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SUMMARY

The absorption characteristics of light and the optical technique of fluorescence microscopy to enhance metallographic interpretation are presented. Characterization of thermally sprayed coatings by optical microscopy suffers because of the tendency for misidentification of the microstructure produced by metallographic preparation. Gray scale, in bright-field microscopy, is frequently the only means of differentiating the actual structural details of porosity, cracking, and debonding of coatings. Fluorescence microscopy is a technique that helps to distinguish the artifacts of metallographic preparation (pullout, cracking, debonding) from the microstructure of the specimen by color contrasting structural differences. Alternative instrumentation and the use of other dye systems are also discussed. The combination of epoxy vacuum infiltration with fluorescence microscopy to verify microstructural defects is an effective means to characterize advanced materials and to assess structural integrity.

INTRODUCTION

The underlying principles of fluorescence microscopy (ref. 1) and the equipment necessary to perform the analysis of specimens are presented. Fluorescence attachments for microscopes and the dyes used with them are commercially available. Reference 1 does not discuss the theory of how molecules absorb light or fluoresce and how these processes govern the appropriate selection of equipment necessary to effectively perform the technique. Presented herein are some of the fundamentals of spectroscopy, the interaction of light with molecules, and the selection of appropriate dyes and associated optical components to optimize the viewing in a fluorescence microscopy experiment.

THEORY

The processes by which molecules absorb energy in the form of light and emit energy in the form of fluoresced light are illustrated in figure 1, a Jablonski diagram (ref. 2). The energy diagram is divided into various electronic energy levels (the ground and various excited states) and vibrational levels within the various electronic states. Under normal conditions, the electrons capable of absorption of ultraviolet or visible light energy reside in the lowest vibrational energy level of the ground state or reside, to a small extent by thermal processes, in the second lowest vibrational level of the ground state. Upon absorption of the correct amount of energy, the electron is promoted to the appropriate vibrational level of an excited state. The molecule now possesses too much energy and immediately begins to dissipate the energy by any number

of processes. The first process occurs rapidly and is called vibrational relaxation, which is a nonradiative, or non-light-emitting, process leaving the molecule in the lowest vibrational level of the first excited state. The molecule may then internally convert, another nonradiative process, to the various vibrational levels of the ground state and, subsequently, more vibrational relaxation returns the electron to the lowest vibrational level of the ground state. This process is the primary one in competition with fluorescence, which is the return of the electron from the lowest vibrational level of the excited state to the various vibrational levels of the ground state, with the accompanying release of light energy, or fluorescence. The electron then returns to the lowest vibrational level of the ground state, as discussed previously. Because the vibrational levels are so closely spaced in most molecules (with respect to the energy required for the electron transitions between electronic states), the resulting spectrum, a plot of absorbed or fluoresced light intensity versus the energy of the light, resembles broad Gaussian distributions. It is obvious from this diagram that the fluoresced light is generally at a lower energy level than that of the absorbed, or excitation, light. The absorption (or excitation) spectrum overlaps to a small degree the fluorescence spectrum because of the possibility of excitation from the second lowest vibrational level of the ground state to the lowest vibrational level of the excited state. Fluorescence then occurs by transition from the lowest vibrational level of the excited state to the lowest vibrational level of the ground state. The relationship of the energy of the light involved in the transitions to the wavelength of the light wave is given according to

$$E = h\nu \tag{1}$$

where E is the energy of the light wave, h is Planck's constant, and ν is the frequency of the light wave. The wavelength of light λ and the frequency are related to the speed of light c:

$$c = \lambda\nu \tag{2}$$

The intensity of fluorescence F is given by

$$F = A\Phi_f P_o K \tag{3}$$

where Φ_f is the efficiency of the molecule for conversion of absorbed light into fluoresced light, P_o is the intensity of the exciting light, K is the efficiency of the instrument for collection of the fluorescence, and absorbance A is defined by

$$A = \varepsilon bC \tag{4}$$

where ε relates to the probability of a molecule absorbing a light wave, b is the depth of the light penetration into the sample, and C is the concentration of absorbing molecules.

