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Evaluation of Excitation Light Sources for Incident Immunofluorescence Microscopy

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A variety of fluorescent excitation light sources were compared using a standard fluorescein solution or a bacterial conjugate with immunofluorescent microscopy. Quantitative data were obtained with microscope photometric apparatus. Both the quantitative data and comparative conjugate titering suggest that the 450-W xenon arc excited significantly more fluorescence than did the more commonly used 250-W mercury arc or the 100-W halogen lamp. The conjugate could be diluted 4 to 32 times more using the 450-W xenon. Additional advantages of 450-W xenon excitation include sufficient energy of wavelengths between 470 to 490 mm, thus permitting narrow-band excitation resulting in less autofluorescence and the ability to perform fluorescent-antibody procedures without the darkening of ambient room light.

Recent advances (3) in immunofluorescence technology have resulted in new methods that have expanded the applications of this technique (5–7, 9, 17). One aspect has been development of new filters and their use with incident-light microscopes, permitting activation of a fluorochrome at the surface of the specimen with light of a wavelength close to the emission peak (16). Thus, autofluorescence is considerably reduced and maximum brightness of the fluorescent image is obtained. Such developments have increased the value of careful selection of the light source for fluorescent-antibody (FA) excitation.

Since the beginning of FA work, high-pressure mercury arcs (HBO200) routinely have been employed for excitation. Such mercury arcs were used because their intense spectral-line emission (see Fig. 1) in the ultraviolet region (300 to 420 nm) produced good fluorescence with a contrasting black background (2–4, 13). However, since fluorescein's greatest fluorescence occurs with excitation in the blue spectrum (460 to 500 nm), it is not surprising that blue-light excitation procedures have attracted considerable interest (4, 13, 14). Research in FA filter technology has led to the development of blue-light excitation filters relatively specific for fluorescein (14, 16, 18). With these new filters, combined with a xenon light source (Fig. 1), stronger and more specific fluorescein excitation is possible. The 450-W xenon light source (XBO450) appears to be particularly attractive but has not been tested for immunofluorescence microscopy.

Faulk and Hijmans (3) and Ploem (16) have compared several light sources for FA use, but they did not include the 450-W xenon source. The purpose of the present study was to compare the 450-W xenon source with five others for fluorescein excitation (see Table 1). Photometric measurements were done to assess fluorescent intensity and fading. In addition, the working titer of a fluorescein conjugate was determined with selected light sources to see which light source-filter combination allowed the greatest dilution.

MATERIALS AND METHODS

Microscope. A Leitz Orthoplan microscope equipped with a Leitz fluorescent incident Ploem illuminator (14, 16) was used for all experiments. A Leitz (model 500) mirror housing permitted rapid comparison of light sources as it could accommodate several lamp housings. The following objectives (Leitz) were used: P1 Apo 40/0/74 (high-dry) and P1 Apo Oil 100/1.32 (oil-immersion).

Light sources. The high-pressure mercury arcs HBO100 and HBO200, the high-pressure xenon arcs XBO75, XBO150 and XBO450 (Osram), and the halogen 12 V, 100-W halogen lamp (Philips) were evaluated (Fig. 1 and Table 1). All light sources were operated on stabilized current, with the exception of the XBO150. Direct current power supplied were used for the XBO75, HBO200, XBO450, and HBO100. The power supply for the XBO450 (Electropower model 255) was adjusted to 25 A. All light sources, except the XBO450, were mounted on the upper back aperture of the mirror housing. After centric and focusing adjustments were completed, the collector

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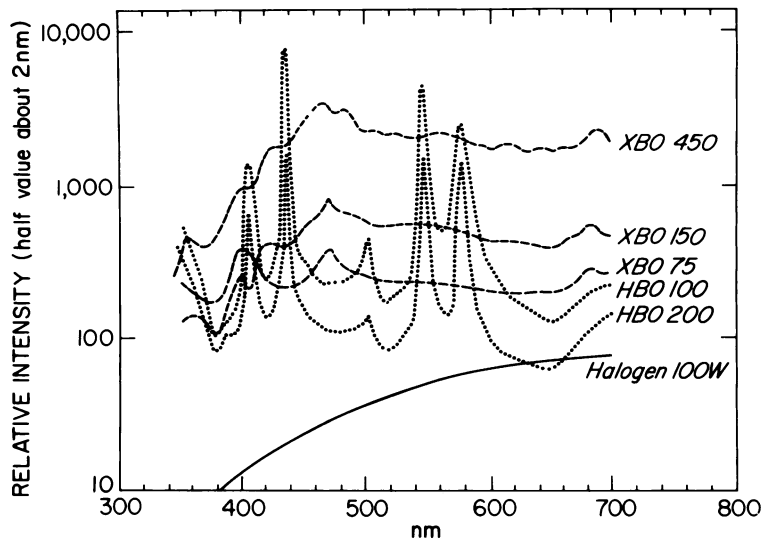

