Automation of the Indirect Fluorescent-Antibody Test for Toxoplasmosis

G. I. KAUFMAN, J. S. REMINGTON, AND H. C. WATERS

The Perkin-Elmer Corporation, Norwalk, Connecticut 06852, and Stanford University School of Medicine, Palo Alto, California 94301

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The feasibility of automating the decision-making tasks of the medical technician in the determination of the results of the indirect fluorescent-antibody test for toxoplasmosis was investigated. Two approaches were studied: (i) macroscopic measurement of fluorescence from a large number of organisms (full-field illumination measurements), and (ii) microscopic measurements of fluorescence and morphology of individual parasites (pattern recognition). The macroscopic approach was studied utilizing an argon-ion laser in incident illumination with a Leitz Ortholux microscope and a phototube mounted so as to measure green fluorescence. Due to similar amounts of fluorescence from negative polar reactions and positive rim reactions, the macroscopic technique was concluded to be not feasible. The microscopic approach was studied utilizing a high resolution optical pattern recognition instrument. The results indicate that an object area measurement is sufficient to distinguish the presence of Toxoplasma gondii organisms from debris and overlapping organisms when studying the red fluorescence (due to Evans blue counterstain). Dark reactions were determined by the absence of green fluorescence. The differentiation of the rim and polar reactions was determined by the measurement of the ratio of green fluorescing area (due to fluorescein isothiocyanate) to red fluorescing area on the same organism. Clinically important titer information was also demonstrated to be obtainable. It was concluded that complete automation of the indirect fluorescent antibody test for toxoplasmosis is feasible with existing technology.

In 1966, Walton, Benchoff, and Brooks (13) described an adaption of the conventional indirect fluorescent-antibody method (IFA) to the diagnosis of toxoplasmosis. This method has been adopted on a world-wide basis and is presently available through state laboratories in the United States. In 1966, a modification (8, 9) of the conventional method was described for the express measurement of immunoglobulin M (IgM) antibody for diagnosis of acute congenital and acquired toxoplasmosis. This latter method has proven valuable for diagnosing congenital toxoplasmosis and a number of other infections which are often difficult to diagnose in the newborn, e.g., cytomegalovirus, toxoplasma, rubella, herpes simplex, syphilis.

What is now needed is a method whereby all newborns can be screened for congenital infections which are major causes of mental and motor retardation, epilepsy, blindness, etc., in the United States as well as the rest of the world. The world-wide experience with the IFA method and its usefulness in recognition of congenital infections in the newborn (1, 2, 4, 10) served as an impetus to our work on automation of this method. The feasibility of automating the chemical preparation of the IFA procedure has been demonstrated (3). However, reading slide preparations is still left to the trained observer. It was our purpose to demonstrate the feasibility of replacing the human observer by automated instrumentation in the toxoplasma IFA test.

MATERIALS AND METHODS

Toxoplasma IFA test methodology. The indirect fluorescent antibody test as applied to toxoplasma is a two-step procedure (13). In the first step, adherent trophozoites on a slide are allowed to react with human serum. In the second step, fluorescein isothiocyanate (FITC)-conjugated antihuman globulin and Evans blue counterstain are allowed to react with the product of the first step. If toxoplasma antibodies are present in the serum, fluorescein-labeled organisms counterstained with Evans blue will be the end result. If no toxoplasma antibodies are present, only Evans blue counterstaining will occur. Under the conditions
of the test method, the FITC fluoresces primarily green and the Evans blue primarily red under ultraviolet (UV) excitation. To obtain quantitative information regarding the titer of antibodies in a particular patient's sera, a series of fourfold dilutions from 1:16 to 1:16,384 were made. For the purpose of this study, any dilution of serum where more than 50% of the organisms exhibited fluorescing rims was considered positive. Figure 1 illustrates the three types of reactions which are observed in the conventional test. The positive rim reaction (Fig. 1A) is one in which the rims of the organism fluoresce brightly with a green fluorescence while the body of the organism fluoresces red. A negative polar reaction (Fig. 1B) is one in which one of the poles of the organism fluoresces brightly with a green fluorescence. Polar reactions (12) occur in approximately 30% of seronegative individuals. Here again, the body of the organism fluoresces red. The negative dark reaction is one in which the rims of the organism display no green fluorescence and the body of the organism fluoresces red (Fig. 1C). The role of the technician is to: (i) identify the presence of T. gondii parasites on the slide and (ii) to identify the type of reaction as polar, rim, or dark.

