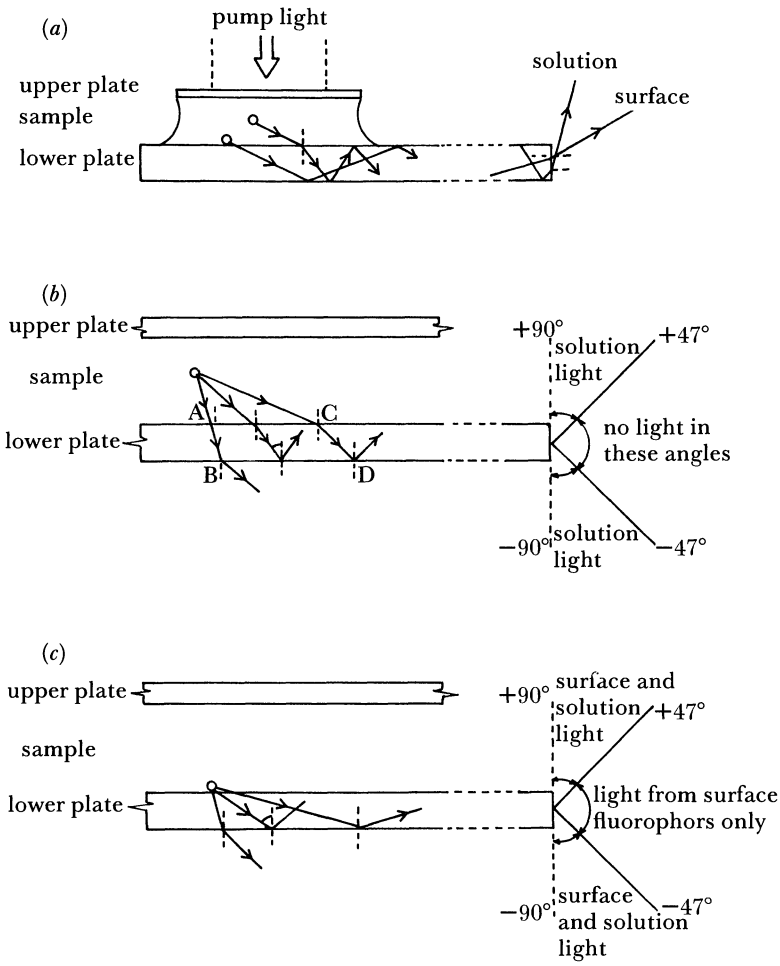

FIGURE 3. Schematic diagram of ray paths of emitted fluorescent light in the FCFD. (*a*) Schematic cross-section of FCFD. (*b*) Ray paths for light emitted by fluorescently labelled conjugate molecules in solution between the upper and lower plates. (*c*) Ray paths for light emitted by fluorescently labelled conjugate molecules bound by antibody on the surface of the lower plate.

The amount of solution fluorescence that appears as a function of output angle is affected by several factors:

(i) The angular-emission polar diagram of the fluorophor. (This will usually be homogeneous, but for particular pump-light polarization and molecule-immobilization conditions it may have an angular dependence.)

(ii) The transmission and reflection coefficients at the sample–glass and glass–air boundaries for the given angle.

(iii) The distribution of energy between the lower plate and the upper plate and solution for a given angle. (This results from the fact that light may be refracted into and out of the lower plate, in the central portion of the CFD. An equilibrium distribution of energy between the two plates and the sample exists after a number of interface transitions.)

(iv) The absorption and scattering properties of the glass, because the optical path-lengths of rays that emerge at very large angles to the plane of the lower plate will be significantly larger than those of rays that emerge at smaller angles.

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(v) The surface quality of the plate and its overall flatness; deficiencies in these areas will lead to scattering of light or merging of the emissions from different angles.

(vi) The edge quality and perpendicularity of the end of the lower plate. Prismatic or rippled ends will also cause angular merging.

In addition to these effects, surface roughness and bulk inhomogeneities in the glass plates will result in scattering of pump light and solution fluorescence, and also of substrate fluorescence. These mechanisms will result in some light being present at all output angles at the end of the guide. A typical curve showing the fluorescence from a solution of fluorescein in a CFD is shown in figure 4*b*. The clear cut-off angle at around 47° is easily seen; the relatively small level of background signal for angles less than 47° comes from inefficiently filtered scattered pump light and from scattering and fluorescence in the glass plate. The aim is to minimize the amount of light present at small angles, as this is a factor in limiting the sensitivity range of any assay system.