FIG. 1. *Comparison of the intensity and spectral distribution of various light sources suitable for fluorescent microscopy (10).*

TABLE 1. *Technical data on light sources suitable for fluorescent microscopy*[a]

W	Type	Light flux (lumens)	Mean luminous density (stilbs)	Life expectancy (h)	Approximate cost/h ($)	Light stability
450	XBO 450	13,000	35,000	2,000	0.11	Excellent
150	XBO 150	3,000	15,000	1,200	0.09	Excellent
75	XBO 75	1,000	40,000	400	0.40	Excellent
200	HBO 200	9,500	33,000	200	0.30	−50% decreases at 175 h (4)
100	HBO 100	2,200	170,000	200	0.34	Good (18)
100	Halogen	2,800		40	0.15	Good

[a] Adapted from data provided by the manufacturers (11). Cost of illumination per hour was estimated using life expectancy data.

lens was trimmed slightly to give maximal readings and uniform field illumination and to more fully utilize the emission from arcs with either large or small electrodes. All lamps were operated for 15 min to reach stable emission prior to any data collected. Only mercury arcs with less than 10 h of operation were employed.

Filters. All lamp housings were equipped with 4-mm, BG-38 red-absorbing filters and the following heat-absorbing filters (as recommended by the manufacturer): 2-mm KG-1 for the HBO-100, HBO-200, and the 100-W halogen lamps; Calflex B1/K2 for the XBO-75, XBO-150, and XBO-450. For excitation, a KP500 filter consisting of two Leitz KP490 short-wave pass interference filters (λ_{max} = 495, T_{max} = 90%, and 0.1% T at 525 nm) was used. For the narrow-band excitation, a $\lambda > 480$-nm filter was added to the KP500 filter. The incident illuminator contained TK510 dichroic mirror and a K515 suppression filter. Additional suppression filters studied were the K510, the K530, and the S525 fluorescein selection filter (type AL; λ_{max} = 530 nm, T_{max} = 68%; and 1 half-width = 2.1 nm).

Photomultiplier attachment. A Leitz MPV microscope photometer, an EMI photomultiplier tube with S11 cathode, a Knott type NSHM power supply, a Kipp AL3 galvanometer (with appropriate resistance), and a Beckman type RB Dynograph pen recorder were employed. The power supply and meter were permitted 15 min to reach operating stability. Full scale on both the galvanometer and the recorder pen was equal to 10^{-2} mV.

Standard fluorescein solution. The fluorescein solution was prepared from fluorescein diacetate according to the method of McKinney et al. (12). Early observations revealed that in the 1- to 2-μM concentration range (molecular weight, 416), the observed fluorescence was proportional to the concentration. Factors held constant included room temperature and concentration and pH of the fluorescein diacetate solution. Appropriate background readings were obtained with a control solution and were subtracted from the fluorescence results. The fluorescein diacetate stock solution was stored in the dark at 4 C in a polyethylene bottle between experiments.