Our sample preparation procedure consisted of the following. Parallel sets of slides were prepared at the same time in Palo Alto using the standard procedure (9). One set was shipped the same day in dry ice to the Perkin-Elmer Laboratory in Norwalk, Conn. The other set was read in Palo Alto before and after freezing. Slides were defrosted 1 h before use. (Reproducibility studies showed that no sample degradation occurred for periods of up to 3 days in shipment.) The fluorescein-conjugated antiserum to whole human Ig (Lot #F3-14-67) was purchased from Hyland Laboratories, Costa Mesa, Calif.

Basic approaches to automation of the slide reading of the IFA-T. gondii test. We studied two basic approaches in our efforts. These approaches are discussed below, first conceptually and then as to their experimental design.

One approach is that of full-field illumination. In this procedure measurements are performed of the total fluorescence from a macroscopic area of the slide which contains a large number of organisms. Figure 2 outlines how an automated instrument would operate. The underlying assumptions for this approach to be successful are: (i) in a positive test, organisms fluoresce more than in a negative test; (ii) total background fluorescence will be low when compared to total positive test fluorescence; (iii) negative-dye test polar reaction fluorescence can be eliminated or is sufficiently reduced so that it does not constitute a false positive result; (iv) antigen and antiserum can be reproducibly and uniformly fixed on a selected area; (v) reagents can be standardized.

There is a more general approach to this problem which would be valid for all IFA tests. This is the pattern recognition approach which is our second approach. In this procedure each individual fluorescing object in the microscopic field is studied and identified by image processing. Figure 3 outlines how an automated instrument would operate. In the case of T. gondii the image processing would be such as to try and distinguish negative polar reactions from

![Fig. 1. Typical toxoplasma indirect-fluorescent antibody test. Results as viewed without fluorescent filters. A, Positive rim reaction; B, negative polar reaction; C, negative dark reaction.](image-url)
Gondii organism (20 times.

The measurement of fluorescence was standardized. A Keithly millimicrovolt 620B voltmeter which served both as amplifier and signal indicator, and a Perkin-Elmer Model 165 strip-chart recorder. The phototube was mounted on the monocular and operated at 1,800 V with a terminating 500 kΩ load resistor. An argon-ion laser operated at 488 nm was used for the illumination source. A more detailed description of this instrument and the advantages of argon-ion laser illumination have been discussed in a previous article (7).

In order to simulate the two-channel system as described in Fig. 2 the same field was measured twice, once with a green filter and once with a red filter (Fig. 4).

The procedure of obtaining data was as follows. The power density of the incident laser beam on the slide was measured using a calibrated photocell and adjusted to 80 W/cm². This power density was chosen due to its ability to generate sufficient fluorescence for easy photoelectric detection. A neutral density filter was placed in the path of the laser beam which reduced the power density to 8 W/cm². Utilizing this lower power level, individual organisms were centered in the field with little prefading (less than 5%). The neutral density filter was then removed and the initial fluorescence of the organism was measured. Ten different organisms were studied from different positions of each serum dilution tested. The background fluorescence from each serum dilution was obtained by measuring the fluorescence from an empty field (no organisms). The fluorescence values discussed below are the net difference between the fluorescence signal from an organism and the background signal.

Flying spot scanner optical computer instrument. An electromechanical flying spot scanner which scanned in two dimensions was constructed. It

Positive rim reactions. The underlying assumptions for this approach to be successful are: (i) the green fluorescence from rim and polar reactions is much greater than the green fluorescence from negative dark reactions; (ii) background fluorescence is low compared to positive test organism fluorescence; (iii) reagents can be standardized.

Optical instrumentation. The following two instruments were used during this study to determine the feasibility of the procedure described in the full field illumination and pattern recognition approaches.

Full-field microfluorimeter. An experimental microfluorimeter was developed which is suitable for measurements of fluorescence from individual FITC-tagged T. gondii organisms. The instrument was basically a Leitz Ortholux microscope with a Leitz Ploem illuminator. A 40 × 0.85 NA fluorite dry objective and 10 × ocular combination was used at all times. Between the illumination source and the dichroic beam splitter of the Ploem illuminator was placed an aperture stop which reduced the size of the field being illuminated to 25 μm. This was chosen so as to be greater than the largest dimension of a T. gondii organism (20 μm). The fluorescence detection system was composed of a fluorescence filter in front of an RCA no. 7265 photomultiplier tube with an S-20 photocathode response, a Keithly millimicrovolt 620B voltmeter which served both as amplifier and signal indicator, and a Perkin-Elmer Model 165 strip-chart recorder. The phototube was mounted on the monocular and operated at 1,800 V with a terminating 500 kΩ load resistor. An argon-ion laser operated at 488 nm was used for the illumination source. A more detailed description of this instrument and the advantages of argon-ion laser illumination have been discussed in a previous article (7).

