(12) UK Patent Application (19) GB (11) 2 150 704 A

(43) Application published 3 Jul 1985

(21) Application No 8412736

(22) Date of filing 26 Mar 1982

Date lodged 18 May 1984

(30) Priority data
(31) 250528 (32) 2 Apr 1981 (33) US
255128 17 Apr 1981

(60) Derived from Application No 8208957 under Section 15(4) of the Patents Act 1977

(51) INT CL[4]
G02B 5/20

(52) Domestic classification
G2J FN

(56) Documents cited
GB 0702600

(58) Field of search
G2J

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(54) Filter assembly for fluorescence spectroscopy

(57) Apparatus for conducting fluorescent spectroscopy which involves irradiating a test sample with light of a first wavelength which causes the test sample to fluoresce light of a second wavelength and measuring a portion of the fluorescent light; irradiating the test sample with light of the first wavelength at reduced intensity and measuring the light of the first wavelength transmitted through the test sample; and comparing the intensity of the flurescent light to the transmitted light comprises an improved filter assembly which is rotatable between a test position and a reference position. The filter assembly includes a pair of test filters, one passing excitation light at one wavelength and the other passing fluorescent light from a test solution at a second wavelength, a pair of reference filters, each passing light at the first wavelength, and light shielding means to block stray light and prevent it from optically coupling between the filters. A pre-filter minimizes the background transmitted light. Insertion of the filter assembly into a commercially available automated clinical photometer enables the instrument to be used as a filter fluorimeter.

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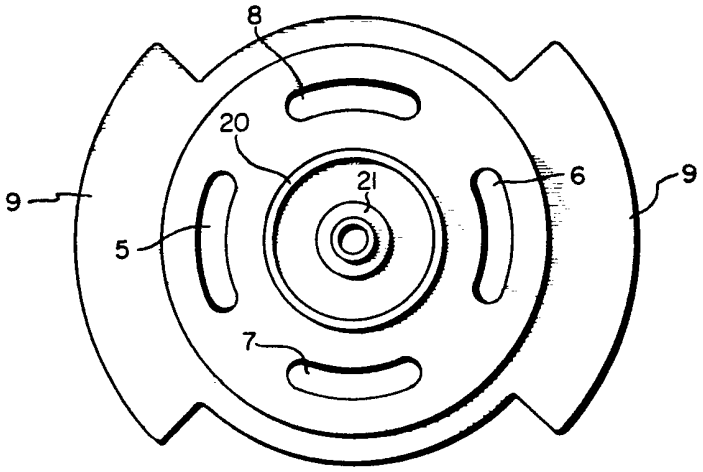
FIG.2