The application of fluorescence is made through an instrument constructed such that two specific regions of the spectrum are isolated—one to probe the sample (absorption) and the other to interrogate it (fluorescence). Two variations of this type of instrument are schematically illustrated in figure 2. Figure 2(a) depicts a fluorometer, an instrument frequently employed by chemists for quantifying trace levels of constituents in samples. The unit consists of a light source and monochromator (M_{ex}) to isolate light capable of being absorbed by molecules of interest within the sample. The light is then allowed to excite the sample. Because of the low concentrations determined, much of the light is not absorbed by the sample (eq. (4)). Excited molecules in the sample emit fluorescence in all directions, some of which is isolated by the emission monochromator (M_{em}) and is then measured by a sensitive photodetector, usually a high-sensitivity photomultiplier tube (PMT). The fluorescence microscope (fig. 2(b)) is remarkably similar, with two exceptions. First, relatively high concentrations of the fluorescing dye are employed, causing all of the exciting light to be absorbed at the surface of the sample; hence, the b term of (eq. (4)) is small. Because the fluorescence is proportional to P_o (eq. (3)), the higher the intensity light source employed, the more fluorescence observed for a given concentration of dye. A second major difference is the method of light detection, in that the photodetector is replaced by the eye. In this respect, the eye is a superior detector because it responds not only to light intensity but also to different colors and different hues of the same color. This response provides two-dimensional information and, in some cases, even three-dimensional information regarding the structure of the material under investigation. Drawbacks associated with using the eye include rapid fatigue associated with making correct light intensity determinations and differing responses to different colors, as indicated in figure 3. The eye is considerably more sensitive to green, yellow, and orange than it is to other colors in the visible spectrum; hence, the fluorescence experiment is optimized by viewing in this region of the spectrum. The same effect is noted with photographic film, much of which is optimized for response in the green region of the spectrum.

The function of the monochromators is to isolate a single color or, in practice, a single region of the spectrum. Figure 4 shows the two types of filters employed for isolating excitation light (short-pass filters) and fluorescence light (long-pass filters). The solid curves represent the ideal filter and the combined solid-plus-dashed curves represent what is actually available. The function of the short-pass filter is to allow transmission of those wavelengths which excite sample molecules from the light source to the sample and to block transmission of all light with a wavelength similar to that of the fluoresced light. The long-pass filter blocks excitation light reflected from the sample and allows transmission of the fluoresced light through to the detector (i.e., these two filters are respectively wavelength matched to the absorption or excitation and fluorescence emission characteristics of the

fluorescent molecules in the sample). In true fluorescence mode (fig. 5(a)), the combination of short- and long-pass filters is such that none of the excitation light is transmitted through to the detector.

In figure 5(b), the filter combination is such that a small portion of the excitation light is allowed to pass through to the detector, an artifact known as "crosstalk." The problem with crosstalk in a fluorescence instrument is that the detector is unable to distinguish between the crosstalk and the fluorescence wavelengths. Low concentrations of fluorescent materials in combination with the facts that Φ_f is typically much less than 1 and the collection efficiency of the unit K is very much less than 100 percent produce fluorescence which can be orders of magnitude smaller than the directly reflected crosstalk. This effect is beneficial in fluorescence microscopy as it allows direct viewing of the specimen at the crosstalk color and simultaneous observation of the fluorescence experiment occurring at a different wavelength (i.e., color) provided that the intensity of light from the two experiments is approximately comparable. One of the commonly overlooked disadvantages in using filter monochromators is that they allow light to be transmitted in undesirable regions of the spectrum, as indicated by transmission associated with the dashed lines in figure 4. This effect is frequently minimized or eliminated by virtue of the fact that the light source may not produce light in the region where undesirable transmission would occur; also, in the event that light did get through both the excitation and emission filters, the detector might not be sensitive to that wavelength anyway. For example, the short-pass excitation filter shown in figure 4(a) also transmits violet, which will also transmit through the long-pass emission filter. Even though the intensity of the violet may be comparable to the intensity of orange fluorescence transmitted by the emission filter, the eye response (fig. 3) would be swamped by the orange intensity. This effect can also be employed to generate useful crosstalk similar to that which was previously discussed in association with figure 5(b). A combination of the red transmission of the excitation filter in figure 4(a) with the solid-line profile of the emission filter would produce a system similar to that illustrated in figure 6(a) This degree of crosstalk would cause the crosstalk intensity to swamp the fluorescence intensity, even though the eye response is poorer to the crosstalk than to the fluorescence. First, the fluorescence is typically much lower in intensity because of nondirectional fluorescence emission as opposed to the direct reflectance of the crosstalk. Second, a tungsten bulb, for example, will produce higher intensity red output than the blue that used to excite the sample (eq. (3)). The amount of crosstalk generated by this effect can also be adjusted by using two excitation filters that have different transmission and blocking ranges, as seen in figure 6(b). This system is a modification of the one in figure 6(a): a second short-pass filter was added (short-pass #2) to block in the region where the undesirable red light begins to be transmitted by the first short-pass filter.