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Flying spot scanner optical computer instrument. An electromechanical flying spot scanner which scanned in two dimensions was constructed. It
utilized an argon-ion laser which was operated at 3 mW output at 488 nm. The optical path of the laser beam is displayed in Fig. 5 which is a schematic diagram of the instrument where, for clarity, the action of the scanning mirror is shown in only one plane.

The instrument included a modified Vickers microscope model M15 which was operated in the incident illumination mode. A 100×1.30NA oil immersion objective focused the beam to a diffraction limited spot of 0.25 μm and power density of 100 W/cm² on the specimen.

The horizontal scanning motion was generated by a vibrating mirror galvanometer, and the vertical motion was generated by tilting the galvanometer. The area of each scanned image was a field 16 by 16 μm. The instrument has been described in detail by Slomba et al. (11).

The fluorescence signal from the sample passes through the dichroic filter (10× ocular), fluorescence filter, and pin hole prior to impinging on the RCA no. 7265 S-20 photomultiplier tube which is mounted on the monocular.

The signal information is then amplified using Tektronix types D and L plug-in amplifiers operating in series. The video information, in analogue form, is transmitted with the addition of suitably generated synchronized timing pulses for analysis to a Varian 620 digital computer. The computer divides the analogue signal into 32 grey levels and stores it in an image array of 64 by 64 points for further processing. The phototube voltage is chosen so that the largest fluorescent signal corresponds to the 32nd grey level whenever this was less than 2,200 V. If this condition was not met the voltage was set to 2,200 V. This procedure was chosen so as to simulate an automatic gain control which would be necessary on an automated machine due to variations in fluorescence between reactions.

In order to simulate the two channel system as described in Fig. 3 a single channel was used where the same field was scanned twice, once with a green filter and once with a red filter (Fig. 4). The data obtained were stored on magnetic tape so that processing of the images could be done at leisure. Details of the processing techniques used have been discussed elsewhere (5).

**RESULTS**

**Full-field illumination system.** The crucial problem in this procedure is to verify that there is a measureable difference in green fluorescence from negative polar and positive rim reactions. Our approach was to measure the net green fluorescence from individual organisms from different reactions and titers.

A serum dilution of 1:16 was chosen and sera from 63 individuals were tested, 33 with known positive dye-test titers ranging from 1:8 to 1:16,000,10 negative in the dye test but with polar reactions in the IFA test, and 20 negative in the dye test with no polar stain. The results are shown in Fig. 6. It appears that the fluorescence signal was greater with higher titered sera when screened at a single dilution of 1:16 and a fixed irradiation intensity. The reason for the apparent lack of response in the sera with a titer of 1:256 when compared with lower titered sera is unclear and was not studied further. Essentially, the method was able to differentiate between antibody-positive and antibody-negative sera, with the exception of those sera which were negative in the dye test and the IFA test, but which had a polar reaction. The presence of polar staining per se will give a reading using the full-field illumination method which cannot be differentiated from results obtained with sera-containing antibody.

![Diagram of flying-spot laser scanner system.](image)

**Fig. 5.** Diagram of flying-spot laser scanner system.

![Average single organism fluorescence vs. titer at serum dilution of 1:16.](image)

**Fig. 6.** Average single organism fluorescence vs. titer at serum dilution of 1:16.
Flying spot scanner computer system: Organism location. Based on the proposed procedure as described in Fig. 3, the first step was that of locating and identifying the presence of toxoplasma in scanned fields. The identification of the presence of organisms prior to determining the type of reaction was necessary to distinguish negative-dark reaction cases from the empty field situation. To simulate this procedure the red filter was used to observe the fluorescence from the Evans blue counterstain. The fluorescence information from a scanned field was organized in the form of histograms where the range of grey scale values was plotted against the frequency of occurrence. In Fig. 7 are typical histograms from representative fields. In the case where no organisms were present there was only one peak representing electronic noise and nonspecific fluorescence. When organisms were present in the case of rim, polar, or dark reactions there were two peaks present in the histogram consisting of a distinct fluorescent information peak (which is due to the Evans blue counterstain) and the background peak. By displaying only those points in the histogram which are in the information peak (all grey levels brighter than the valley between the two peaks) as ones on a synchronous scan phototube display, binary images of the fluorescing objects were obtained. Each of the objects in the binary images were separately located and their areas were computed. If these values were within preset limits for recognition of whole organisms, we identified the object as a toxoplasma organism. Figure 8 describes the results of such a test on 60 organisms per reaction from the 3 different reaction types, polar, rim, and dark, and 207 other objects consisting of debris and overlapping organisms. The false negative results are due to our choosing the maximum area of the fluorescing material identified as T. gondii organisms to be less than some large individual organisms which are approximately twice as large as the smallest T. gondii organisms. This choice eliminates overlapping small organisms from being called individual organisms. The false negative results were distributed as 6 each in the rim and dark reaction groups and 9 in the polar reaction group.

