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Fluorescence Microscopy of Cereals

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FLUORESCENCE MICROSCOPY OF CEREALS[1]

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Abstract

The fluorescence microscope is one of the most sensitive instruments available for morphological and microchemical analysis of biological material, and especially of cereal grains. Recent innovations in illuminating systems, fluorescence chemistry, and specimen preparation have combined to provide significant improvements over conventional bright-field microscopy in both specificity and sensitivity. A variety of relatively specific fluorescent markers has been devised for routine and high resolution detection of all major cereal components. Several examples of useful fluorescent markers are described, including appropriate methods for specimen preparation, fluorescence analysis, and photography.

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Introduction

Mature cereal grains contain a variety of nutritionally and industrially important constituents, including proteins, carbohydrates and lipids, as well as minor compounds such as phytin, vitamins, phenolic compounds (e.g. cinnamic acids, flavonoids, lignin), aromatic amines, nucleic acids, etc. All of these components are synthesized, packaged and stored in specific tissues, making the three major grain fractions (the bran, germ and starchy endosperm) chemically and morphologically distinct from each other. In short, the mature cereal grain is highly compartmented, and each morphological entity possesses distinct chemical characteristics.

In addition to obvious differences in chemistry and morphology among tissues in a single kernel, there are pronounced structural differences between equivalent tissues of different genera or cultivars, depending upon the genetic and/or environmental background of the parent plants. As these dissimilarities reflect chemical differences and thus determine the nutritional and processing characteristics of cereals, it is important to identify the extent of this variation to provide a sound basis for further improvements. Typically, tissue heterogeneity is best elucidated by applying simple microscopic techniques which define both the structural *and* chemical characteristics of grain components. Fluorescence microscopy is well suited to this task and, in combination with various specimen preparation procedures, provides considerable chemical information, simplicity, high resolution, and application to both intact and processed cereal grains. This overview outlines the advantages of the fluorescence microscope and illustrates methods for identification of specific cereal components.

The Fluorescence Microscope

The fluorescence microscope is one of the most sensitive chemical instruments available for cereal analysis, and it is also one of the simplest and most flexible. A suitable fluorescence microscope is a bright-field microscope fitted with a high intensity, broad-spectrum illuminator, and two filter systems for (a) providing excitation, or illumination, of the

Table 1. Spectral Characteristics of Fluorescence Filter Combinations

Combination	Exciter Filter (transmission (max., nm)	Barrier Filter (transmission max., nm)
FC I	365	>418
FC II	450-490	>520
FC III	546	>590

specimen (exciter filters), and (b) eliminating unwanted (excitation) illumination from the fluorescent image (barrier filters). Exciter filters commonly transmit in the ultraviolet, blue, or green regions of the spectrum and are inserted between the illuminator and specimen to maximize the excitation of the fluorescent compounds under investigation. Barrier filters are inserted between the specimen and the detector or ocular to remove all wavelengths, including excitation, shorter than that of the induced fluorescence. Thus the fluorescent image is viewed on a dark or black background and the high contrast provides the most important characteristic of the fluorescence microscope - sensitivity. As little as 10^{-18} moles of fluorescent material can be detected by microspectrofluorimetry (von Sengbusch and Thaer 1973). Spectral properties of filter combinations suitable for cereal analysis (designated FC I, II and III) are given in Table I. Others are available and may be used for special applications.

For most purposes, including cereal analysis, the fluorescence microscope should be equipped with an epi- (or incident-) illuminator such that the excitation illumination is reflected (by dichroic mirrors) through the microscope objective onto the *upper* surface of the specimen (conventional, sub-stage condensers illuminate the lower surface of the specimen). Fluorescence microscopy was introduced over seventy years ago, but it is the relatively recent development of epi-illuminators which has increased dramatically both the efficiency and application of fluorescence microscopy, for several reasons. First, because the epi-illuminator excites the top surface of the specimen, there are few problems with relatively thick sections, such as loss of intensity and resolution due to diffusion and absorption of fluorescence within the specimen. The latter has been a common problem with older fluorescence microscopes equipped with sub-stage condensers. Second, because the objective acts as the condenser, excitation occurs only in the area of the specimen being examined, and fading of fluorescence under high intensity illumination is restricted to the area of view (sub-stage condensers illuminate large areas of the specimen). Third, epi-illuminators dramatically increase the excitation intensity at the specimen surface with each increment in objective power. Therefore, fluorescence intensity, and hence sensitivity, increases as resolving power increases. This intensity improvement which is characteristic of epi-illuminators, combined with the availability of improved, high speed films now permits a satisfactory color or black and white photography of fluorescent objects in a few seconds, a fraction of the time previously required with sub-stage illuminators. All photographs included in this paper were recorded on Kodak High Speed Ektachrome (ASA 400).