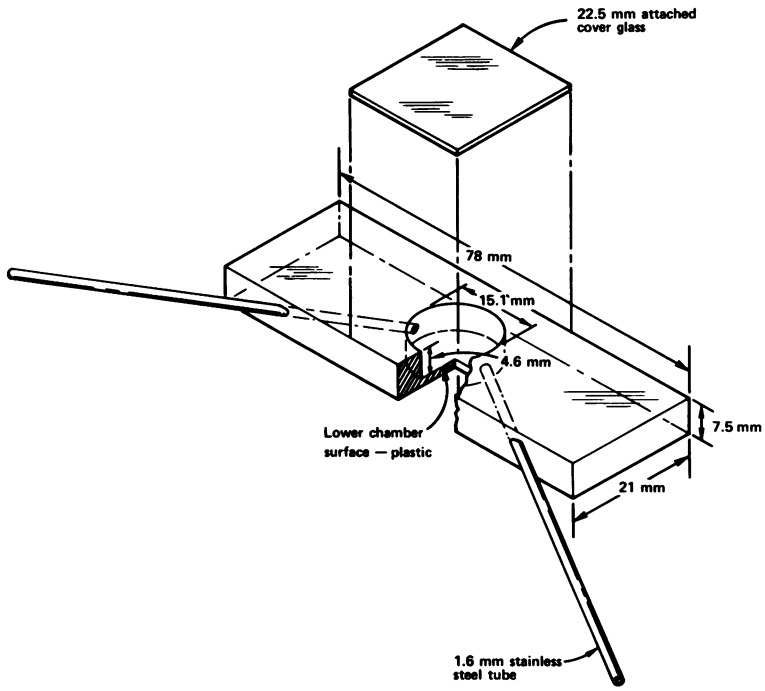

FIG. 2. *Microscope slide with perfusion chamber. The upper surface of the chamber consists of a cover glass attached to the slide to provide a hermetical seal. Adapted from Koch (10–11).*

Microscope slide with perfusion chamber. To collect data on the relative fluorescence with each combination of filter and light source, a perfusion chamber on a slide was constructed to eliminate the fading which normally occurs during FA quantitation. This slide was constructed of plastic with a cylindrical chamber (see Fig. 2). The top of the chamber was formed by attaching a 22.5-mm² cover glass (0.17 mm thick) securely to the chamber with nonfluorescent adhesive. Fluorescein or control solution was pumped (3 ml/min) to the chamber from a reservoir not exposed to light and held at 22 C (±0.7 C). The chamber allowed multiple measurements without fading, as well as the changing of light sources, fluorochromes, or filter combinations without the difficulties of using individual wells.

Microscope slide with 3-mm wells. Fluorescent measurements also were obtained with 1 μM fluorescein diacetate solution placed in the wells of a slide constructed according to Jongsma et al. (8). Loaded wells were carefully covered with separate cover glasses. Focusing was accomplished by initially closing the field diaphragm in the incident illuminator to permit sharp focusing of the visible diaphragm. The intensity of the fluorescence was recorded for 90-s periods while the two lights being compared were alternately selected. The emission of individual lights thus remained stable, whereas the specimen excitation was interrupted only for a fraction of a second during the switching of the mirror. Each light source was compared with the xenon 450-W arc.

Conjugate titering. The working titer of an FA conjugate was determined using the 450-W xenon arc and the 200-W mercury and 100-W halogen lamp for excitation. This was done in a room with moderate illumination.

RESULTS

Examination of the emission spectral curves and technical properties of light sources compatible with fluorescent microscopes (see Fig. 1 and Table 1) revealed that at least six lights warranted evaluation. These lamps included the 100-W halogen, the 75-, 150-, and 450-W xenon arcs, and the 100- and 200-W mercury arcs. Examination of Fig. 1 disclosed that, in the region of maximal fluorescein absorption (460 to 500 nm), the 450-W xenon's intense continuous emission was unsurpassed.

To choose the best suppression filter to be used in the experiments, the K510, K530, and S525 filters were evaluated with the 450-W xenon source (Fig. 3) using fluorescein diacetate solution in the 3-mm well slide. The initial unadjusted intensity, including background light, was about equal for the three suppression filters examined. Unexpectedly, the intensity decreased very little during the 90-s observation period with the K510 filter, in contrast to either the K530 or S525 filter. This can be ex-

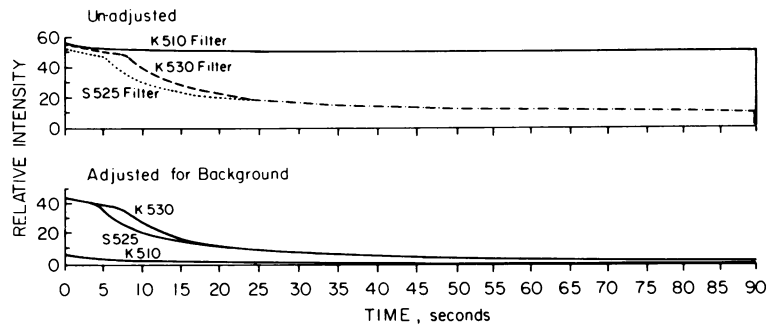

FIG. 3. *Effect of suppression filters on fluorescent intensity and fading. Measurements were obtained with a 40× objective.*

plained by the fact that the K510 filter transmits a large quantity of unwanted excitation light. The results after adjustment for background light (Fig. 3) reveal that the rate of fading with the K530 filter was only slightly different than that of the S525 filter. Since the transmission characteristics of the S525 filter was more compatible with the fluorescence spectrum of fluorescein, this filter was selected for all remaining experiments.