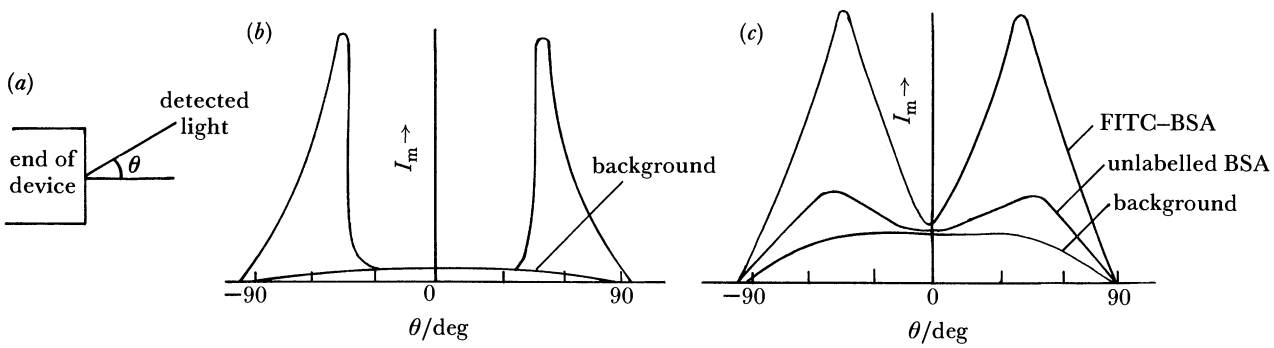

FIGURE 4. The measured intensity, I_m, of fluorescent light (peak wavelength *ca.* 510 nm) emitted from the end of the lower plate, as a function of the measurement angle, θ as defined in (*a*). (*b*) Fluorescence obtained from a solution containing 0.5 μg ml^{-1} of fluorescein isothiocyanate (FITC) and corresponding to a total of 5 pmol of FITC within the device. Note the absence of fluorescent light in the range $\theta = -47°$ to $\theta = +47°$ and the background signal due to breakthrough of scattered pump light and fluorescence in the glass plate. (*c*) The angular emission from the lower glass plate when a layer of fluorescently labelled protein (FITC-labelled BSA) has been immobilized on the surface. The emission at angles between $\theta = -47°$ and $\theta = +47°$ is seen clearly. Also shown are the background signal from the lower glass plate alone (this is the same signal as in figure 4*a* and shows the change in vertical scale factor) and the signal when unlabelled BSA is immobilized on the surface.

(*b*) *Surface fluorescence*

When fluorescent molecules are bound close to the surface of the lower glass plate, they can emit light into a broader range of angles than would be possible from solution (figure 3*c*). This is a well-understood result of the fact that light travelling within an optical waveguide is not totally confined to the higher refractive index medium but decays away exponentially over a short distance, of the order of the wavelength of light in the lower refractive index medium bounding the guide (see, for example, Harrick 1979). For a glass plate surrounded by water, the light rays that are 'guided' (i.e. not refracted out of the plate) are those that emerge at angles of less than 47°. If a fluorescent molecule bound to the surface of the glass plate emits light, then a proportion will be coupled into and guided in the glass plate, and will emerge at angles less than 47° to the plane of the plate. Light will also be found at angles larger than 47° and will result from a similar process to that of solution fluorescence. The coupling into the small angles is known as evanescent-field coupling (El-Hang Lee *et al.* 1979; Carniglia *et al.*

1972). Figure 4*c* shows the angular emission from the lower glass plate when a layer of fluorescently labelled protein (BSA) has been immobilized on the surface. The emission at angles less than 47° on both sides is seen clearly. Also shown are the light emission from the glass plate alone and the signal when a layer of unlabelled protein is on the surface of the plate. A small amount of solution fluorescence will also radiate into the small angles as a result of evanescent-field coupling. Because the concentration of solution fluorophors near the surface of the plate is, however, normally small compared to that of the bound molecules, this effect is usually minimal.

Optical assay method

The fact that the angular emission from the end of the lower glass plate of the CFD changes significantly, depending on whether fluorescent molecules are freely in solution or bound to the surface, gives a potential method for monitoring the binding to the solid phase during the course of an immunological reaction and without any need for washing or separation steps. During the course of an assay the angular-emission profiles of the light emerging from the end of the glass plate would look initially as shown in figure 4*b* and, assuming all protein eventually binds to the surface (unlikely in practice as a result of a finite affinity constant), would end as shown in figure 4*c*. At intermediate times the profile will be a linear superposition of the two basic curve shapes, depending on the relative numbers of molecules in solution and on the surface. If the quantum efficiency of the fluorescent molecules in the two states is the same, then the peak intensity at the critical angle (47°) will remain constant throughout the assay, because the evanescent field at this angle has an infinite decay length. In practice it is found that the quantum efficiency of surface-bound fluorophors is often somewhat lower than that of those in solution. The reason for this phenomena is not well understood but does not fundamentally affect the ability to use the waveguide phenomena in a non-separation assay.

The simplest way to monitor the change in the emission profile is to measure the integrated intensity in the angle range from −47° to +47°. Measurements of the light at angles greater than 47° can give a useful normalization signal, as already mentioned (figure 2).

