Use of Tunable, Pulsed Dye Laser for Quantitative Fluorescence in Syphilis Serology (FTA-ABS Test)

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A pulsed dye laser was used as an excitation source in a fluorescent treponemal antibody absorption (FTA-ABS) test. A high precision in quantitative fluorescence was obtained with this high-power excitation source coupled to an electronic detection system and a storage oscilloscope by standardization of fluorescence evaluation and through elimination of human error. One 0.4-μs pulse exposure was sufficient to record fluorescence intensity data on the oscilloscope. Absence of fading of fluorescence after repeated excitation permitted multiple readings of the same microscope field. Almost 100% reproducible results were obtained for the FTA-ABS test with 40 samples. Electronic detection of fluorescence and the high sensitivity obtained with laser excitation raise doubts about the relative value of quantitative immunofluorescence in the FTA-ABS test.

Detection of antibodies by immunofluorescence techniques is being employed increasingly in diagnostic microbiology since its discovery by Coons et al. (3). The fluorescence emitted by most bacterial cells when excited by xenon or mercury arc lamps is so low that long periods of exposure are necessary for adequate fluorescence monitoring. This long exposure to light in the ultraviolet (UV) region photodecomposes the conjugated dye into free dye, causing fluorescence fading. Also, it is extremely difficult to quantitate precisely the intensity of fluorescence by human eye particularly when the interpretation is influenced by the presence of nonspecific or autofluorescence, leading to a reduction in the signal-to-noise ratio.

In the fluorescent treponemal antibody absorption (FTA-ABS) technique described by Deacon et al. (4), the patient's serum is graded for the presence of syphilitic antibodies by the visual, microscope quantification of the intensity of fluorescence produced by the conjugated fluorescein bound to Treponema pallidum cells.

Mansberg and Kusnetz (11), by using flying spot scanning techniques, observed that the signal-to-noise ratio in fluorescent dye preparations could be increased by employing certain excitation sources and optical absorption filters, and proposed the use of a continuous-wave (CW) laser because of its stability, monochromaticity, and high power output.

Numerous lasers are now available which produce different power levels and wavelengths corresponding to the exact need of a researcher. The continuous lasers are of low power and fixed-frequency output. On the other hand, the pulsed laser's output is of short duration and therefore delivers a high peak of power per pulse. The possibility of multiple frequency selection with a single dye laser source was first reported by Sorokin and Lankard (13). Different organic dyes can be used as lasing materials to obtain different wavelengths by rotating a grating inside these so-called tunable lasers.

Kaufman et al. (10) used fixed-wavelength CW argon and He-Cd lasers to excite fluorescein isothiocyanate (FITC)-stained Escherichia coli and found that the intensity of fluorescence was at least 100 times stronger than the one produced by the routine arc lamps. Kaufman and Nester (9) used the same continuous argon laser and a scanner to identify the treponemes in the FTA-ABS test. Bergquist (1) used a Ploem illuminator (12) and a pulsed dye laser to excite FITC-stained, polymerized human immunoglobulin G microspheres of 5- to 50-μm diameter.

The treponemes employed in the FTA-ABS test are very thin spirochetes varying from 6 to 14 μm in length and 0.25 to 0.30 μm in width. The present study was undertaken to investigate the possible advantages of using a tunable, pulsed dye laser in a substage illumination.

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system coupled to electronic fluorescence recording equipment for precise qualitative and quantitative interpretation of the FTA-ABS test.

MATERIALS AND METHODS

Antigen. Redi-fix slides (Aerojet General Corp., El Monte, Calif.; lot no. 053471) containing methanol-prefixed Treponema pallidum cells were used for the FTA-ABS test as described by Deacon et al. (4). The slides were preserved at -20°C in the dark until required for use.