8
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21
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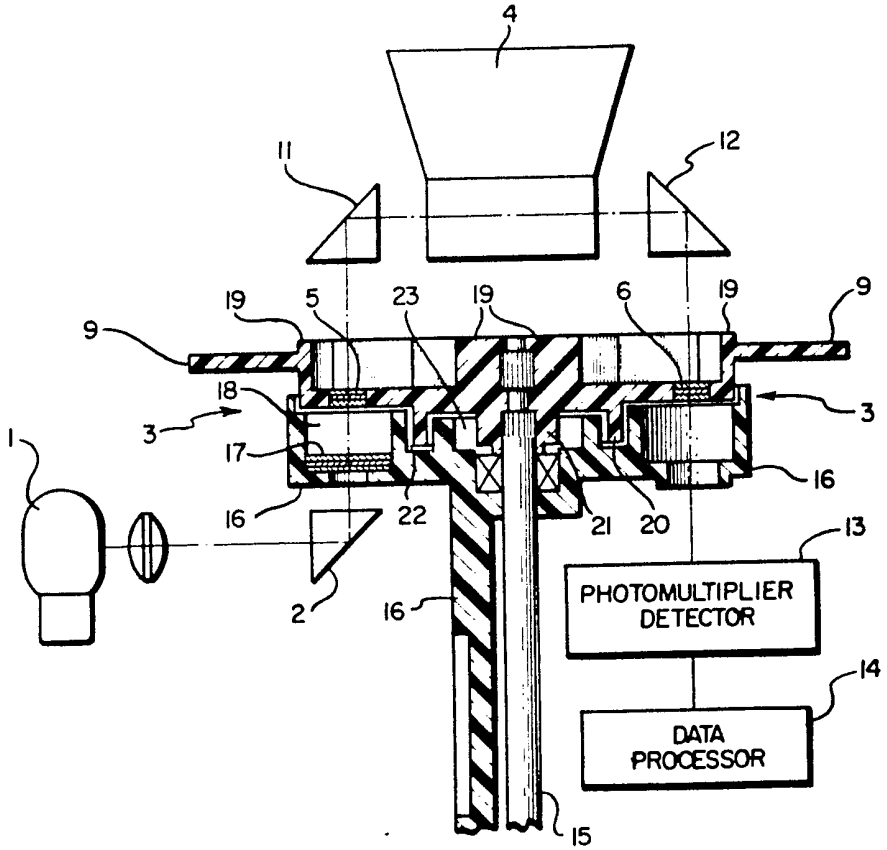
FIG.1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23
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21
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PHOTOMULTIPLIER DETECTOR
2
16
14
DATA PROCESSOR
15

FIG.3

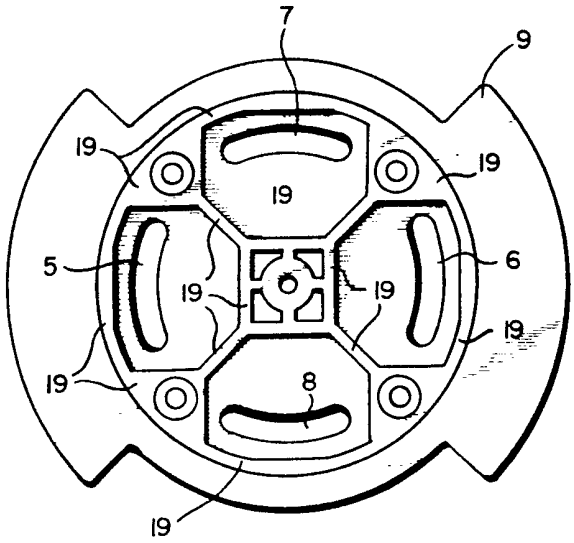
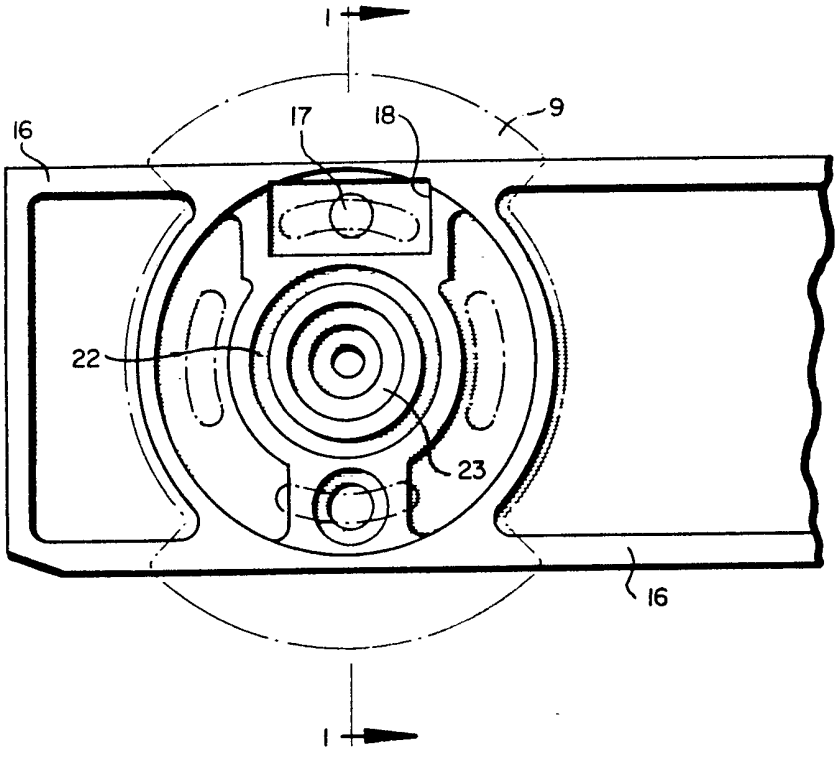