Experimental details

In the following sections the experimental work carried out to demonstrate a competition immunoassay in an FCFD is described in detail. It was decided, in view of the relatively large quantities of antibody needed in the early stages of setting up manufacture protocols and the short time available for procuring sufficient supplies, that an assay for human immunoglobulin G (hIgG) would be attempted despite the possible problems, such as those of cross-reactivity and affinity in such a system. The use of affinity-purified material ensured a high level of potential binding sites; for this system, this is more important than the possible loss of a small fraction of higher-affinity antibodies during its preparation. The conjugate reagent would be fluorescein-labelled hIgG. Various antibodies have been used, as described below, but all have been polyclonal and none is ideal for this type of assay.

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(*a*) *Automated angular-intensity measurement system*

As has already been discussed, the eventual instrument for use in conjunction with FCFDs will probably use a flash lamp and integrate broad ranges of output angles (figure 2). Some work on this basic instrument concept has been carried out and a laboratory baseboard development unit is being developed. However, for the work on understanding the optical, immunological and biochemical properties of CFDs, it has been necessary to look at the detailed angular profiles of the light emitted from the end-face of the CFDs. To do this, a computer-controlled, automated system for angular-data acquisition, display and processing was constructed. A schematic diagram of the main elements is shown in figure 5.

The apparatus shines filtered blue light on to a 5 mm diameter area in the centre of the CFD. This light is modulated by a mechanical chopper. The light emerging in an angular wedge of less than 1° is collected by an optical system, which is filtered so it will only accept green

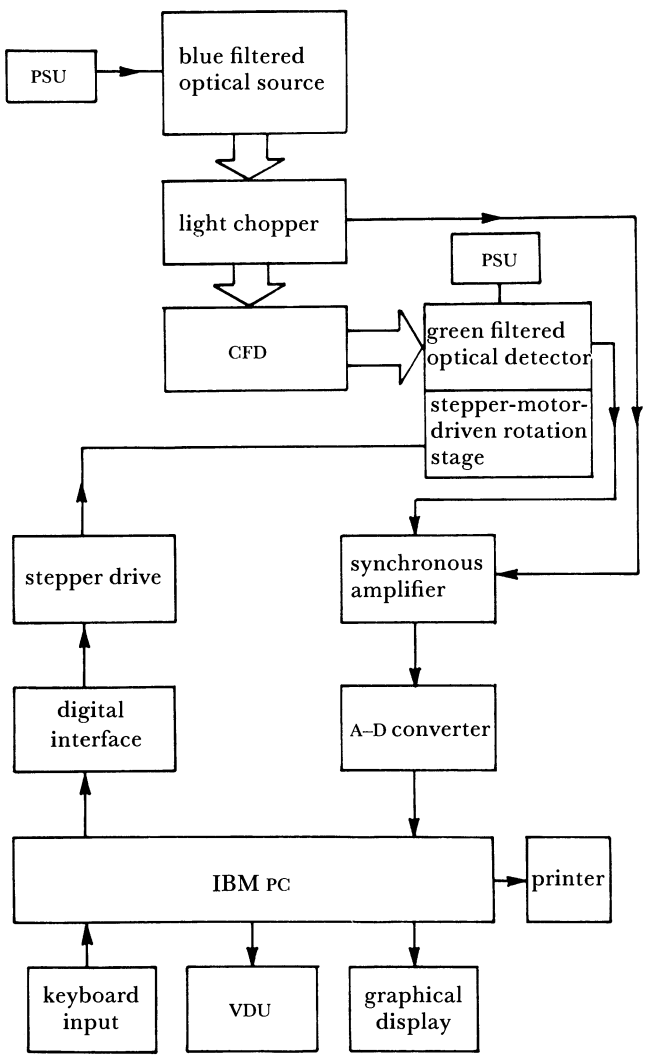

FIGURE 5. Block diagram of the automated system for acquisition of angular-intensity data, as used in the experimental measurements.

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light, and is detected by a photomultiplier tube. The modulated component of the light falling on to the tube is measured with a synchronous lock-in amplifier. The complete optical detection system is mounted on a rotation stage driven by a stepper motor, which is controlled via the digital interface of an IBM personal computer (PC). The analogue data from the lock-in amplifier are read into the PC by means of an analogue-to-digital converter card. The data are logged, filed on floppy disk or displayed graphically as required.

For direct assay measurements, the relevant data are those for the integrated intensity in the inner range of angles corresponding to surface-bound fluorescence. In practice it has been found helpful to use the average of two ranges corresponding to ($-40°$ to $-20°$) and ($+24°$ to $+44°$). The reason for the slight offset of the origin is to counteract a fixed geometrical error in the present apparatus. The angles near the origin ($-20°$ to $+24°$) are not used, because they are susceptible to receiving scattered light from the end of the CFD gap, especially when there is considerable solution fluorescence. It has been found helpful to use an average of two integrals centred on the origin, because some of the errors introduced by prismatic end-faces on the optical waveguide, or mispositioning of the CFD, may be compensated in practice.