Specimens. Twenty-five known control proficiency testing sera furnished by the Center for Disease Control (CDC), Atlanta, Ga., were received through the Laboratory Center for Disease Control, Ottawa, Canada. In addition, nonspecific control (Aerojet, lot no. 30271), reactive 1+ and 4+ controls (Aerojet, lot no. 10571), and sorbent controls (Aerojet, lot no. 71410) were also included in the study. A limited number of unknown sera received in the laboratory were also evaluated for the presence of syphilitic antibodies.

Conjugate. FITC-conjugated rabbit antihuman gamma globulins (Sylvania Co., Willburn, N.J.; lot no. 091472-AL) were used at a titer of 1:1,000.

Equipment. The equipment (Fig. 1) consisted of optical, excitation, and detection systems. A trinocular Leitz microscope M.P.V. 2 (variable photomultiplier microscope) with incident and transmitted illumination facilities was used. Routine observations were done with a planachromat objective (40×); numerical aperture, 0.65) and 10× oculars using a dry, darkfield condenser only in substage illumination. A halogen lamp (Sylvania, 100 W, FCR) was used in substage illumination for specimen location and focusing. The excitation sources used in the present study were a UV bulb (Osram G.M.B.H., Berlin, Germany; HBO 100) in incident illumination (2, 8) and a pulsed dye laser (Synergetics Research, Princeton, N.J.; Chroma beam model 1070) in transmitted substage illumination. A helium-neon CW laser (Metrologic Instruments, Inc., Belmawr, N.J.; model 360) with low output was used for optical alignment of the pulsed dye laser. The detection system for energy measurement consisted of a photomultiplier, with a rise time of 10 ns, connected in parallel to a digital voltmeter (Knott, Electronic, Germany; model 166) and a storage oscilloscope (Tektronix Beaverton, Ore.; model 7313) complete with modules 7B50, 7A22, and 7A15A, with a rise time of 0.5 ns. A 35-mm camera, loaded with an Anscocrome color film (GAF, Mississauga, Ontario, Canada; ASA 500), fitted to the trinocular head of the microscope was used for photographic recording. To avoid interference from static electricity, the whole laser unit was kept in a Faraday box.

Methodology. After alignment, the emission band width of the laser beam, ranging in wavelengths from 430 to 490 nm (1, 5, 10), produced after excitation of the dye solution (75 mg of 7-diethylamino-4-methyl coumarin [Eastman Kodak, Rochester, N.Y.] per liter of ethanol) was reduced to a wavelength of 470 nm by an optical grating (Littrow-mounted 1,200 lines per

mm; blazed at 500 nm). The pulsed dye laser was operated at 23 kV and the photomultiplier was operated at 0.6 kV. The sensitivity of the oscilloscope was adjusted to 100 mV/division. Barrier filter K490 was used to absorb the laser excitation light and to permit only the passage to oculars of greenish-yellow fluorescence produced by the laser-excited objects. With the help of a special device in the trinocular head of the microscope, it was possible to direct the fluorescence from the ocular pathway to the photomultiplier or to the camera.

The processed treponemal antigen slides were focused under darkfield, substage illumination from the halogen lamp. The slides for UV incident illumination were processed and interpreted as mentioned previously (8). A green fluorescence was seen after excitation of the treponemes with each laser pulse lasting

![Diagram](image-url)
only 0.4 μs. To standardize data interpretation, fluorescence emitted by three treponemes focused within the adjustable entrance diaphragm of 4 mm² of the photomultiplier was recorded on the oscilloscope after laser excitation. A reading of the adjacent field of the same surface area without treponemes was also taken to record the nonspecific background fluorescence. The difference in total signal (treponemes plus background) and noise (background) was recorded as the specific treponemal fluorescence.

To establish accuracy, a total of six fields per slide, i.e., three with treponemes and three without treponemes, was examined. Ten pulse readings per field were recorded on the oscilloscope. Thus, the intensity of fluorescence for each slide was reported as the difference of means of 30 pulse readings with nine treponemes under focus and of 30 pulse readings of three backgrounds without treponemes.