In order to efficiently correlate the structural information obtained from the bright-field observation of the specimen to the porosity information obtained from fluorescence viewing, it is necessary to view both modes simultaneously. The use of crosstalk permits simultaneous viewing with both modes. The bright-field mode appears at the crosstalk color and the fluorescence mode appears at the same color as the fluorescence of the dye incorporated into the infiltrated epoxy.

PROCEDURE

Specimen Preparation

The thermally sprayed tungsten carbide coating on titanium or stainless steel substrate (88-12) was first mounted in an epoxy resin by the vacuum infiltration method (ref. 1). After curing, the specimens were metallographically prepared by the procedure outlined later in the Reagents and Materials section. The specimens were provided by General Electric Aircraft Engine Division. This procedure produced a uniform microstructure which correlated to the processing parameters employed in the manufacturing of the coatings. Some of the specimens exhibited a large amount of pullout, indicating a cold plasma jet, and other specimens showed a fully dense microstructure. The specimens were examined by optical microscopy in bright-field and by fluorescence methods to determine the microstructure. With the bright-field method, it was difficult to determine if porosity or pullout was present in the microstructure. Pullout occurs if the metallographic procedure is too aggressive for the given material or, in the case of thermally sprayed coatings, if the individual particles are weakly bonded together.

The following procedure was followed for the metallographic preparation of the thermally sprayed tungsten carbide cobalt coated specimens:

(1) Mount the specimen in fluorescent epoxy (epofix, two-part epoxy) and vacuum infiltrate epoxy into specimens.

(2) Polish at 150 rpm with a 200–N force using

(a) 150-grit Al_2O_3 wheel (until planar)

(b) 6-μm diamond on a Petro M lapping disk for 9 min

(c) 6- and 3- μm diamond on a synthetic hard cloth for 1 min each

(d) 0.5-μm colloidal silica plus 10 percent V:V of 30 percent hydrogen peroxide on a chemically resistant cloth for 1 min

The fluorescence method helps to distinguish between pullout and filled porosity, but it cannot distinguish between isolated nonconnecting porosity and pullout. The fluorescence method, using filters to differentiate between the color of the specimen and the fluorescent dye, enhances the differences in the microstructure. A systematic study of fluorescent dyes and filtration systems (both commercial systems and systems

constructed from commercially available filters) was undertaken to enhance viewing conditions such that both the bright-field and fluorescence information could be acquired simultaneously and conditions could be improved for either color or black and white photography.

Reagents and Materials

The dyes and solvents were reagent quality and were used as received. Fluorescent dyes were mixed with a dilute resin to make a saturated solution of dye in resin and allowed to stand for 48 hr, at which time the resin was decanted into a clean container. The epoxy was blended in a ratio of 6:1 Struers, Inc., Epofix epoxy resin to hardener prior to vacuum infiltration of epoxy into the specimens. The fluorescent dyes examined were rhodamine B and 6 G, rose bengal, DCM, amber dye, and epodye. (See fig. 3 for spectral characteristics of dyes. Solid vertical lines indicate the wavelength range where dye molecules absorb light with high efficiency; hatched lines indicate spectral ranges where they fluoresce with high efficiency.) For spectrophotometric determinations, the appropriate amount of dye-containing resin was diluted to read on the scale with ethanol in 1–cm quartz cells. The absorption samples were further diluted 1:100 with ethanol for reading in the spectrofluorometer in 1–cm^2 quartz cells. The absorption and fluorescence spectra were obtained in the normal manner. The spectrophotometer was blanked with an appropriate ethanolic resin mixture. The fluorescence for an appropriate blank was used to correct spectral data for the dyes examined.

Instrumentation

The Struers Abraplan was used for the initial grinding; all other preparation steps were done with the Struers Abrapol. The microscope used for all specimen examination and photographic work (except the true fluorescence figure) was the Reighert-Jung MeF_3, which was equipped with both tungsten and 450-W high-intensity xenon sources and a camera. All photographs presented herein were taken under xenon lighting with either the 4×5 Polaroid 545 Land film holder or the FT–1 Konica 35-mm camera. The film used was Polaroid 53 black and white or 59 color and 35-mm Kodak Ektar 125, respectively. This unit was used with either the B5 fluorescence module (red filter) system or a similar module block that was fitted with excitation filters of 520, 540, or 560 nm and emission filters of 520, 540, or 560 nm. The microscope used to obtain the true fluorescence photographs was the Olympus PMG3, equipped with a 150-W high-intensity xenon arc light source and a PMG3 DMB blue excitation filter (LECO filter system).