Measurements of the integrated light-intensity at large angles, corresponding primarily to solution fluorescence (e.g. $-64°$ to $-44°$ and $48°$ to $68°$), can give a useful measure of the amount of fluorescent material present in a CFD. Furthermore, given a known concentration of fluorophor and a constant illuminated area, variations in this outer-angle integral will give a measure of the variations in the thickness of the CFD gap. It has been found that the outer angle integrals usually vary by as little as 3%; this result indicates that there is a high degree of reproducibility in the gap dimension from cell to cell.

With the system, a precise angular profile of the light emerging can be acquired and displayed in less than two minutes. In addition to recording information on the experiment, experimental conditions, and the measured data, the computer program also computes the integrated intensities over selected ranges of angles, thus simulating the functionality of the eventual instrument. The inclusion of rapid graphical presentation of the data has enabled certain cell conditions to be recognized by characteristic 'signature' angular-emission profiles.

(*b*) *FCFD fabrication and reproducibility*

Demonstration of the FCFD concept relies on the ability to make reasonable numbers of reproducible-performance devices even at early stages in the development program. Although single devices have been made from individual pieces of glass of a similar size to microscope slides (25 mm × 75 mm) throughout the program, key results have come from batch measurements of large numbers of devices cut from square plates of side 7 in. It is therefore logical to begin with a description of the batch-manufacture method which has been evolved so far.

The principal steps in the manufacture of a batch of FCFDs are shown schematically in figure 6. Two 7 in square plates of 1 mm thick glass are used to make a 'sandwich' of CFDs. For experimental purposes, the present device dimensions are 25 mm × 50 mm, so a sandwich yields 18 devices. In production, the glass plates will be larger and the individual devices are likely to measure 10 mm × 20 mm; hence a single sandwich should yield over 500 devices and possibly several thousand devices. The fact, however, that very large numbers of sandwiches may be needed to supply the predicted demand means that the reproducibility of all physical and chemical properties from sandwich to sandwich, as well as across each plate, needs to be as good as possible.

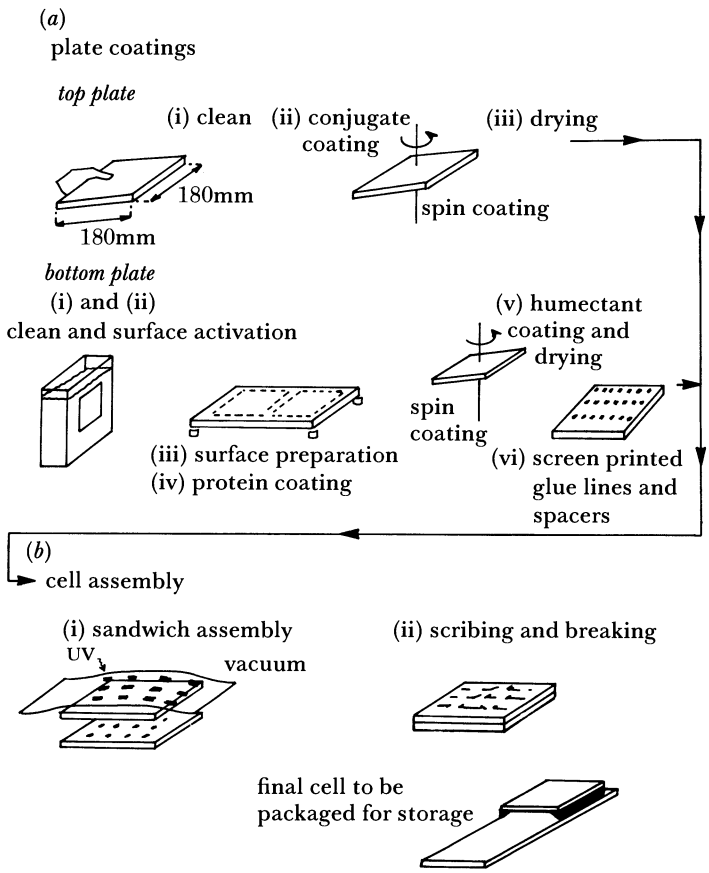

FIGURE 6. Schematic diagram of FCFD fabrication process.

Detailed protocols have been devised to cover the main steps involved in treating and assembling the plates; these will be dealt with in future publications. For the present it should suffice to indicate that the polyclonal anti hIgG (Unipath Ltd) was purified by affinity chromatography on hIgG (Sigma) on Affigel (Biorad). It was immobilized by using glutaraldehyde (Polysciences) to link it covalently to a layer of gamma-aminopropyltriethoxysilane (APS, Aldrich) coated onto the surface of the glass plate. The antibody layer was preserved in the dry state by a spin-coated layer of humectant. The conjugate was prepared from fluorescein isothiocyanate (Sigma) and hIgG (Sigma). Phosphate and Tris buffers at pH between 7.2 and 8.5 were employed. The chemicals (Analar) were obtained from Fisons.

