Fluorescence Polarization Immunoassay II. Analyzer for Rapid, Precise Measurement of Fluorescence Polarization with Use of Disposable Cuvettes

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This instrument, developed for quantitating fluorescence polarization immunoassays, automatically measures both polarization components and computes a polarization value corrected for background and optical bias. An electronically switched liquid crystal provides a non-mechanical means of rotating the plane of polarization in the excitation optics. The electronic design features digital integration and microprocessor controlled functions. Readings are made in disposable 12 × 75 mm round culture tubes, in less than 10 s. Results are precise to 0.001 polarization unit, linear from 10^-7 to 10^-10 mol of fluorescein per liter, and correlate well with those obtained with other "high-performance" instrumentation.

Clinical applications of fluorescence polarization immunoassays have been encumbered by the lack of simple, low-cost, fast, and reliable instrumentation (1). Drawbacks of previously available fluorescence polarization instruments include their lack of automated data reduction, the necessity to use precision cuvettes, the cumbersome and unreliable manual rotation of polarizers, and the use of high-power light sources. One attempt to overcome these problems has been reported (2). Most systems described in the literature and available commercially have been designed for research applications rather than for the clinical laboratory, where ease of use, low cost, reliability, and speed are essential.

A bench-top analyzer has been developed to meet these requirements and bring fluorescence polarization immunoassays to the clinical laboratory. This FPA (fluorescence polarization analyzer) features digital output, hard-copy printout, an electro-optical system designed to read samples in disposable culture tubes, automated data reduction, and an electronically switched liquid crystal that provides a non-mechanical and automatic means of rotating the plane of polarization of the excitation light.

Materials and Methods

Specialized Apparatus: General Description

The analyzer consists of a filter fluorometer, a data processor, a control panel, and a printer. The filter fluorometer utilizes a 50-W tungsten/halogen lamp, interference filters in the excitation and emission optics, and a photomultiplier tube. The interference filters have been selected for use with fluorescein; other filters may be used with minimal effort in changing over. The plane of polarization is automatically rotated with a liquid crystal. Samples are inserted manually into the test tube well, and a slide cover is used to shield the fluorometer from ambient light. A microprocessor controls all system functions and data processing. It executes programs stored in permanent memories, which provide several modes of operation. The hard-copy printer provides a record of test results. The control panel, filter fluorometer, data processor, and printer are integrated into a portable benchtop instrument that is 28 cm high, 48 cm wide, and 40 cm deep.

The degree of polarization of a fluorescent sample is determined from the equation (3):

\[
P = \frac{(I_{VH} - I_{HV})}{(I_{VH} + I_{HV})}
\]

where \(I_{VH}\) is the photomultiplier signal when vertically polarized light excites the sample and the vertical component of the emitted light is analyzed, and \(I_{HV}\) is the photomultiplier signal when horizontally polarized light excites the sample and the vertical component of the emitted light is analyzed. Thus, by sequentially exciting the sample with vertically and horizontally polarized light and by analyzing the vertical component of the emitted light, the polarization of the fluorescent sample is evaluated.

Options in the software of the microprocessor allow the operator to (a) monitor the output of the tungsten-halogen light source; (b) operate in the ratio mode by dividing the fluorescent signal by the signal from the light source, thus compensating for variations in the output of the light source; (c) automatically set the gain of the photomultiplier tube by using a representative sample from the fluorescent samples to be measured; (d) manually set the gain of the photomultiplier tube; and (e) perform blank subtraction to compensate for interference from severely icteric samples. With the flexibility designed into the software, the fluorescence polarization analyzer is well suited for both a research tool and a clinical laboratory instrument.

The FPA requires 2 mL of sample, contained in a 12 × 75 mm glass culture tube. The tubes we used in the FPA were borosilicate disposable culture tubes manufactured by Kimble or Corning and obtained from American Scientific Products. Each sample-containing culture tube is manually inserted into the FPA's sample well, the slide cover is closed, and a reading of fluorescence polarization is automatically performed and printed in 10 s. The optics and electronics that were designed and assembled to perform the rapid and precise automatic measurement of fluorescence polarization are discussed in detail below.