Magnification of objective. Most FA microscopy is performed using 100× oil immersion objectives (1). In the present study, there was considerably more variation with this objective than with the 40× objective measurements (Table 2). Consequently, the 40× objective was used for most of the remaining experiments with the 3-mm wells. Figure 4 shows that relative intensity and the rate of fading observed with 3-mm wells using either the 100× or 40× objectives with the XBO450 lamp. Although the initial intensity was of almost equal magnitude, the fluorescence with the 100× objective faded more rapidly but was greater after 90 s.

Comparison of light sources. (i) Perfusion chamber. In contrast to the results observed with the 3-mm wells, measurements with the perfusion chamber were stable and reproducible with either the 100× or 40× objectives (see Table 3).

(ii) Wells (3 mm). In Fig. 5 the fluorescence observed with the 450-W xenon source is compared with the three most commonly used light sources. The initial fluorescence observed with the 100-W halogen lamp was only 1/20 of that with the 450-W xenon. Even after fading, the 450-W xenon produced 10 times greater fluorescence than the halogen lamp. This difference was observed whether or not the xenon preceded or followed the halogen light for alternating periods of 5 s each (Fig. 5, dotted line). The results from alternating the 200-W mercury and the 450-W xenon sources are also depicted.

TABLE 2. *Effect of the excitation light source and microscope magnification on the relative fluorescent intensity and variation from 0.1 μM fluorescein diacetate solution in 3-mm wells on a microscope slide*[a]

Light source	Relative intensity					
	40×[b]			100×[b]		
	n	Mean	Coefficient of variation	*n*	Mean	Coefficient of variation
XBO 450	20	4.4	0.6	10	3.6	1.8
XBO 150	20	3.1	2.1		1.7	
XBO 75	20	4.5	0.9		3.3	
HBO 200	20	1.5	1.1	6	1.2	7.9
HBO 100	20	4.7	0.3		3.9	
Halogen, 100 W	9	0.18	0.7		0.04	

[a] The measurements were obtained with a S525 suppression filter and were adjusted for background illumination. Coefficient of variation was not calculated for light sources where only duplicate readings were recorded.
[b] Magnification.

The 450-W xenon excited considerable more fluorescence. Even at the end of the observation period, when fading was maximal, the 450-W xenon produced more than three times the fluorescence of the 200-W mercury arc. The fluorescence produced by the 150-W xenon arc and the 200-W mercury were found to be quite similar (Fig. 5). The major difference was the variation observed with the alternating current-operated 150-W xenon. The effects of the 60-cycle current and line voltage transients are visible in Fig. 5. The initial intensity of the 150-W xenon fluorescence was 75% of that produced by the 450-W xenon.

In Fig. 6 the 450-W xenon is compared with either the 100-W mercury or the 75-W xenon.

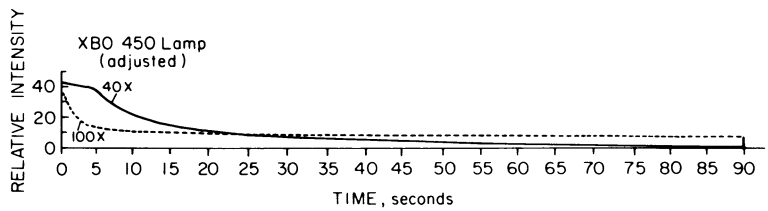

FIG. 4. *Effect of objective magnification on fluorescent intensity and fading.*

TABLE 3. *Effect of the excitation light source, microscope magnification, and excitation band width on the relative fluorescent intensities obtained with 0.1 μM fluorescein diacetate solution circulated through a microscope perfusion chamber*

Light source	Relative intensity			
	40×[a]		100×[a]	
	Wide band (KP500, BG38)	Narrow band (KP500, BG38) (λ >480)	Wide band (KP500, BG38)	Narrow band (KP500, BG38) (λ >480)
XBO 450	4.7	3.3	4.4	2.4
XBO 150	3.5	0.9	2.7	0.8
XBO 75	4.6	1.9	4.2	2.3
HBO 200	3.2	0.6	2.2	0.6
HBO 100	4.8	1.5	4.7	2.3
Halogen, 100 W	0.21	0.08	0.04	0.01

[a] Magnification.