(*c*) *Competition assay design*

Experience in assay development invariably shows that those types of test rarely conform exactly to the types of second-order rate reaction or diffusion models that would be expected from first principles. However, the CFD is, by most assay standards, potentially a very well-defined and characterizable system. Some simple predictions of the reaction kinetics and of the likely relations between antibody loading, conjugate concentration and assay range have therefore been made, to illustrate the way in which parameters may be altered to shift the midpoint of the assay curve.

(i) *Kinetics*

The first question is how fast any assay will come to completion within a CFD, bearing in mind that there is a second-order rate reaction (or more complicated reaction) at the solid-phase surface, and diffusion limits the speed at which protein can migrate to the surface and bind. The relative timescales (approximately the time taken to completion) of the diffusion process, t_d, and reaction process, t_r, are given by

$$t_d = d^2/D; \quad t_r = (k_1 C_0 + k_2)^{-1},$$

where d is the gap width, D is the diffusion constant, k_1 and k_2 are the forward and reverse rate constants, respectively, and C_0 is the original concentration of material in the cell.

For proteins ($M_r = 10^5$), $D \approx 10^{-11}$ m^2 s^{-1}, $k_1 \approx 10^5$ M^{-1} s^{-1} and $k_2 \approx 10^{-4}$ s^{-1}. (The rate constants are actually those measured for solution conditions and will not necessarily be the same for surface-bound material.) This means that, for a 1 mm gap dimension, diffusion will dominate for all sample concentrations, although for high concentrations the solid phase is likely to be saturated long before diffusion allows equilibrium of the solution. A true equilibrium will only be reached in 10^5 s. For a typical CFD gap of 0.1 mm, the diffusion timescale is 10^3 s (i.e. reaction completion in 17 min) and this means that reaction effects will become significant for concentrations less than about 10 nM. For the narrower gaps possible with the CFD (e.g. thickness, $d = 10$ μm) reaction rates can be dominant even at 1 μM concentrations.

For species of low molecular mass (*ca.* 300), such as haptens, diffusion is faster ($D = 10^{-10}$ m^2 s^{-1}) and reaction rates are substantially different ($k_1 = 10^8$ M^{-1} s^{-1}; $k_2 = 10^{-1}$ s^{-1}). The result is that, except under conditions of very narrow gaps and very low concentrations, diffusion will dominate. For a 0.1 mm gap, the diffusion timescale is 100 s.

(ii) *Relative concentrations*

By manipulating the normal equilibrium constant equation for second-order rate reactions, it is possible to derive an expression for the proportion of active antibody binding to antigen at the end of a reaction, as a function of the original ratio of concentrations of antigen to antibody. The expression contains one further factor, K, which is the dimensionless product of the affinity constant of the antibody and the original concentration of antibody. Some calculated results are shown in figure 7. Because for any competition assay there must be more antigen present than antibody, only antigen:antibody ratios greater than unity are shown. If a saturation curve is established within the CFD format by using a radioisotope then the original concentration of antigen required to give maximal binding can be found and the amount of material bound to the substrate will, for a system based on IgG–anti-IgG, be equivalent to the effective active-antibody concentration originally on the solid-phase surface. An equivalent volume concentration of antibody can be calculated by dividing by the capillary gap distance.

As an example, if 250 μg ml^{-1} of antigen (IgG) is found to be needed to cause saturation, and the amount of antigen bound in a 0.1 mm thick CFD is 50 ng cm^{-2}, the effective concentrations of antigen and antibody (IgG) will be 1.67 μM and 33 nM respectively. Thus the ratio needed to give maximal saturation is around 50. From figure 7, a system that becomes saturated at a ratio of 50 has $K = 3.3$; hence the effective affinity constant of the antibody is around 10^8. A system with this level of affinity will show substantial binding for antigen concentrations that approach the effective concentration of the antibody. This means that the

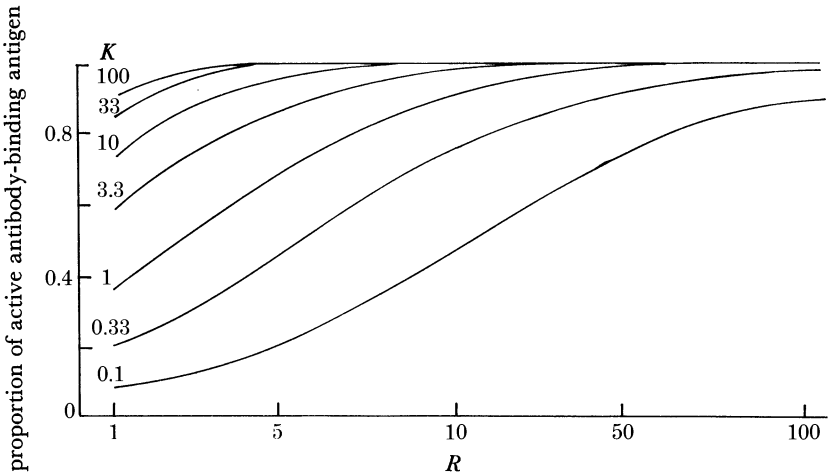

FIGURE 7. Second-order rate-reaction calculations for antibody–antigen interactions. The dimensionless parameter K is the affinity constant of the antibody multiplied by the initial antibody concentration (equivalent molar concentration in the CFD sample volume); R is the ratio of the initial antigen concentration to the initial concentration of active antibody.

level of conjugate used in a competition assay within a CFD can, in principle, be relatively low, so long as the optical-signal level is satisfactory.