Several conventional (bright-field) microscopic techniques also may be combined readily with epi-fluorescence systems for increased flexibility. Added information may be obtained by viewing a specimen simultaneously or sequentially using phase-contrast or polarizing optics in conjunction with fluorescence optics. Similarly, many fluorescent stains are also visible in normal bright-field illumination and it is often desirable to examine stained specimens with both fluorescence and bright-field optics. For example, in bright-field illumination, Congo Red imparts red coloration to a variety of cereal carbohydrates, but in the fluorescence mode only mixed linkage β-glucans are fluorescent. Most research microscopes are capable of combining fluorescence epi-illuminators with the usual range of conventional optical systems. The instrument used in this study is a Zeiss Universal microscope including a III RS epi-illuminating condenser with three standard filter systems (see Table I), and bright-field, polarizing and phase-contrast optics. Specialized techniques such as microspectrofluorimetry (Fulcher *et al* 1972), immunofluorescence (Fulcher and Holland 1971; Craig *et al* 1979; Gibbons 1980), and image analysis using fluorescence systems (Munck *et al* 1980) are beyond the scope of this discussion.

Sample Preparation

Preparative methods which are common to other light microscope analyses are generally also useful for fluorescence analysis. These include most fixation procedures, cryotomy, and paraffin, epoxy or methacrylate embedding. The usual criteria regarding good fixation and embedding apply equally to fluorescence analysis as they do to other forms of microscopy.

For routine analysis of grains, it is often sufficient to simple cut relatively thin (10-20 μm) sections by hand using a clean razor blade and examine the sections directly for autofluorescence (see following section) or after the application of specific fluorescent dyes. Hand-cut sections offer the advantages of speed and minimal disruption or extraction of important constituents (e.g. lipids, vitamins) which might normally be lost to fixation, dehydration or embedding media. Some cereal grains (e.g. oats) are relatively soft and easily cut with a razor blade while others (e.g. hard wheats) are best softened before cutting by soaking in water at ice temperature. The epi-illuminating fluorescence condenser permits reasonable resolution of structures in hand-cut sections.

Similarily, ground or other processed

cereal samples (e.g. flours, concentrates) may also be readily examined for microchemical characteristics by fluorescence. Small amounts of powdered material should be mixed in one or two drops of fluorescence-free immersion oil under a cover glass for detection of primary fluorescence. For analysis after staining however, the nature of both the specimen and the fluorescent dye must determine the appropriate technique. Unlike conventional bright-field dyes, which are always deeply colored, some fluorochromes are not fluorescent until complexed with specific substrates. 8-Anilino-1-naphthalene sulfonic acid (ANS) for example, is not fluorescent in aqueous solution, but is intensely fluorescent when bound to hydrophobic sites on proteins (Weber and Laurence 1954; Gates and Oparka 1982). Thus, proteinaceous structures in flours or protein concentrates can be detected readily by mounting the sample directly in the aqueous fluorochrome solution under a cover glass; protein residues are intensely fluorescent and no extraneous fluorescence is evident. In other cases, excess fluorochrome must be washed from the sample after staining to minimize background fluorescence.

For high resolution fluorescence analysis, it is usually necessary to apply fixation and sectioning procedures which provide relatively thin sections (0.1-2.0 μm). Again, there are several techniques available, but the most useful of these employ glutaraldehyde fixation followed by glycol methacrylate embedding. Because mature grains are low in moisture (>15%) and extremely dense, the most critical preparation step is fixation. It is normally desirable to prolong fixation times to allow adequate fixation and hydration of all tissues; apparently, tissues which are not sufficiently hydrated subsequently will not embed properly. A typical preparation schedule requires fixation of 1-2 mm thick slices of grain in 5% (w/v) glutaraldehyde in 0.1 m sodium phosphate buffer (pH 6.8-7.2) for 2-3 days on ice. Acrolein or formaldehyde may be included in, or substituted for, this fixative solution for special purposes, but it is essential that heavy metals such as osmium or permanganate *not* be used; many heavy metals quench primary fluorescence in plant tissues, or interfere with the reaction of fluorescent dyes.

After fixation, cereal segments are dehydrated through methyl cellosolve, ethanol, *n*-propanol, and *n*-butanol and infiltrated with glycol methacrylate (GMA) monomer, and polymerized following the procedure of Feder and O'Brien (1968). Polymerized blocks containing tissue segments are cut to the desired thickness on glass knives using a Porter-Blum MT-1 ultramicrotome or similar instrument. Sections are then affixed to glass slides.