With the least-squares method, a linear standard curve was obtained by plotting 540 treponemal fluorescence readings of nine known control sera (six from CDC and three from Aerojet) representing nonreactive, borderline and reactive 1+, 2+, 3+, and 4+ FTA-ABS tests (Fig. 2). Each time a new conjugate is used, a new standard curve must be established with known control sera; thus, the fluorescence grading becomes independent of the conjugate used.

RESULTS

Fluorescence stability. To determine the stability of fluorescence, three different processed slides, with sera rated 4+, 3+, and 1+ for the FTA-ABS test, were exposed to pulses from the laser and recorded by the oscilloscope. The same field on each slide containing treponemes and the adjacent field without treponemes were each exposed to 10 laser pulses during a period of 30 s. Treponemal and background fluorescence produced by each pulse are plotted in Fig. 3. The total time of exposure to the laser beam per field was 4 μs. An interval of 3 s between two pulses was necessary for the laser to regain the initial voltage of 23 kV after discharge. There was no loss in the intensity of fluorescence in the three specimens, despite repeated excitation of the conjugated fluorescein by the laser (Fig. 3). A good signal-to-noise ratio permitted a precise interpretation of each slide.

Reproducibility. Two slides from each of 40 unknown samples were prepared and processed for the FTA-ABS test under identical conditions. The results were fully reproducible in 87.5% of the cases, whereas the remaining 12.5% showed one-step variation in the intensity of fluorescence (Table 1). If we ignore this one-step variation due to reagents and physicochemical errors, the reproducibility was almost 100%.

Comparative study of known sera from CDC. Two slides processed for FTA-ABS test with each of the 25 known CDC control sera were examined, one with the UV incident light and the other with the transmitted, pulsed laser beam. Positivity from laser excitation was evaluated with the standard curve (Fig. 2). As shown in Table 2, 19 out of 25 sera were found reactive for FTA-ABS with laser, whereas only 17 were found reactive with UV incident light. There was a disagreement between the CDC and laser data in 2 out of 25 (8%) cases; one borderline and one nonreactive CDC sera were evaluated as nonreactive and 1+, respectively, by the laser. The same two sera were evaluated as nonreactives with the UV incident method.

A higher percentage of complete agreement between the laser and UV incident method (72%) was obtained than between the laser and CDC method (60%) (Table 3). Each method agreed with the other in 92% of the cases, including complete and partial agreements.

DISCUSSION

The inability of the human eye to evaluate precisely the low-fluorescing objects leads occasionally to erroneous quantitative grading. The reproducibility of weakly reactive or borderline data becomes extremely important in the early stages of disease when antibody titer is low. The
spheres 13 to 468 times bigger than the treponemes, might have recorded a nonspecific fluorescence with incident laser excitation, as no filters were used during photography. In our studies with substage laser excitation, better contrast and less nonspecific fluorescence were obtained with absorption filters in place while data were being recorded on an oscilloscope, digital voltmeter, or photographic color film.

One-step variations in reproducibility experiments (Table 1) could be due to fluctuations in line voltage, difference in the total surface area occupied by treponemes of various lengths, number of conjugated antibody molecules occupying the receptor sites on the respective trepo-