If a different assay range is required, the methods available are to change the affinity of the antibody (i.e. use a different antibody), to adjust the quantity of antibody on the solid phase and to adjust the amount of conjugate present. Experience from the development of other assay systems suggests that antibody affinity is the most important factor, but this may not be valid for the CFD system and will be investigated experimentally.

(iii) *Development of the waveguide solid phase*

To reach the point where non-separation assays in the CFD were possible it was necessary to proceed in sequence through sets of experiments that had the following points as their aim:

(i) Getting a suitably large and uniform amount of protein onto the glass surface (ideally close to a packed monolayer) and keeping it there.

(ii) Making sure that the protein layer had sufficiently high binding capability (i.e. number of antibody binding sites) to obtain a detectable signal, and that this protein layer could be humectant-coated, dried and stored before successful use.

(iii) Ensuring that reproducible amounts of labelled antigen (conjugate reagent) would bind to the first protein (antibody) layer.

The criteria for successful completion were established and achieved for each stage; the details of this considerable volume of work will be the subject of a future publication, along with consideration given to alternative methods.

(*d*) *Demonstration of competition assay for human immunoglobulin G (hIgG)*

Once binding within the CFD had been established work proceeded to demonstrate a competition assay within the cells. Ultimately, fixed doses of conjugate reagents will be dried in each CFD and will be rehydrated during sample introduction. Previous work at our laboratory has shown that dried conjugate can be redissolved without loss of activity, therefore the first

priority was to establish competition binding when a premixed sample of antigen and conjugate was introduced into the CFD.

It was necessary to estimate the maximum binding capability of the CFD; this was established as about 50 ng cm^{-2}. It was found that this saturation level of binding could be achieved when conjugate levels of around 250 μg ml^{-1} were used. For conjugate concentrations around 25 μg ml^{-1} (250 ng cm^{-2} for 0.1 mm CFD gap) 30–50% binding was achieved. In other competition assays it has not been found necessary to have saturating levels of conjugate; an initial conjugate concentration of 25 μg ml^{-1} was therefore chosen. This level was a good compromise between the need to maximize the possible bound signal without encountering the problems associated with very high solution-signal levels.

It was also necessary to confirm that the kinetics of the reaction were sufficiently fast and that humectant coatings would not cause problems. Results of a kinetics experiment that used fluorescent measurements to yield a measure of the amount of protein bound are shown in figure 8. It was difficult to catch the very rapid binding in the first minute of incubation, but it can be seen that most of the binding occurs in a period of about 10 min, confirming the results expected from diffusion calculations. If faster time periods are required, reducing the CFD gap will quadratically reduce the time to completion. Reaction speeds can also be improved by the addition of solvent-partitioning additives, such as polyethylene glycol.

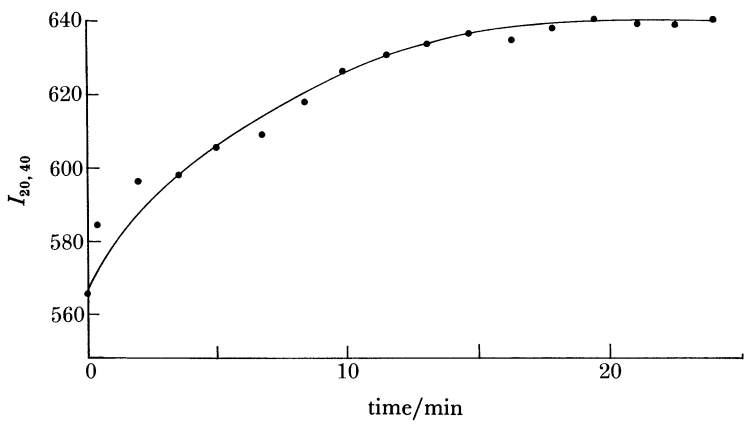

FIGURE 8. Experimental data showing conjugate (25 μg ml^{-1} FITC-labelled IgG) binding kinetics. Note non-zero ordinate value at origin. $I_{20,40}$ is the integrated inner-angle ($20° < |\theta| < 40°$) fluorescent intensity, from bound conjugate (arbitrary units).