FIG.4



SPECIFICATION

Filter assembly for fluorescence spectroscopy

This invention relates to chemical analysis techniques, and more particularly to a method and apparatus for analyzing substances by exciting with a band of radiant energy and monitoring the fluorescence generated by such an excitation.

United States Patents 3,664,744; 3,748,044; 3,831,618; 3,811,780; 3,900,289; 3,833,304 and 3,817,425, describe in detail a bichromatic spectrophotometer and related apparatus. An Operation Manual entitled *ABBOTT VP BICHROMATIC ANALYZER* (1978) available through Abbott Laboratories Diagnostic Division, 1921 Hurd Drive, Irving, Texas 75062 further described bichromatic analyzers and the details of their operation.

The present invention seeks to include a movable filter assembly which converts this type of bichromatic spectrophotometer into a fluorescent spectrophotometer with a sensitivity level sufficient to perform precise fluorescence immunoassay measurements on extremely diluted solutions.

In order to rapidly and accurately analyze the concentration of a particular substance present in a chemical specimen, such as blood, serum and urine, chemists have extensively relied on photometric measurements using filter photometers and monochromatic servomechanism spectrophotometer systems.

The increased need for sensitivity and specificity in optic detection and the advent of new immunotagging techniques shifted emphasis to fluorimetric measurements and instrumentation. Thus, dedicated filter fluorimeters and spectrofluorimeters have been developed and are commercially available. However, all these prior instruments suffer from a serious deficiency—the inability to change from one mode of measurement, for example photometry, to another, for example fluorimetry, in the same instrument. This deficiency prevents the covering of a wide concentration range.

Since dedicated fluorimeters use primarily right angle illumination geometry and absorption spectrophotometers use primarily straight through illumination geometry, converting one mode of measurement to the other requires substantial hardware changes, such as the insertion of mirrors to divert the beam or the use of an auxiliary light source. Both modifications further require substantial cumbersome operator interaction and calibration.

As an example of such a prior, cumbersome multipurpose fluoro/spectrophotometric apparatus, in *Analytical Biochemstry* 42, 494–504, 1971, Britton, Chance, D. Mayer, and V. Legallais, there is described a dual-wavelength spectrophotometer and fluorimeter using interference filters which measure a fluorescence/absorbance difference ratio using two light sources and three detectors. In U.S. Patent 3,811,777, there is described apparatus for measuring the fluorescence intensity of tissue material and correcting it or separately measuring reflectance measurements on the same sample. This technique is not applicable to dilute solutions, as encountered in immunoassay measurements because of the small penetration of the exciting beam in the solution and the low reflectance signal level. Further, it is not convertible into an absorbance measurements mode as in the technique described in this specification. The present application describes techniques which alleviate these problems and enable either fluorescence or absorption to be measured on a dilute solution on the same light path, using the same light source and detector, thus minimizing hardware complexity and operator intervention.

Another problem encountered in prior fluorescence intensity instruments is the dependence of the detected intensity on sample geometry and position. Thus, very accurate sample repositioning is very crucial for obtaining accurate correlation between fluorescence intensity and concentration. C.A. Parker, Photoluminescence of Solutions, Elsevier, Amsterdam, 1968, p. 220–234, teaches that straight through, in-line illumination is much less critically dependent on the exact position of the cuvette holding the specimen than frontal illumination, and is preferred to right angle excitation in precise measurement of fluorescence intensity of solutions contained in cylindrical cells or cuvettes with optically imperfect surfaces. It is desired, therefore, to make use of these advantages of in-line excitation geometry.