All absorption spectra were recorded on a Shimadzu model UV–160 spectrophotometer, operated in the normal fashion. All fluorescence spectra were recorded on a Perkin Elmer, Model MPF–44B, spectrofluorometer which was operated in the normal manner.

RESULTS AND DISCUSSION

This work systematically examined various combinations of excitation and emission filter systems and the degree of direct crosstalk allowed between them. During the examination, we also varied the region of the spectrum where the fluorescent dyes excite and fluoresce in order to find the optimum system for simultaneous direct and fluorescence viewing of the sample. Fortunately, because the response characteristics of the eye parallel those of the color and black and white film employed, conditions were optimized for both direct viewing and photographic recording of the work. Figure 3 also gives the approximate wavelength ranges for excitation and fluorescence observation of the dyes surveyed.

Epodye is commercially available and is moderately inexpensive; it excites and fluoresces in a good spectral region and, more important, is relatively soluble and chemically stable in the epoxy resin. Figure 7 presents two methods of viewing (in black and white) a specimen mounted in epodye-epoxy: bright field, or direct (fig. 7(a)), and true fluorescence (fig. 7(b)) The filtration system used is similar to that shown in figure 5(a). Evident from the bright-field viewing is that the sample contains a great deal of porosity throughout from left to right; however, the fluorescence viewing indicates that a good deal of the leftside porosity is pullout resulting from the metallographic preparation. Furthermore, this specimen indicates the need for simultaneous viewing of both experiments in order to easily distinguish pullout regions of the specimen from true porosity regions. Figure 8 shows the direct and fluorescence views of a similar specimen preparation; the two crosstalk modes used were those discussed in association with figure 6(b), representing viewing with a B5 module (red filter system), and figure 5(b). With epodye, the combination of the blue excitation-yellow emission (green crosstalk, later referred to as the green filter system) is a superior fluorescence viewing in that the details of the bright-field experiment are considerably more apparent under both visual inspection and color photography. Unfortunately, the details of the direct viewing of the specimen are not as clear in the fluorescence view as they appear in the bright-field view.

In terms of the less expensive and easier to use black and white photographic recording, the blue-yellow filter system is also superior to the B5 module, as demonstrated in figure 9 The color view of fluorescence provides an indication of the degree of true porosity as distinguished from pullout and is unambiguous when compared to bright-field viewing. The black and white view of the fluorescence also demonstrates this effect in that the bright-field information appears in gray, the pullout in black, and the porosity in white (e.g., lower

right corners). The same effect is noted when different dyes are employed, as seen in figure 10 where the epodye was replaced with rhodamine B. The use of rhodamine 6G produced unusual results in that the color photographs achieved greater contrast when the B5, or red, module was used; however, the black and white photographs of fluorescence came out better when the green filter system was used. This effect may be attributed to subtle differences in the sensitivity of the various films to different regions of the spectrum.

We examined specimens prepared in all the dyes listed in figure 3, and they all appear to be suitable for the filtration systems employed, but with varying degrees of acceptability. This finding is not totally unexpected in that the dyes have broad excitation and emission ranges with respect to the ranges covered by the excitation and emission filters investigated. Other chemistry problems restrict the use of the various dyes. The dye DCM is moderately soluble in the resin and retains spectral quality with respect to excitation and fluorescence; however, when hardener (a chemical base) is added, the dye molecule is deprotonated and unacceptable changes occur in both the excitation and emission spectra. Rose bengal and the rhodamines are not as soluble as some of the other dyes, resulting in lower florescence levels than anticipated. In an attempt to improve the solubility of rhodamine B, the acetate salt was synthesized to replace the chloride salt. This effort improved the solubility only slightly, but the acetate altered the pH of the system once the hardener was added.

The final problem encountered in this study was photobleaching of the dyes caused by prolonged exposure to high-intensity light. When a low-intensity tungsten light source was used, the effect of photobleaching, loss of fluorescence, was unnoticed. The high-intensity (450-W) xenon caused rapid, irreversible bleaching of all dyes. For example, after a 3-min exposure to the high-intensity xenon, the amber dye lost half of its fluorescence intensity, and no fluorescence was observed after 6 min. Of all the dyes tested, the epodye appeared to be least susceptible to this process. On the other hand, the tungsten source was not intense enough to take high-quality photomicrographs with either filter system, especially the green system when color film was used.