The next step was to establish that the conjugate binding was specific by inhibiting it with excess quantities of specific antigen. Measurements were made of the inner-angle integrated intensities (the angle ranges chosen were approximately $-40°$ to $-20°$ and 20° to 40°) for conjugate alone in a CFD and for conjugate with saturation concentrations of antigen (1–3 mg ml^{-1}). Both sample and conjugate were mixed in phosphate-buffered saline containing 'Tween' (PBSAT) at pH 7.2. It was found that the signals measured decreased markedly when antigen was present. When measurements were made after about 20 min (sufficient time for equilibrium to be established) initial measurements showed a signal reduction from around 595 to 530 (arbitrary units). In addition, scatter on the data was larger than desirable and the relatively large baseline on the measurements indicated unwanted background light-scattering

effects, which we have sought to reduce. The solutions were then washed out of the CFDs with the same buffer; the change in bound signal was then clearer and represented a decrease from 300 to 170 when antigen was present. Although the aim is to measure binding with solution material present, it has also been found helpful to measure the binding when the CFD has been washed through to give a useful comparison of the binding measurement. Very little of the bound conjugate is removed during the washing and subsequent incubation in buffer, and in general it was found that the variability in measured results was smaller for washed cells than for those with solution present. An initial assay curve was recorded for both solution measurements and washed-out cell measurements with conjugate and antigen in PBSAT buffer. Once the basic assay was established, various experiments were done to check whether changes in the assay conditions could improve the range or reduce the background levels before further assay runs were attempted. In addition a range of supplementary investigations was undertaken, to identify and quantify assay parameters.

Assay results

Competition-assay measurements for various concentrations of hIgG were made with cells from three different plates (FO21, FO28, FO29). The results obtained by using measurements from washed-out cells are shown in terms of mean and standard errors in figure 9. It can be seen that there is a clear basis for a competition curve and that the midpoint of the assay corresponds to an antigen concentration of 35 μg ml^{-1} (0.23 μM). This is close to the conjugate concentration (25 μg ml^{-1}) and is therefore an indication that the affinities of the antibody layer for the antigen and for the conjugate are very similar. This is a first attempt at demonstrating an immunoassay in the FCFD; the errors represent a combination of all the variability effects within the current CFD production process and measurement errors. It is

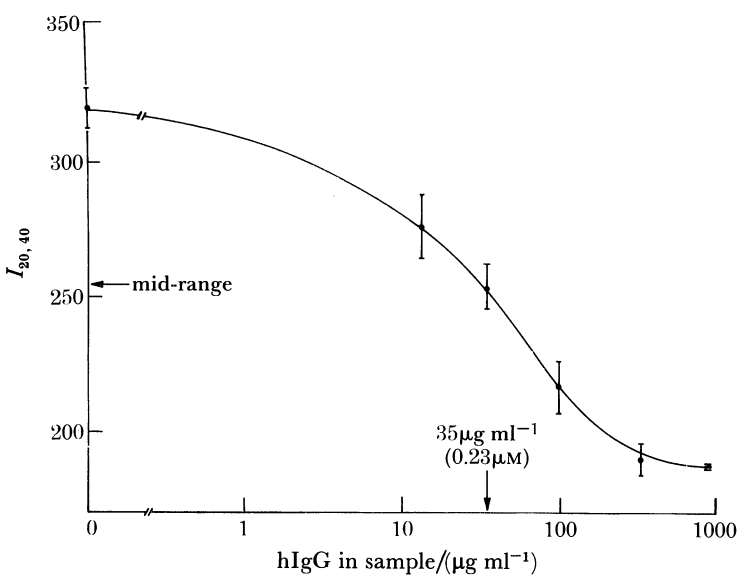

FIGURE 9. Data for a competition immunoassay for hIgG, obtained by using washed FCFD devices. Points plotted are the mean of results from six cells taken from three different batches of plates. Note non-zero ordinate value at origin and mid-range values indicated. $I_{20,40}$ is the integrated inner-angle ($20° < |\theta| < 40°$) fluorescent intensity (arbitrary units) from conjugate competitively bound after 20 min during each assay.

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anticipated that the coefficient of variation of the measurements can be reduced to less than 10%.

The solution (unwashed) measurements taken from plates FO21 and FO28 did not show a clear assay-curve shape and, for each antigen concentration, showed large variability. One possible reason for this was that the practice of filling the CFDs from the end furthest away from the end of the optical waveguide may tend to elute scattering debris into areas where it can disturb the signal. To test this hypothesis, the CFDs from plate FO29 were filled from the other end, thus tending to elute any debris away from the critical regions of the cell. The results were the data shown in figure 10, which have the expected assay shape. The assay range (140 units) is essentially the same as for the washed-out cell measurements, but the baseline value has increased as a result of additional scattering.