According to the present invention, a fluorescence spectrophotometer for investigating the fluorescent characteristics of a test sample, comprises:—

a) a light source providing light of a first wavelength for excitation of the test sample thereby producing fluorescent light of a second wavelength from the test sample;

b) detector means for detecting light of the first and second wavelength;

c) a test sample holder transparent to light of the first and second wavelength;

d) a filter assembly comprising a frame having a plurality of filter members mounted therein, said filter assembly movable between a test position and a reference position, in the test position one filter member allowing the passage of light of the first wavelength from the light source to the test sample and one filter member allowing the passage of light of the second wavelength from the test sample to the detector means and in the reference position one filter member

allowing the passage of light of the first wavelength from the light source to the test sample and one filter member allowing the passage of light of the first wavelength transmitted through the test sample to the detector means;

e) means for directing light from the light source to the test sample and means for directing light from the test sample to the detector; and

f) a means for moving the filter assembly between the test and reference positions operatively associated with the filter assembly.

One disadvantage with the in-line, straight through, excitation geometry in that part of the exciting beam transmitted through the optics will cause a high measurement blank (C.A. Parker, 1968). We discovered one may use multilayered three-cavity filters, sharp cut-off filters, and neutral density filters to achieve high rejection of the excitation beam and achieve sensitivities equivalent to perpendicular excitation. As an example, detectable concentrations of fluorescein of 1.48×10^{-8} M to as low as 7.4×10^{-9} M can be achieved using the techniques described.

While this high level of sensitivity may suffice for many fluorescence measurements, at least another order of magnitude increase in sensitivity is required for precise fluorescence immunoassay measurements. As an example, using straight through excitation geometry, the detected fluorescent light levels are extremely low, requiring sensitivity levels for immunoassay measurements in the order of 10^{-10} M fluorescein. We have now further discovered that the sensitivity of the apparatus just referred to is limited due to the background transmitted light as well as due to the coupling of stray light between filter elements.

Accordingly the invention further provides a fluorescence spectrophotometer for investigating the fluorescent characteristic of a test solution comprising:

a) a light source providing light of a first wavelength for excitation of the test solution thereby producing fluorescent light of a second wavelength from the test solution;

b) detector means for detecting light of the first and second wavelength;

c) a test solution holder transparent to light of the first and second wavelength;

d) a filter assembly comprising a frame having a plurality of filter members mounted therein, said filter assembly movable between a test position and a reference position, in the test position one filter member allowing the passage of light of the first wavelength from the light source to the test solution and one filter member allowing the passage of light of the second wavelength from the test solution to the detector means and in the reference position one filter member allowing the passage of light of the first wavelength transmitted through the test solution to the detector means;

e) said filter assembly including light shielding means for preventing the coupling of stray light between said filter members;

f) means for directing light from the light source to the test solution and means for directing light from the test solution to the detector; and

g) a means for moving the filter assembly between the first and reference positions operatively associated with the filter assembly.

The invention also provides a filter assembly comprising a frame having a pair of test filters and a pair of reference filters mounted therein, members of the first filter pair lying on a circular line 180° apart, one member providing for the passage of light of a first wavelength for excitation of a test solution and one member providing for the passage of fluorescent light of a second wavelength, members of the reference filter pair located on the circular line 180° apart between members of the test filter pair, each member of the reference filter pair providing for the passage of light of the first wavelength and light shielding means for preventing the coupling of stray light between said filter members.