The brilliant arcs of the 100-W mercury and the 75-W xenon resulted in initial fluorescence slightly greater than that of the 45-W xenon. However, considerably more fading occurred during the first 30 s with these arcs than with the xenon 450-W. Similar results were obtained when the 100-W mercury and 450-W xenon were compared using a 100× oil objective (see Fig. 7). Again the 100-W mercury initial fluorescence was slightly greater. After switching to the 450-W xenon, we consistently observed an intensity surge which was not seen when excitation was changed from the 450-W xenon to the 100-W mercury.

Narrow-band excitation. The fluorescent intensities produced by narrow-band and wide-band excitation are presented in Table 3. Since these measurements were obtained using the perfusion chamber, fading was not a significant factor. The 450-W xenon produced approximately twice the fluorescence of the 100-W mercury and the 75-W xenon sources with narrow-band excitation and a 40× objective. It also produced four to five times as much fluorescence as either the 150-W xenon or the 200-W mercury, and 40 times as much fluorescence as the 100-W halogen. It might be expected from the spectral distribution (Table 1) that the additional filtering to produce narrow-band excitation reduced fluorescence less with the xenon arcs than with other lights. This was observed except for the 150-W xenon fluorescence, which had a reduction in fluorescence similar in magnitude to that observed with mercury lamp narrow-band excitation. Another observation was that the change to narrow-band excitation at 100× magnification reduced fluorescence considerably more than did the change to narrow-band at 40×. It seems unlikely that this could be attributed to unwanted excitation light, since both a S525 filter and a K515 filter (built-in barrier filter) were employed in these measurements. The expected exceptions to this occurred with the 2-min arcs, the 100-W mercury and the 75-W xenon.

To further assess narrow-band excitation, and in particular any effect on fading, fluorescence was recorded from 3-mm wells. These measurements were made at 40×, because the absence of two matched narrow-band filters (λ > 480) prevented light sources from being alternated with the same wells, thus requiring sequential measurements with different wells. It was determined that 40× measurements from different wells were reproducible and that the greater variation with 100× measurements might invalidate interwell comparison. The results comparing the fluorescence for both 200-W mercury and 450-W xenon narrow-band excitation are presented in Fig. 8. The 450-W xenon gave considerably more fluorescence than the 200-W mercury and after 40 s of fading, remained twice as intense as the 200-W mercury. Of interest was the finding that background illumination amounted to only 10.1% of total illumination observed with narrow-band excitation, whereas it was 21.8% with wide-band excitation.

Conjugate titering. The results of comparative titering with the XBO450, HBO200, and the

100-W halogen lights are presented in Table 4. The conjugate used was *Streptococcus mutans*, serotype *d* specific conjugate. The 450-W xenon permitted a fourfold greater working dilution than the 200-W mercury light by both investigators. Titers were lower with the 100-W halogen than with either the 200-W mercury or the 450-W xenon. Excitation with a halogen lamp would require this conjugate to be 32 times more concentrated than with XBO450. In comparing the halogen lamp with the 200-W mercury, it was found that halogen illumination required that the conjugate be eight times stronger than that required with the 200-W mercury.

DISCUSSION

The utility of a light source in FA incident microscopy is determined by the interaction of many factors (10, 11). The combined effect of excitation intensity, spectral distribution, angular aperature of the illuminating beam, nature of the fluorochrome being excited, and filter and dichroic mirror characteristics, as well as the entrance pupil size of the microscope objective, all determine the fluorescence observed (3, 4, 8, 9, 16). It has been suggested that intense, narrow-band excitation light specific for given fluorochromes would give optimal excitation.

An important finding was that the 450-W xenon had sufficient energy with narrow-band filtration to produce at least two times as much fluorescence at 40× and at 100× than the other light sources studied. Although the rate of fluorescein fading with 450-W excitation did not appear to be reduced with narrow-band filters (Fig. 8), certain biological specimens showed considerable reduction in fading with narrow-