CONCLUSION

Fluorescence microscopy in the crosstalk mode is used to identify the actual structural details of porosity, cracking, and debonding and to distinguish them from the artifacts that occur during mounting and polishing (pullout, cracking, debonding). Two modes of crosstalk are identified; the crosstalk at a shorter wavelength than that of the fluorescence of the mounting dye appears to be the most promising for simultaneous direct viewing, fluorescence viewing, and/or photographic recording (either color or black and white). Because the eye and photographic films have similar response curves, the optimal viewing conditions are achieved by (1) mounting the specimen in an epoxy with a dye that excites in the blue and fluoresces in the yellow and (2) using a filtration system constructed of a blue short-pass filter and a yellow long-pass filter such that crosstalk appears in the green region of the spectrum.

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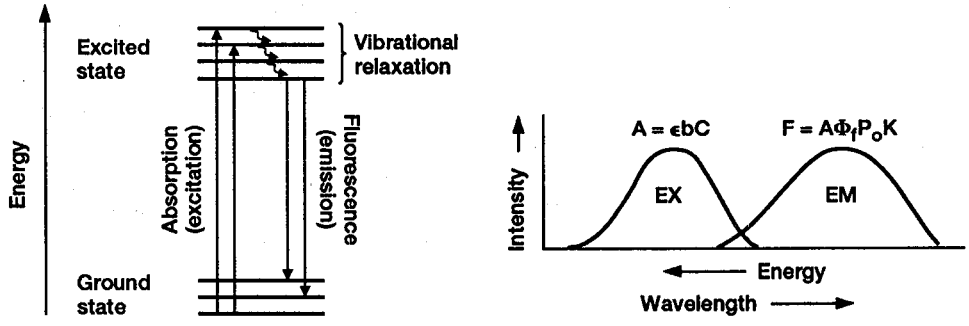
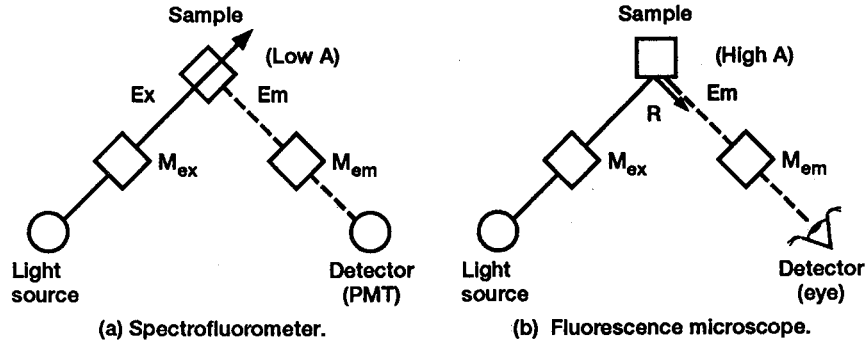


(a) Jablonski diagram of electronic transition. (b) Absorption (excitation) and fluorescence.

Figure 1.—The processes by which molecules absorb light and fluoresce.



(a) Spectrofluorometer. (b) Fluorescence microscope.

Figure 2.—Optical diagram for observation of fluorescence.

Wavelength, λ, nm	Response	Color
400	0.0004	Violet
420	0.004	Indigo
440	0.023	Blue
460	0.060	Blue
480	0.14	Blue
500	0.32	Green
520	0.71	Green
540	0.95	Green
560*	1.0	Green
580	0.87	Yellow
600	0.63	Orange
620	0.38	Red
640	0.18	Red
660	0.060	Red
680	0.017	Red
700	0.004	Red