**Table 1. Reproducibility data of FTA-ABS tests using laser**

| Specimen no. | Slide 1 | Slide 2 |
|--------------|---------|---------|
|              | Signal–noise (mV) | Results* | Signal–noise (mV) | Results* |
| 1            | 207     | R 1−    | 205     | R 1+    |
| 2            | 150     | NR      | 185     | R B     |
| 3            | 167     | R B     | 168     | R B     |
| 4            | 312     | R 2+    | 460     | R 3+    |
| 5            | 200     | R 1−    | 218     | R 1+    |
| 6            | 503     | R 4+    | 643     | R 4+    |
| 7            | 288     | R 1,2+  | 301     | R 2+    |
| 8            | 74      | NR      | 93      | NR      |
| 9            | 249     | R 1−    | 278     | R 1,2+  |
| 10           | 182     | R B     | 213     | R 1+    |
| 11           | 255     | R 1−    | 277     | R 1,2+  |
| 12           | 241     | R 1−    | 300     | R 2+    |
| 13           | 578     | R 4−    | 680     | R 4+    |
| 14           | 264     | R 1,2+  | 325     | R 2+    |
| 15           | 88      | NR      | 108     | NR      |
| 16           | 343     | R 2+    | 300     | R 2+    |
| 17           | 525     | R 4+    | 547     | R 4+    |
| 18           | 298     | R 2−    | 343     | R 2+    |
| 19           | 235     | R 1−    | 205     | R 1+    |
| 20           | 64      | NR      | 143     | NR      |
| 21           | 117     | NR      | 140     | NR      |
| 22           | 578     | R 4−    | 680     | R 4+    |
| 23           | 264     | R 1,2+  | 325     | R 2+    |
| 24           | 88      | NR      | 108     | NR      |
| 25           | 525     | R 4+    | 548     | R 4+    |
| 26           | 589     | R 4+    | 625     | R 4+    |
| 27           | 441     | R 3−    | 467     | R 3+    |
| 28           | 681     | R 4−    | 714     | R 4+    |
| 29           | 420     | R 3−    | 455     | R 3+    |
| 30           | 521     | R 4+    | 614     | R 4+    |
| 31           | 347     | R 2+    | 327     | R 2+    |
| 32           | 460     | R 3−    | 641     | R 4+    |
| 33           | 691     | R 4−    | 666     | R 4+    |
| 34           | 558     | R 4−    | 612     | R 4+    |
| 35           | 545     | R 4−    | 732     | R 4+    |
| 36           | 585     | R 4−    | 533     | R 4+    |
| 37           | 274     | R 1,2+  | 280     | R 1,2+  |
| 38           | 336     | R 2−    | 349     | R 2+    |
| 39           | 374     | R 2,3+  | 398     | R 3+    |
| 40           | 107     | NR      | 92      | NR      |

* NR, Nonreactive; R, reactive; B, borderline.
TABLE 2. Comparative study of 25 known CDC sera with laser and UV incident systems

| Specimen no. | Laser | Oscilloscope readings (mV) | Reactivity results* (UV transmitted) | CDC results* (UV incident) | Quebec lab results* (UV incident) |
|--------------|-------|---------------------------|---------------------------------------|----------------------------|----------------------------------|
|              |       | Signal:Noise | Signal - noise | Resultsa |                         |                                  |
| 1            | 146   | 82          | 64            | 0.00     | NR                     | NR                               |
| 2            | 193   | 76          | 117           | 0.08     | NR                     | NR                               |
| 3            | 298   | 63          | 235           | 1.15     | R 1+                   | R B                              |
| 4            | 232   | 82          | 150           | 0.04     | NR                     | R B                              |
| 5            | 391   | 48          | 343           | 2.17     | R 2+                   | R 3+ R B                        |
| 6            | 344   | 46          | 298           | 1.76     | R 2+                   | R 2+ R B                        |
| 7            | 582   | 57          | 525           | 3.84     | R 4+                   | R 4+ R 1+                        |
| 8            | 678   | 89          | 589           | 4.42     | R 4+                   | R 4+ R 4+                        |
| 9            | 661   | 83          | 578           | 4.34     | R 4+                   | R 4+ R 4+                        |
| 10           | 333   | 69          | 264           | 1.43     | R 1-2+                 | R 1+ R 3+                        |
| 11           | 227   | 89          | 138           | 0.28     | NR                     | NR                               |
| 12           | 617   | 109         | 508           | 3.68     | R 4+                   | R 4+ R 4+                        |
| 13           | 363   | 50          | 313           | 1.90     | R 2+                   | R 2+ R 2+                        |
| 14           | 266   | 59          | 207           | 0.92     | R 1+                   | R 1+ R 1+                        |
| 15           | 315   | 74          | 241           | 1.24     | R 1+                   | R 4+ R 1+                        |
| 16           | 148   | 60          | 88            | 0.00     | NR                     | NR                               |
| 17           | 242   | 60          | 182           | 0.70     | R B                    | R 3+ R B                         |
| 18           | 303   | 48          | 255           | 1.35     | R 1+                   | R 4+ R B                         |
| 19           | 126   | 52          | 74            | 0.00     | NR                     | NR                               |
| 20           | 295   | 46          | 249           | 1.29     | R 1+                   | R 3+ R 1+                        |
| 21           | 325   | 57          | 268           | 1.48     | R 1-2+                 | R 3+ R 1+                        |
| 22           | 352   | 40          | 312           | 1.90     | R 2+                   | R 3+ R 2+                        |
| 23           | 542   | 39          | 503           | 3.63     | R 4+                   | R 4+ R 4+                        |
| 24           | 249   | 82          | 167           | 1.55     | R B                    | R B R B                          |
| 25           | 262   | 53          | 209           | 0.94     | R 1+                   | NR                               |
| Total reactivesa |       |               |               |          | 19 (76%)               | 19 (76%) R 17 (68%)               |