**Determination of reaction type.** In order to determine the type of reaction, histograms were obtained using a green filter. Representative

![Typical histograms and images using red filter channel.](image)

![Results of utilizing area measurements for finding toxoplasma. Positive identification criterion for single organism, S(μm)² ≤ A ≤ 17(μm)². A, Area of organism.](image)
histograms and images of individual organisms from the three different types of reactions are shown in Fig. 9.

In the case of the rim reaction, it was possible to choose a threshold that actually displayed the organisms as rims (a portion of the fluorescence information peak) but this is a very sensitive choice not needed for distinguishing rim from polar reactions. By going lower in threshold (the total fluorescence information peak) we obtained full organisms instead of rims, and polar images remained polar. If no information peak was present, the reaction was termed dark reaction. If a fluorescence peak was present, the rim reaction was distinguished from the polar reaction by use of the morphologic differences; namely, that organisms with rim reaction have a much larger percentage of their area exhibiting green fluorescence than do organisms with polar reactions. An area ratio test was performed comparing the organism area of the green filtered binary image to that of the red filtered binary image. The results of using this test on 45 polar, 48 rim, and 45 dark reaction organisms are shown in the Fig. 10. If the area ratio was 0.7 to 1.2 the reaction was termed positive rim, and in all cases the rim reaction was correctly distinguished from negative and polar reactions. If the area ratio was between 0.1 and 0.3 the reaction was termed polar, and in all cases the polar reaction was correctly distinguished from negative and rim reactions.

**Titer information.** In a blind study between our two laboratories, there was agreement on titters of 1:64, 1:256, and 1:1,024. In order for a field to be considered a positive reaction, at least 50% of 20 organisms identified as toxoplasma had to be identified as exhibiting rim reactions and the green fluorescence had to cause saturation at the maximum phototube voltage. A maximum voltage of 2,200 V was allowed on the photomultiplier tube, but lower amounts were utilized for brighter reactions. The photomultiplier tube voltage was adjusted so that the maximum fluorescence signal reached the maximum grey scale. This simulated an automatic gain control.

**DISCUSSION**

The results described above form the basis for the proposal that an automated pattern recognition instrument capable of replacing the human observer in determining the titer and type of reaction of a toxoplasma IFA test is feasible.

The instrument we envisage as capable of performing this task consists of an automated microscope capable of taking a slide from a cassette, centering it, automatically focusing it, and then scanning the field of interest at a magnification of ×1000 utilizing an ×100 oil objective lens and a 488-nm laser source. The fluorescence is then brought to a beam splitter which divides the fluorescence equally between two channels. One channel leads to a phototube with a filter which passes green fluorescence
and the other to a phototube which passes red fluorescence. Two different images are thus obtained and stored in the computer for processing. The field scanned would be 32 by 32 µm in order to cover more area and the sampling field would be 128 by 128 µm. This would allow one to obtain the most information from a diffraction limited spot of 0.25 µm. The images are then processed as outlined in the above work. After a suitable number of organisms have been identified, the instrument would then shift to the next dilution and repeat the process. Autofocus and automatic stage motion have been used routinely in the Perkin-Elmer laboratory for many years and the instrument is well within technological capability. Such an instrument could process 60 specimens per h on a one-dilution-per-specimen basis.

It may be possible to operate at reduced resolution (0.5 µm) in the same manner as described above since high resolution morphology was never needed. This implies that a lower power dry objective might be used which is a convenient feature from the point of view of: (i) more area being searched per field, (ii) eliminating oil, (iii) increasing the depth of focus.

One of the main attractions of the pattern recognition technique is that other fluorescent antibody test results could be determined by using different fluorescent filters and software programs (i.e., fluorescent treponemal antibody-absorption test (6)). Preliminary results indicate that the same procedure used in this study can be used for determining the IgM-IFA test for toxoplasmosis and here a single dilution is all that is needed for a screening test for pregnant women or newborn infants.

At present the full field fluorescence approach does not appear feasible due to its confusion of negative polar and positive rim reactions causing a large number of false positives. If a chemical approach capable of blocking polar reactions is developed, this approach may become tractable.

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