Optics

Figure 1 is a diagram of the optical system for the FPA. The filament of a General Electric EFM 50-W tungsten/halogen projector lamp is focused onto the entrance aperture of the system (diameter, 3 mm). After passing through a BG-38 heat absorbing glass (Corion Corp., Holliston, MA 01746), the light is collimated by a lens. All lenses in the optics of the FPA are Melles Griot plano-convex lenses, focal length 18 mm and diameter 15 mm. The collimated light then passes through a narrow-bandwidth excitation filter (485-nm center wavelength, 10-nm bandwidth; Corion Corp.) corresponding closely to the absorption peak of fluorescein. Light reflected from the transparent glass beam splitter (Corning cover glass no. 1) is focused onto a UV-215B reference silicon detector (EG&G, Inc., Salem, MA 01970). The reference detector signal is used...
The use of a polarizer/liquid crystal combination to rotate the plane of polarization is unique in fluorescence polarization instrumentation. It is used to excite the sample with vertical and horizontal light by simply applying an electric field (voltage) to the liquid crystal (4, 5). The liquid crystal is located between a fixed horizontal polarizer and the fluorescent sample. When no voltage is applied, the liquid crystal rotates the light impinging on it by 90°, and thus the sample is excited by vertical light. When a voltage is applied to the liquid crystal, no rotation occurs, and horizontal light excites the sample.

The polarization bias of the total optical system is confined to the light path between the polarizer in the excitation optics and the polarizer in the emission optics and may be compensated for by calculating polarization with use of the correction factor (6), K:

\[ P = \frac{I_{Vv} - I_{Hv}(K)}{I_{Vv} + I_{Hv}(K)} = \frac{I_{VH}}{I_{HH}} \]

where \( I_{VH} \) is the photomultiplier signal when vertically polarized light excites the sample and the horizontal component of the emitted light is analyzed and \( I_{HH} \) is the photomultiplier signal when horizontally polarized light is analyzed.

Because the polarization bias is relatively small (\( K = 0.98 \)), \( K \) is routinely assumed to equal 1, as is the case for polarization data from the FPA presented in this paper.

**Electronics**

Analog and digital electronics are outlined in Figure 2. The functional organization of the fluorometer was derived from an existing system, Abbott Laboratories’ QUANTUM I dual-wavelength filter photometer.

The tungsten/halogen lamp is driven by a switched-mode power supply, and the beam’s intensity is monitored by a sil-
sion photovoltaic reference detector after the beam is filtered. The beam is then polarized and the plane of polarization is switched by a liquid crystal responsive to the phase relationship of a two-phase drive.

Measurements are automatically initiated by sensors in the sample chamber; one senses that a test tube is completely inserted in the chamber and the other that the test tube cover is closed. The emission fluorescence intensity is measured by a Hamamatsu R928 photomultiplier tube (PMT) whose gain is controlled by a Model PMT-20 A/N high-voltage power supply (Bertran Associates, Inc., Syosset, NY 11791).

The data-acquisition scheme measures both the excitation beam intensity and the polarized fluorescence intensity. The vertical and horizontal fluorescence intensity signals are amplified on the Analog Circuit Board and converted to a pulse train by a voltage-to-frequency converter. This frequency is then measured by a counter-timer, which uses the system clock as a reference. Any intensity value measured by this scheme is given as the ratio of the frequency-channel counts to the reference clock-channel counts. The polarized fluorescence intensity may be determined by a single acquisition, as described above, or may be digitally filtered by performing multiple acquisitions for a specified integration time at regular intervals. Additionally, concurrent acquisitions of the excitation beam intensity may be performed, allowing normalization of lamp flicker. The measurement options are presented to the software via appropriate entry from the keyboard. Also, reports are generated by the particular mode that is being run.