In a preferred embodiment, the apparatus includes a filter frame movably mounted on a base and having a pair of test filters and a pair of reference filters mounted on the filter frame, with the frame movable between a test position and a reference position. In an in-line or straight through optical geometry, and with the filter frame in the reference position, excitation light from a light source at a first wavelength may be passed through one of the reference filters, a test solution holder, and the other reference filter. With the filter frame in the test position, excitation light may be passed through one of the test filters and to the test solution holder, with the other test filter passing fluorescent light of a second wavelength from the test solution. A pre-filter passing excitation light and blocking fluorescent light is inserted between the excitation light source and the filters. This minimizes background transmitted light and fluorescent light in undesired optical paths to reduce optical coupling between the filter elements. Light shielding or baffle means are provided to prevent the undesired coupling of stray light or reflected light between the filter elements, and thereby significantly increase the instrument's sensitivity.

First baffle members on the top filter frame surface form a separate walled compartment to each filter to prevent stray light from optically coupling between filters across the top of the filter frame. Second baffle members on the bottom filter frame surface prevent stray light optically coupling between filters across the filter frame bottom surface.

In a constructed embodiment of the invention, a detected sensitivity of 1.9×10^{-10} M

fluorescein was obtained.

Further according to the invention, there is provided a method for conducting fluorescence spectroscopy comprising:

irradiating a test sample with light of a first wavelength which causes the test sample to fluoresce light of a second wavelength and measuring a portion of the fluorescent light;

irradiating the test sample with light of the first wavelength at reduced intensity and measuring the light of the first wavelength transmitted through the test sample; and

comparing the intensity of the fluorescent light to the transmitted light.

One constructional arrangement according to the invention will now be described by way of example with reference to the accompanying drawings, in which:–

Figure 1 is a schematic view of an improved fluorescent spectrophotometer with a sectional view of a filter assembly having a pre-filter and light shielding or baffling means in accordance with the present invention;

Figure 2 is a bottom view of a filter wheel in the filter assembly;

Figure 3 is a top view of the filter wheel in the filter assembly;

Figure 4 is a top view of a carriage or base with the filter wheel shown in dashed lines.

Referring to the drawings, there is illustrated a light source 1 and a filter assembly 3 having a filter wheel 9 and filters 5, 6, 7 and 8 mounted therein. Filter wheel shaft 15 rotatably mounted in a carriage or base 16 is molded integrally into filter wheel 9 and is used to couple the filter wheel to a drive motor. A test solution holder 4 and a detector such as photomultiplier detector 13 feeds detector output information into a data processor 14.

A pre-filter 17 is mounted in a cavity 18 formed in carriage 16 to minimize background transmitted light. A series of continous raised ridges 19 (see Fig. 3) provided on the top surface of filter wheel 9 form walled compartments which respectively surround each of the filters 5, 6, 7, 8 to block and prevent stray light from optically coupling between the filters at the filter wheel top surface. Also, respective annular ridges 20, 21 project downwardly from the bottom surface of filter 9 (see Figs. 1 and 2) to block stray light below the filter wheel from optically coupling between filters. This stray light may consist, in part, of excitation light reflected from many surfaces below the filter wheel. Ridges 20, 21 are mated with corresponding respective annular grooves 22, 23 provided in carriage 16.

In operation, in the test position, light from light source 1 is directed by prism 2 to filters 17 and 5, each of which is a 500 nm narrow band interference filter which serves to pass light of an excitation wavelength to prism 11 which directs the excitation light to the test sample 4. Fluorescing light from the test solution is directed by prism 12 to a 530 nm sharp cut-off filter 6 which passes light to the detector 13. Rotation of filter wheel assembly 9 through 180° places filter 7 and 8 (500 nm narrow band interference and neutral density filters) in the path of the light as it travels from the light source to the detector thereby producing a reference signal.

A cartridge containing filter assembly 3 and carriage 16 can be conveniently inserted into an Abbott VP™ bichromatic absorption spectrophotometer to convert it to a fluorescence spectrophotometer. One may utilize the principles of the invention to adapt other spectrophotometers to enable fluorescence measurements.