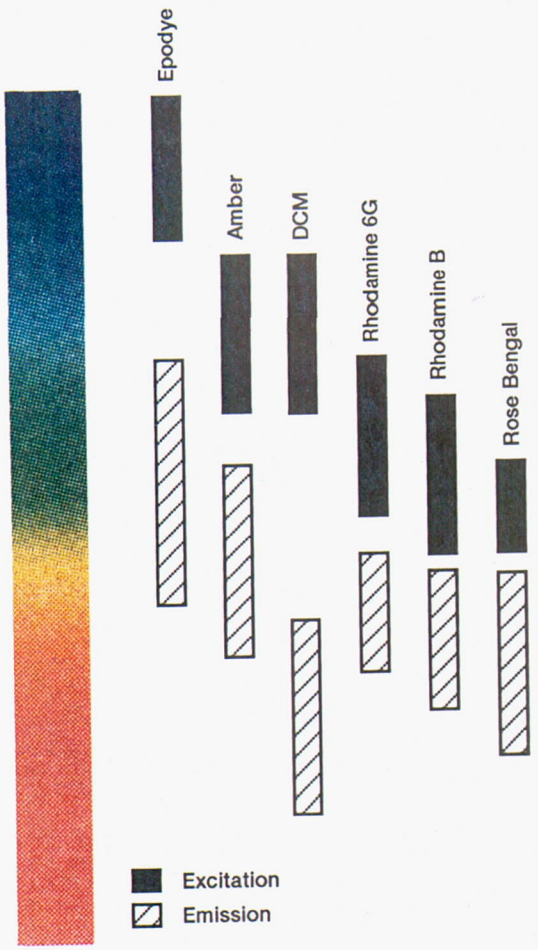
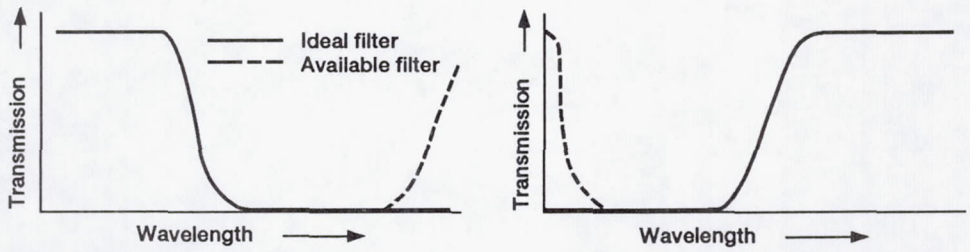
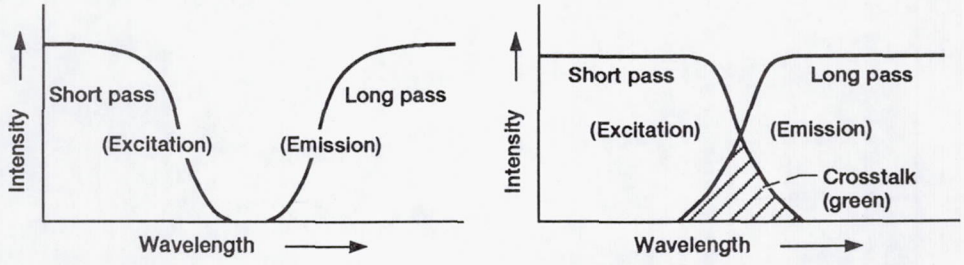


Figure 3.—Relative response of the cones of the eye to light of various wavelengths (left) and spectral characteristics of fluorescent dyes (right). *The response of the eye at 550 nm is taken as unity, and the response at all other wavelengths is calculated relative to that at 550 nm.



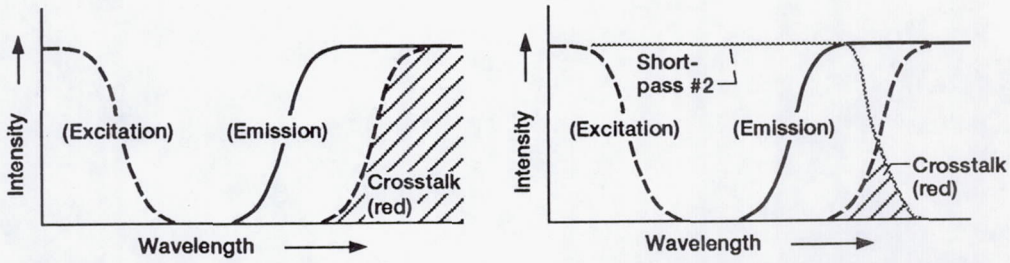
(a) Short-pass filter (excitation). (b) Long-pass filter (emission).

Figure 4.—Filter monochromators used in fluorescence instrumentation.



(a) True fluorescence. (b) Crosstalk-contaminated fluorescence.