Results

Precision

Fluorescein was diluted in buffer (0.1 mol/L sodium phosphate, pH 7.5, containing 0.1 g of bovine gamma globulin and 0.1 g of sodium azide per liter) to the following concentrations: $1 \times 10^{-7}$, $1 \times 10^{-8}$, $1 \times 10^{-9}$, and $1 \times 10^{-10}$ mol/L. Reproducibility of the instrument was determined by reading 20 sam-

Polarization of fluorescein as a function of concentration

Squares: not blank subtracted; $\times$: data corrected by blank subtraction

Table 1. Precision of Fluorescence Polarization Analyzer

<table>
<thead>
<tr>
<th>Conc. of fluorescein in buffer, mol/L</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-7}$</td>
<td>0.0070</td>
<td>0.00029</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>0.0073</td>
<td>0.00018</td>
</tr>
<tr>
<td>$10^{-9}$</td>
<td>0.0076</td>
<td>0.00045</td>
</tr>
<tr>
<td>$10^{-10}$</td>
<td>0.0067</td>
<td>0.00084</td>
</tr>
</tbody>
</table>

n = 20 at each concentration.

Sensitivity

Sensitivity is defined as that concentration of fluorescein which can be distinguished from buffer with 95% confidence. The concentration of fluorescein for which the total intensity ($I_{vis} + 2I_{Hv}$) is two standard deviations from the mean of the total intensity of the buffer is the lowest concentration detectable. The standard deviation and mean of the total intensities for five replicates of buffer were determined. Using replicates of fluorescein diluted in buffer in concentrations varying from $10^{-9}$ to $10^{-12}$ mol/L, we generated a curve of total intensity vs concentration and determined the lowest detectable concentration or sensitivity to be $0.5 \times 10^{-11}$ mol/L.

Linearity

We determined the linearity of the fluorescence polarization analyzer by measuring the polarization of fluorescence at the following fluorescein dilutions: $10^{-7}$, $0.5 \times 10^{-7}$, $10^{-8}$, $0.5 \times 10^{-8}$, $10^{-9}$, $0.5 \times 10^{-9}$, and $10^{-10}$ mol/L. Fluorescein was chosen because of its low polarization; the values will be more affected by stray light than with higher-polarization fluorophores. Figure 3 shows the polarization of fluorescein as a function of concentration. The squares depict data that were not corrected by blank subtraction. Dependency of polarization on concentration in the uncorrected data is evident and is due to stray light and scattered light, which effectively increase the observed polarization value. With use of uncorrected data, the fluorescence polarization analyzer is linear to $10^{-9}$ mol of fluorescein per liter. Data corrected by blank subtraction, with a sample of buffer as the blank, are shown. Blank subtraction effectively linearizes the data at all measured concentrations. The average blank subtracted or corrected value for the polarization of fluorescein dissolved in buffer and measured at room temperature was approximately 0.007, which disagrees with the published value (7) of approximately 0.02. This apparent discrepancy is ascribable to the small polarization bias in the instrument, which can be compensated for by using the polarization correction factor, $K$, which equals 0.98, giving a corrected value of 0.017. Because the analyzer was designed primarily as a read-out device for fluorescence polarization immunoassays, such polarization corrections are not used routinely, because all samples in an experiment contain the same tracer concentration, usually
about 10⁻⁹ mol/L, and only relative polarization values are required.

Correlation with Reference Instrument

The FPA was compared with a commercially available fluorescence polarization instrument (Model MPF-43A; Perkin-Elmer Corp., Norwalk, CT 06856) for gentamicin immunoassay (1), using 24 samples containing various concentrations of gentamicin and using both instruments as read-out devices (Figure 4). Various concentrations of gentamicin were used to obtain a range of polarization values at the same fluorescein concentration. Linear regression analysis of the data indicated that results from the instruments correlated well. A slope of 0.947, y-intercept of 0.002, and correlation coefficient of 0.994 were calculated, with use of a least-squares fit to a straight line.

Discussion

Applications of fluorescence polarization have been hampered in the past by complexity and cost of the requisite instrumentation. The development of a simple, reliable, and high-performance fluorescence polarization analyzer has made research in fluorescence polarization immunoassays in our laboratories routine and the use of these assays in the clinical laboratory practical. Advances embodied in this analyzer include: (a) polarization readings are automatically obtained in real time in 10 s; (b) the incident plane of polarization is automatically rotated by an electronically switched device, the liquid crystal; (c) a microprocessor is used to calculate polarization values and control the operation of the instrument, and (d) samples are read in disposable culture tubes.

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References