The signal obtained from a conventional bichromatic spectrophotometer converted to a fluorescence spectrophotometer by the apparatus of the present invention is proportional to the logarithm of the ratio of the fluorescence intensity to the reference intensity. In the case of very dilute solutions, the signal is linear with concentration over one order of magnitude in change in concentration, and in the case of larger concentration ranges the signal is proportional to the logarithm of the concentration over several orders of magnitude of concentration change.

The neutral density filters are selected to adjust the intensity of the excitation light transmitted through the test solution to the detector in the reference mode, and therefore, adjusts the sensitivity range of the measurements.

Those skilled in optics will recognize a variety of light sources, detectors, and filter combinations suitable for achieving the purposes of this invention. A variety of prisms, mirrors, lens, and collimators are suitable means for directing light from the light source to the test sample and from the test sample to the detector. Similarly, a wide variety of data handling techniques are available for processing electrical signals resulting from the test (fluorescing light) and reference (excitation light transmitted through the test sample) beams.

The filters in filter assembly 3 for measuring fluorescing substances are listed in Table I, it being understood that filter 17 is the same as filter 5 in each instance. Thus, pre-filter 17 has optical transmittance and blocking characteristics matching those of excitation filter 5, and therefore increases the blocking characteristics of the excitation filter at the fluorescence wavelength.

TABLE 1

5	6	7	8	Fluorescing Substance
490 nm	515 nm	490 nm	490 nm	Fluorescein
405 nm	450 nm	405 nm	405 nm	Umbelliferone
340 nm	460 nm	340 nm	340 nm	α-naphthol, NADH
366 nm	470 nm	366 nm	366 nm	8-anilinonephthalene
319 nm	445 nm	319 nm	319 nm	Homovanillic acid/H_2O_2

Those skilled in the optic arts will recognize the use of narrow band-pass filters, cut-off filters and the like for reference and fluorescence.

As an example for using this invention in fluorescence immunoassay measurements, a constructed embodiment of the invention was employed in the determination of theophylline in human serum samples. Filter assembly 3 included a pre-filter 17 at 405 nm, an excitation filter 5 at 405 nm, a fluorescence filter 6 at 460 nm, with a broad band extending from 440–470 nm, and reference filters 7, 8 at 405 nm. The pre-filter, excitation and refeence filters were narrow band interference filters. This filter assembly was placed in an Abbott VP™ bichromatic analyzer available from Abbott Laboratories, Irving, Texas.

Theophylline was determined by using the following methods and reagents. The reagents were commercially available from Ames Division, Miles Laboratories, Elkhardt, Indiana 46515.

The theophylline in the solution was reacted with a reagent containing an antibody to theophylline and an enzyme, β-galactosidase. A theophylline derivative labeled with a substrate for this enzyme, β-galactosyl-umbelliferone-theophylline conjugate was added to the mixture. This drug derivative is non-fluorescent under the conditions of the assay; however, hydrolysis catalyzed by β-galactosidase yields a fluorescent product. When antibody to theophylline reacts with the labeled theophylline, it protects it, making it virtually inactive as a substrate for the β-galactosidase. Competitive binding reactions are set up with a constant amount of the theophylline labeled reagent, a limiting amount of antibody, and the clinical serum or plasma sample containing theophylline:

labeled theophylline + antibody + theophylline ↓↑ (antibody/labeled theophylline) + (antibody/theophylline) —β-galactosidase→ fluorescene produced in proportion to the theophylline level in the serum sample

Standards and samples were prediluted 1:51 using a 1:20 diluted bicine buffer as specified by the kit manufacturer. 100 u1 aliquotes of each prediluted standard and sample was placed in the sample cups in the multicuvette assembly of the Abbott VP™ bichromatic analyzer. The enzyme/antibody reagent provided by the manufacturer in a concentrated form was diluted 1:30 using bicine buffer and was loaded in the reagent reservoir. The fluoregenic drug reagent supplied by Ames was loaded in an auxiliary reagent reservoir. The Abbott VP™ bichromatic analyzer was set up with a dispense ratio of 1:26 (10 ul sample + 250 ul reagent). The auxiliary reagent dispenser was to 10.22 ul at station 21. Temperature in the incubator water bath was 30°C. The cuvette and the sample processing module were covered with black plastic covers. The instrument was set to run in an end point mode after priming the reagent and auxiliary manifolds.