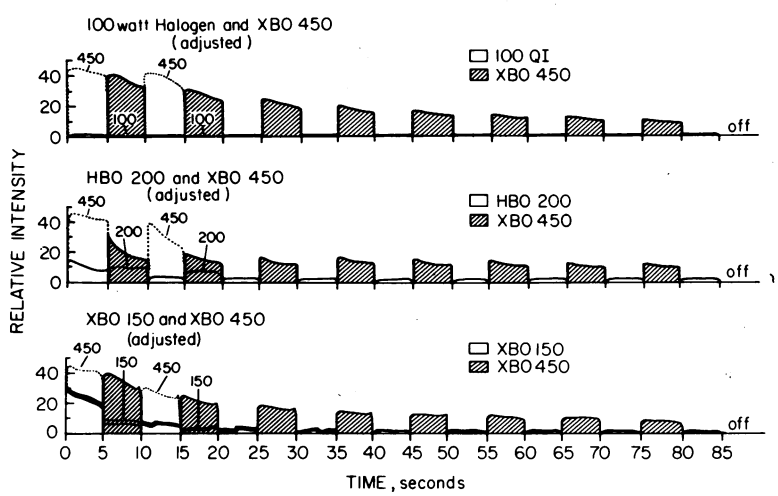

FIG. 5. *Effect of the excitation light source on fluorescent intensity and fading is represented by solid lines. The results obtained when the lamp order is reversed (XBO 450 first) during the initial 20 s are superimposed as dotted lines.*

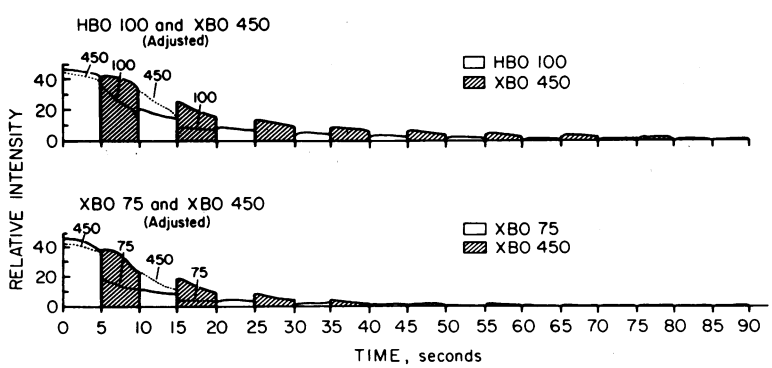

FIG. 6. *Effect of the excitation light source on fluorescent intensity and fading is represented by solid lines. The results obtained when the lamp order is reversed (XBO 450 first) during the initial 20 s are superimposed as dotted lines.*

band excitation. Thus, it appears that narrow-band excitation offers sufficient advantage to justify the slight decrease in intensity with the 450-W xenon. However, the reduction in intensity with the commonly used HBO200 mercury arc suggest that this source is less suitable. The 100-W mercury was an exception and performed very well as a narrow-band excitation source at 100×. It should be remembered that the small configuration of the 100-W mercury precludes its use in transmitted FA excitation.

Direct conjugate titering in the present study (see Table 4) supported the premise recently stated by Faulk and Hijmans (3) that "the use of high-intensity light sources will also be of value if the present trend to use more dilute conjugates continues." The present data support this concept.

The fourfold or greater conjugate dilution permitted with the 450-W xenon, as opposed to that permitted with the 200-W mercury or the 100-W halogen, was significant. In estimating any potential cost benefit with 450-W xenon excitation, the principal consideration should be what work load would offset the additional cost. Since only a research-grade microscope will accommodate all of the light sources evaluated, and since frequently such a microscope of this quality is required for certain diagnostic procedures, then the extra expense for the 450-W xenon light source will involve the lamp, lamp housing, and the power supply. Although formidable (about $4,000), the combined benefit of lower lamp cost per hour and additional conjugate dilution (averaging from four- to 32-fold) for the 450-W xenon over the popular light sources could equal the additional equipment outlay over time.

Fading of fluorescence has been a major problem in quantitating fluorescence and is of concern in most FA work (16). In fluorescein-stained specimens, the rate of fading has been reported to be inversely related to the concentration of the fluorochromes and directly related to the exposure time and excitation intensity (4). Therefore, one would prefer to use conjugates with a high ratio of fluorescein to protein and short exposures with moderate excitation. This has encouraged the use of the 200-W mercury arc and, more recently, the 100-and 150-W halogen lamps.