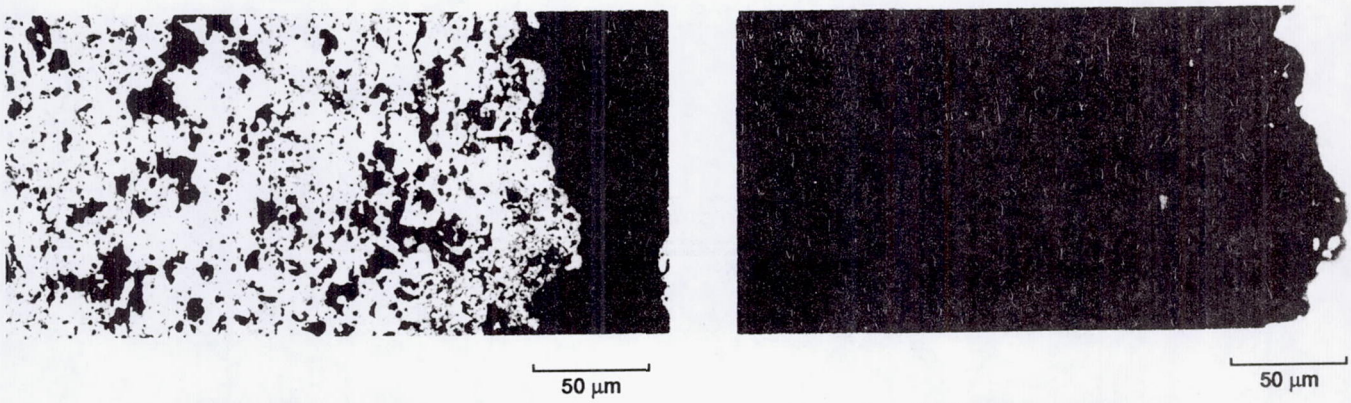
Figure 5.—Combination of excitation and emission filters employed for viewing fluorescence. Short pass transmits blue light and long pass transmits yellow, orange, and red. True fluorescence (a) will not transmit green whereas the "crosstalk" mode (b) transmits substantial green intensity.



(a) Two-filter combination and actual transmission characteristics of the excitation filter.

(b) Three-filter combination where the excitation monochromator exhibits the actual characteristics of two short-pass filters.

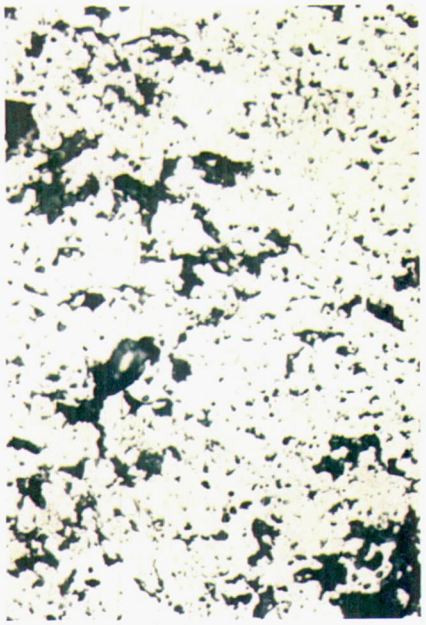
Figure 6.—Combinations of excitation and emission filters which produce crosstalk at longer wavelengths than fluorescence wavelengths.



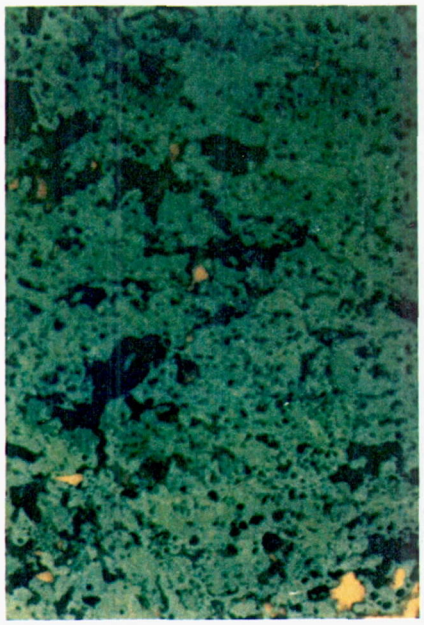
(a) Bright field (direct).

(b) True fluorescence.

Figure 7.—Two methods of viewing (in black and white) a specimen mounted in epodye-epoxy. A LECO filter system was used.



(a) Bright field (direct).