Signal from the 10th revolution (27 minutes) incubation time were plotted against standard concentration of theophylline. Theophylline concentration in the solutions was determined from the plot. This data was correlated with enzymatic immunoassay data performed on a spectrophotometer in an absorbance mode using EMIT™ reagents from Syva Company. The date correlated well between the two methods.

It may be particularly noted that a significant advantage of this invention resides in that since both fluorescence and reference signals are seen by the same detector, prior problems in transients and fluctuations in the light source are eliminated.

Rather than the rotating filter wheel described herein, the principles of this invention may be applied to provide other moving filter assemblies, such as a sliding, vibrating or reciprocating filter assembly wherein the two test filters and the two reference filters may be sequentially and repetitively inserted in the optical path.

CLAIMS

1. A filter assembly comprising a frame having a pair of test filters and a pair of reference filters mounted therein, members of the test filter pair lying on a circular line 180° apart, one member providing for the passage of light of a first wavelength for excitation of a test solution and one member providing for the passage of fluorescent light of a second wavelength, members of the reference filter pair located on the circular line 180° apart between members of the test filter pair, each member of the reference filter pair providing for the passage of light of the first wavelength and light shielding means for preventing the coupling of stray light between said filter members.

2. A filter assembly according to Claim 1, wherein said light shielding means includes walled compartment means forming a respective walled compartment above said frame for each of said filters to block and prevent stray light from coupling between filter members.

3. A filter assembly according to Claim 2, wherein said walled compartment means includes a continuous wall extending above said frame and respectively surrounding each of said filter members.

4. A filter assembly according to Claim 2, wherein said light shielding means further includes at least one continuous ridge projecting below said frame to block and prevent stray light from coupling between filter members.

5. A filter assembly according to Claim 4, wherein said filter assembly includes a pre-filter mounted intermediate the light source and said filter means and allowing the passage of light and the first wavelength while substantially blocking the passage of light of the second wavelength.

CLAIMS

New claims or amendments to claims filed on 21 Dec 1984

Superseded claims All

New or amended claims:—

1. A filter assembly comprising a frame having a pair of test filter members and a pair of reference filter members mounted therein, members of the test filter pair lying on a pitch circle 180° apart, one member providing for the passage of light of a first wavelength for excitation of a test solution while substantially blocking the passage of light of a second wavelength and the other member providing for the passage of fluorescent light of the second wavelength while substantially blocking passage of light of the first wavelength, members of the reference filter pair being located on the same pitch circle and also 180° apart but on a different diametral line from the members of the test filter pair so as to alternate in circumferential succession with the members of the test filter pair, each member of the reference filter pair providing for the passage of light of the first wavelength while substantially blocking the passage of light of the second wavelength, and light shielding means for preventing the coupling of stray light between said filter members.

2. A filter assembly according to Claim 1, wherein said light shielding means includes walled compartment means forming a respective walled compartment for each of said filter members to block and prevent stray light from coupling between filter members.

3. A filter assembly according to Claim 2, wherein said walled compartment means includes walls continuous with one another extending from said frame and respectively surrounding each of said filter members.

4. A filter assembly according to Claim 2 or Claim 3, wherein said light shielding means further includes at least one continuous range projecting from said frame at the side thereof remote from said walled compartment means to block and prevent stray light from coupling between filter members.

5. A filter assembly according to any one of the preceding claims, wherein said filter assembly includes a pre-filter mounted intermediate the light source and said filter members and allowing the passage of light of the first wavelength while substantially blocking the passage of light of the second wavelength.

Printed in the United Kingdom for Her Majesty's Stationery Office, Dd 8818935, 1985, 4235.
Published at The Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.