Jongsma et al. (8) have observed that fluorescein-stained cells faded faster than did 50 μM fluorescein solution under similar conditions. Ploem (16) observed that the fluorescence of fluorescein-stained leukocytes decreased 50% during the first 0.33 s of excitation. Both investigators were using stained cells, which suggests that the fluorochrome concentration would be

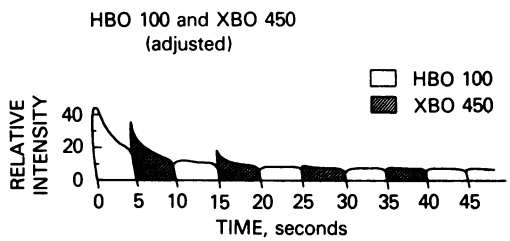

FIG. 7. *Comparison of the fluorescent intensity and fading produced by the HBO 100 and XBO 450 arcs. The objective magnification is 100×.*

TABLE 4. *Effect of the excitation light source on the titer of an FA conjugate as determined by two investigators*[a]

Titers	Investigator I			Investigator II		
	100-W halogen	HBO 200	XBO 450	100-W halogen	HBO 200	XBO 450
1:128	3+	4+	4+	3+	4+	4+
1:256	2–3+	4+	4+	2+	4+	4+
1:512	1–2+	4+	4+	1+	4+	4+
1:1,024	1+	3+	4+	1+	3+	4+
1:2,084	1+	2–3+	4+	±	3+	4+
1:4,096	±	1+	3+	–	2+	3+
1:8,192	±	1+	2–3+	–	2+	3+

[a] The conjugate is against *S. mutans* strain SL-1 (serotype *d*) and is labeled with fluorescein isothiocyanate to give a fluorescein to protein ratio of 18. FA criteria are as described by Cherry (2; personal communication).

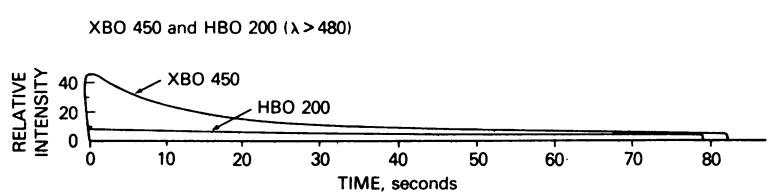

FIG. 8. *Comparison of the fluorescent intensity and fading produced with narrow-band* (λ = *480 to 500 nm*) *blue excitation. The objective magnification is 100×.*

low and result in rapid fading. Ploem (16) used a digital voltmeter and storage oscilloscope to record the transient initial fluorescent intensity. The pen recorder used in the present study was filtered to such an extent that transient signals of extremely short duration could not be recorded accurately. But, since in most routine FA microscopy one needs several seconds to identify or enumerate objects, initial fluorescence transients are principally of interest in microfluorometry.

In the present study the effect of intensity on the rate of fading was evaluated in two ways. The 3-mm well data presented in Fig. 3–8 collectively suggest that fluorescein at a concentration of 1.0 μM fades quite rapidly during the first 3 s when observed at 100× and relatively slowly at 40× during the initial 5 to 10 s. Perhaps the most important finding was that, in spite of the fading produced by the intense XBO450 excitation, substitution of less intense excitation consistently resulted in lower fluorescence. Likewise, when the excitation was instantly increased by substituting the intense XBO450 arc, the fluorescence was generally observed to jump, regardless of whether the specimen had only begun to fade or had already faded to a lower level. This suggests that intense excitation in routine FA microscopy is beneficial.

The selection of appropriate suppression filters becomes particularly important when a high-transmission excitation filter transmits wavelengths close to the emission peak of the fluorochrome. In the present study, the difference observed between the K510 filter and the S525 filter was considerable. Analysis of the background illumination with a calibrated interference wedge (T_{max} = 30 to 35%; half-width = 10 to 11 nm) suggested that background light occurring with the K510 was primarily of the wavelength 480 to 510 nm, with a detectable amount in the red region. A practical solution to reduce the red background light would be to use a total of 8 mm of BG38 filtering. Although background illumination can be helpful for visual orientation, the K510 allowed too much background for microfluorometry and other demanding applications.

Readings obtained with the perfusion chamber slide were highly reproducible with either 100 or 40× objectives (see Table 2). Although a large interwell variation occurred in measurements from the 3-mm wells, particularly with the 100× oil objective, each experiment presented in Fig. 5–8 involved individually only one well; hence, the differences in fluorescence observed as light sources were alternated should be representative of actual differences. Since the cover glass on 3-mm wells was not attached with cement, lofting of the cover glass during focusing might have affected readings. Because the 40× measurements were more reproducible than similar 100× readings, it was suspected that the major component to this variation related to manipulative difficulties associated with the use of oil. The decision to use 40× measurements for most fading data from the 3-mm wells was made with full awareness that noticeable reflections would occur from the cover glass and that minute arc lamps (XBO75 and HBO100) have advantages with 100× objectives (14, 16).