* NR, Nonreactive; R, reactive; B, borderline.

* Borderlines included as reactivates.

TABLE 3. Agreement between the data obtained for 25 known samples for FTA-ABS by three different methods

| Methods                  | Agreement |
|--------------------------|-----------|
|                          | Total     | Completea | Partiala | None    |
|                          | No.       | %        | No.       | %       | No.       | %        |
| Laser and CDC            | 23        | 92       | 15        | 60      | 8         | 32       | 2         | 8        |
| Laser and Quebec Laboratory | 23       | 92       | 18        | 72      | 5         | 20       | 2         | 8        |
| CDC and Quebec Laboratory | 23       | 92       | 13        | 52      | 10        | 40       | 2         | 8        |

* Nonreactives and reactivates with identical results.

* Reactives showing one- step (or more) variations in quantitative fluorescence.

...nemes, and variation in the intensity of the nonspecific fluorescence of the adjacent fields.

Once the stability of the laser fluorescence readings and lack of fading were ascertained, quantifying treponemal fluorescence required only two pulses per slide (one signal and one background fluorescence), making this technique much more practical.

Discrepancies between the laser, CDC, and UV incident data (Table 2) raise a question about the validity of the visual interpretation of the quantitative fluorescence and relative importance of reporting the FTA-ABS reactive in terms of different grades of intensities of fluorescence (14), especially when considering its low value in the evaluation or follow up of syphilis before or after treatment. Disagreement between the laser and CDC data could be explained by factors such as visual interpretation, transport and storage conditions of sam-
amples, reagents, etc.; nevertheless, what becomes important to know is the relative significance of borderline or weakly positive reactions during various stages of the disease and in healthy individuals. Would it be more meaningful to pool all the quantitative data and report the serum simply as reactive, or rather follow the rise in titer of the FTA-ABS-reacting antibodies with further grading of fluorescence intensities now possible with high-power, pulsed laser beams?

A better correlation in the quantitative fluorescence was obtained between the laser and UV incident illumination (Table 3) than between the laser and UV substage transmitted illumination (CDC). This may be due to the fact that the intensity of fluorescence with transmitted laser and UV incident illumination is much higher, thus rendering possible more precise evaluation than with UV substage transmitted illumination.

The coaxial flashlamps used in the pulsed dye laser are at present costly and last only for 2 ms, i.e., 5,000 pulses, although it is quite simple to modify them to triple their lifetime. In our experience, it was found feasible and economical to manufacture one's own flashlamps. Usual precautions should be taken while working with high-energy lasers (6, 7). To reduce the measurement time per sample, studies are in progress towards completely automating the fluorescent-antibody technique employed in the serodiagnosis of various diseases.

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