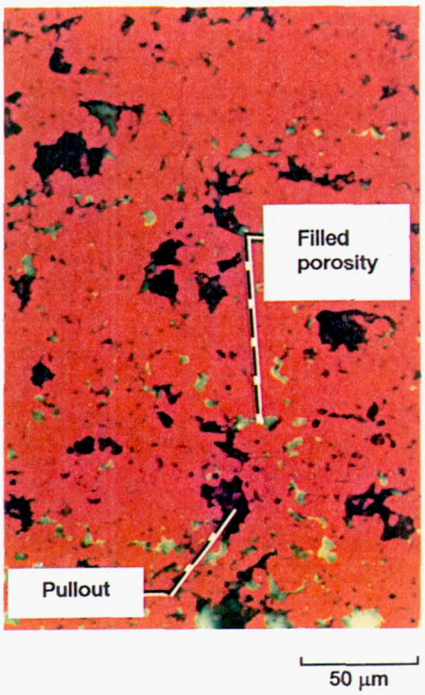


(b) Fluorescence. Epodye with B5 module (red filter system).

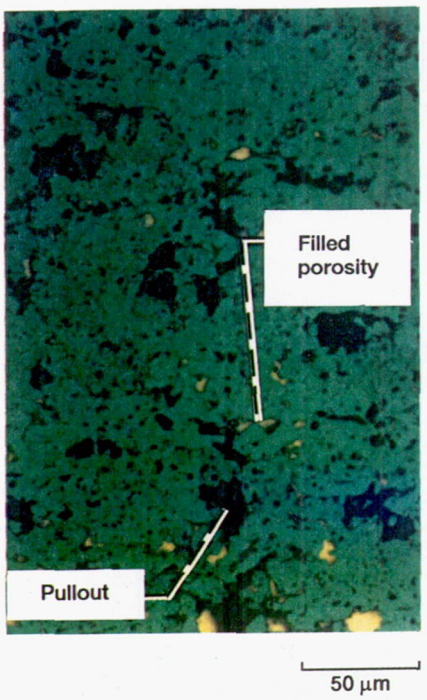
(c) Fluorescence. Epodye with blue excitation and yellow emission (green filter system).

Figure 8.—Bright-field and fluorescence viewing of specimen.

(a) Bright field (direct).

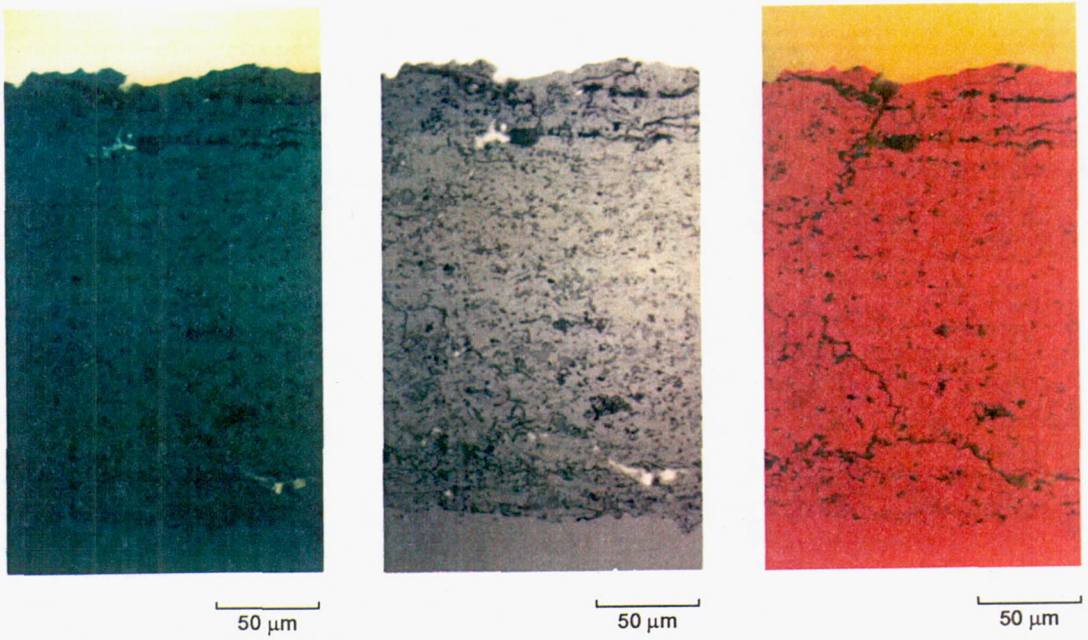


(b) Fluorescence. Blue excitation and yellow emission (green filter system).



(c) Fluorescence (black or white).

Figure 9.—Bright-field and fluorescence viewing of specimen mounted in epodye-epoxy.



(a) Green filter system.

(b) Black and white photograph with green filter system.

(c) B5 module.

Figure 10.—Fluorescence views using green filter system and B5 module with specimen mounted in rhodamine B-epoxy.