Goldman has reported that "when direct current is used the emission of the xenon lamp is concentrated in a relatively small spot at the cathode" (4). In contrast, alternating current produces a shift of the emission between the electrodes as each becomes the cathode. Thus, direct current gives a smaller emission configuration. An interesting finding was that the small electrode in the HBO100 was the cathode and the large electrode in the XBO75 was the cathode. Hence, it appears that reversing the polarity of the 75-W xenon might give a smaller configuration and hence a more brilliant arc.

To our knowledge there has been no previous report quantitatively comparing the utility of the 450-W xenon with conventional light sources for FA excitation. Although the present study was principally exploratory, sufficient data were obtained to suggest specific advantages and applications in FA work for the XBO450 or other intense excitation sources. The results described above clearly support the premise that the 450-W xenon is capable of intense fluorescein fluorescence, permitting (i) additional conjugate dilution, (ii) satisfactory narrow-band excitation, and (iii) diagnostic work to be performed in environments with normal ambient illumination.

LITERATURE CITED

1. Cherry, W. B. 1970. Fluorescence emission with special reference to standardization in immunofluorescence, p. 127–136. *In* E. J. Halborow (ed.), Standardization in immunofluorescence. Blackwell Scientific Publications, Oxford.
2. Cherry, W. B., M. Goldman, and T. R. Carski. 1960. Fluorescent antibody techniques in the diagnosis of communicable diseases, p. 10–11. Public Health Service publication no. 729. U.S. Government Printing Office, Washington, D.C.
3. Faulk, W. P., and W. Hijmans, 1972. Recent developments in immunofluorescence. Progr. Allergy 16:9–39.
4. Goldman, M. 1968. Fluorescent antibody methods, 4th ed. Academic Press Inc., New York.
5. Hebert, G. A., B. Pittman, R. M. McKinney, and W. B.

Cherry. 1972. The preparation and physiochemical characterization of fluorescent antibody reagents. Center for Disease Control Bulletin. U.S. Department of Health, Education, and Welfare, Washington, D.C.
6. Hijmans, W., and H. R. E. Schuit. 1972. Immunofluorescence studies on immunoglobulins in the lymphoid cells of human peripheral blood. Clin. Exp. Immunol. 11:483–93.
7. Hijmans, W., H. R. E. Schuit, Y. Teiko, and I. Schechter. 1972. An immunofluorescence study on synthesis of antibodies of single specificity. Eur. J. Immunol. 2:1–4.
8. Jongsma, P. M., W. Hijmans, and J. S. Ploem. 1971. Quantitative immunofluorescence; standardization and calibration in microfluorometry. Histochemie 25:329–343.
9. Kawamura, A., Jr. 1969. Fluorescent antibody techniques and their applications, 1st ed. University Park Press, Baltimore.
10. Koch, K. F. 1971. Light sources for fluorescence microscopy. 1) FITC immuno-fluorescence. Leitz Wetzlar Sci. Tech. Inf. 2:50–52.
11. Koch, K. F. 1972. Fluorescence microscopy: instruments, methods, applications. Leitz Wetzlar Sci. Publ. 2:50–52.
12. McKinney, R. M., J. T. Spillane, and G. W. Pearce. 1974. Fluorescein diacetate as a reference color standard in fluorescent antibody studies. Anal. Bacteriol. 2:474–476.
13. Nairn, R. C. 1969. Fluorescence microscopy and photomicrography, p. 61–94. *In* R. C. Nairn (ed.), Fluorescent protein tracing. The Williams & Wilkins Co., Baltimore.
14. Ploem, J. S. 1967. The use of vertical illuminator with interchangeable dichroic mirrors for fluorescence microscopy with incident light. Z. Wiss. Mikrosk. 68:129–142.
15. Ploem, J. S. 1970. Quantitative immunofluorescence, p. 63–73. *In* E. J. Halborow (ed.), Standardization in immunofluorescence, Blackwell Scientific Publications, Oxford.
16. Ploem, J. S. 1971. A study of filters and light sources in immunofluorescence microscopy. Ann. N.Y. Acad. Sci. 77:414–429.
17. Van Boxtel, C. J., C. P. Engelfriet, and T. E. W. Feltkamp. 1973. Immunofluorescence microphotometry for the detection of platelet antibodies. I. Standardization of the method. Scand. J. Immunol. 2:217–219.
18. Van der Ploeg, M., and J. S. Ploem. 1973. Filter combinations and light sources for fluorescence microscopy of quinacrine mustard or quinacrine stained chromosomes. Histochemie 33:61–70.