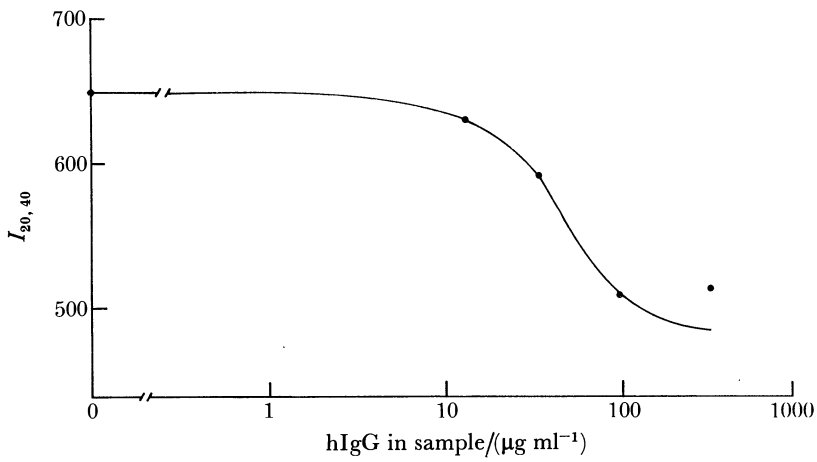

FIGURE 10. Data from unwashed FCFD devices for the assay of figure 9. These data were obtained from one set of the devices used in figure 9 before they were washed out.

The protocols for spin coating humectant layers were then run under clean (class 100) conditions; although the point-to-point variability for solution measurements was found to improve, the effect was still larger than optimum. It now seems likely that the variability results from differential quenching of the quantum efficiency of the fluorophors located near the waveguide surface.

A feature of both the solution and washed-cell measurements is that the observed signal, as measured by the present equipment for angular-intensity data acquisition and analysis, has a distinct range but is superimposed on a baseline signal caused by background effects. In effecting comparison with some conventional assay systems, it is important to realize that this baseline signal can be removed by simple appropriate optical and electronic methods and does not necessarily limit the sensitivity of the assay method. In particular, the flash-photometer system measures the background light signal in the inner range of angles as well as the total fluorescence signal, and corrects both signals for variations in flash-lamp intensity. By subtracting an appropriately scaled version of the normalized background light signal from the normalized fluorescence signal, a final output is obtained which has a baseline of zero and is corrected for background effects over the entire assay range. This type of processing is not convenient with the experimental apparatus described but is simple to achieve in a photometer, which makes an integrated angular-intensity measurement.

Conclusions

This paper has documented the first phase of a development programme aimed at producing novel immunosensors. Demonstration of an assay for hIgG represents a major achievement, as the non-separation assay format is completely new, the manufacture process is quite unlike anything used for conventional immunoassays, and a different type of photometer is required for readout. The development of a non-optimized research prototype has allowed identification of the areas of the format and process which need improvement, and in many cases has quantified the deficiencies. In the next phase of the programme, improvements in each of the key areas will be attempted. These are likely to include:

(i) Reductions in variability of the protein immobilization and activity across individual plates, and from plate to plate.

(ii) The use of alternative immobilization methods to improve uniformity and to investigate fluorescence quantum efficiency effects.

(iii) A reduction in the physical size of CFDs to allow more cells per plate.

(iv) Modification of the cell construction materials to reduce light scattering and unwanted fluorescence effects.

(v) The use of solution phase immunological reactions in conjunction with solid phase 'capture' antibodies.

(vi) Investigation of assays for analytes of lower molecular mass.

(vii) Realization of a laboratory flash photometer with the basic functionality of a production unit.

Work is under way on many of these items and will be reported in future publications as the research programme progresses. (See *Note added in proof*, overleaf).

The FCFD programme has been a multidisciplinary collaboration. Most of the detailed experimental work and CFD manufacture was carried out by Mr R. H. Greenhalgh, Ms I. Jonrup, Mr C. L. Mangan, Mr M. Tinkler and Ms S. Whiteley. Their help is gratefully acknowledged. We also acknowledge and thank Unipath Ltd. for their support of this research.

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Discussion

J. D. R. Thomas (*Applied Chemistry Department, UWIST, Cardiff, U.K.*). It is gratifying that an industrialist should speak about a development for commercial exploitation. Professor Shanks mentioned the need for cheap devices for large-volume markets and his device for immunoglobulin G is most interesting. What is this device like for accuracy and precision, and how do the data it produces compare with the laboratory method for IgG?

I. A. Shanks. The IgG assay reported in this paper is, as I have said, the first attempt at an immunoassay with this new device and was attempted primarily to demonstrate the feasibility of the FCFD concept. As can be inferred from the error bars in the calibration curve (figure 9) the accuracy and precision achieved leave a lot to be desired. This performance has been substantially improved on in our later work; these improvements, and the details of how they were achieved, will be reported in due course.

Note added in proof (*7 April 1987*). All the improvements listed have been put into practice and recent results indicate that assays for several analytes can be performed which show clinically useful precision, reproducibility and range. Furthermore, the results have been obtained in unwashed devices containing all the required predosed reagents.

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