GMA is compatible with most aqueous staining solutions. The plastic is very hydrophilic and, unlike many epoxy-based embedding resins, does not interfere with staining reactions. The plastic also permits penetration by enzymes, allowing selective removal of cellular components by specific enzymes (Fulcher *et al* 1977).

Other methacrylate-based procedures may also be employed where it is necessary to minimize extraction of cellular components (especially lipids) during embedding. Hargin *et al* (1980) have adapted a "modified GMA" embedding procedure (Pease 1973) for cereal studies which is particularly useful for retaining neutral lipids (spherosomes) *in situ*. The fixed material is embedded directly in an aqueous mixture of urea/glutaraldehyde/glycol methacrylate and works well provided that water levels in the mixture remain relatively high (>35%). As with the previous GMA procedure, the modified-GMA method readily produces sections of the requisite thickness (0.2-2 μm).

Fluorescence Microchemistry

(a) Autofluorescence

Many plant tissues contain a variety of substances which are naturally fluorescent (autofluorescent) under the appropriate excitation wavelengths and may be detected microscopically without further manipulations or staining procedures. Phenolic compounds are the most common autofluorescent materials in cereal grains and generally occur in cell walls of many tissues. For example, the numerous trichomes which occur on the surface of an oat grain are intensely autofluorescent using FC I (Fig. 1). The identity of the autofluorescent substance is unknown, but it is readily detectable under short wavelength excitation. The intense blue autofluorescence which is characteristic of the aleurone cell walls of wheat (Fig. 2) and all major cereal grains is produced by high concentrations of ferulic acid, a low molecular weight derivative of cinnamic acid (Fulcher *et al* 1972). Ferulate derivatives also occur in the seed coat (Fig. 2) and embryo, but not in significant quantities in the starchy endosperm of mature grains. Characteristically, fluorescent phenolic compounds undergo a significant shift in fluorescence emission spectra to longer wavelengths in alkaline conditions. Thus, exposure of sections to high pH (e.g. ammonia vapor) prior to examination often produces a dramatic color shift, as shown by ammonia-treated barley aleurone cells (Fig. 3). Based on autofluorescence characteristics alone, there is considerable potential for more precise identification of cereal components *in situ* by microspectrofluorimetry, and for monitoring concentrations of specific grain substances in industrial systems, such as flour milling.

(b) Induced (secondary) Fluorescence

Although many cereal compounds may be autofluorescent, most are not and must be converted to fluorescent compounds by chemical treatment. This may be accomplished by applying fluorescent dyes or stains (fluorochromes) or by inducing a specific reaction in sections in order to produce a fluorescent product. Specific fluorescence procedures have been developed at Ottawa Research Station for most major cereal components and several examples follow. Some of the procedures employ common diachromes (colored, bright-field stains such as Congo Red) which are also intensely fluorescent under

appropriate excitation wavelengths. Others, such as Calcofluor and ANS, are colorless in visible light and are used only as fluorochromes. In some instances it has been necessary to adapt spot test or chromatographic spray reagents for histochemical use (e.g. for niacin and aromatic amine detection). Complete details of several of the following procedures have been described recently by Fulcher and Wong (1980), Hargin *et al* (1980), Fulcher *et al* (1981). Other procedures (e.g. for phytin and aromatic amines) represent new tests or improvements to earlier methods and complete details for their use are included.

(i) Phytin

Cereal phytin is a crystalline deposit of *myo*-inositol hexaphosphate, usually containing calcium, magnesium, and potassium. It occurs in the aleurone layer and scutellum of all cereals, and is the primary mineral reserve in the grain. In the past, phytin has been typically identified in grain sections by polarizing optics (the crystals are birefringent) or bright-field stains such as Toluidine Blue O (Jacobsen *et al* 1971; Fulcher *et al* 1981). These methods have been useful but both have limitations to their specificity. Many other grain components are birefringent in polarized light, and the typical red color of phytin after Toluidine Blue O staining is not specific to phytin.

In contrast, Acriflavine HCl provides improved specificity for high resolution fluorescence detection of phytin crystals. Sections are stained for 5-15 minutes in 0.01% (w/v) Acriflavine HCl in water adjusted to pH 3.1 with HCl. They are rinsed in ethanol until all traces of excess dye are removed, air-dried and mounted under a cover-glass in fluorescence-free immersion oil. Using this simple procedure, phytin crystals become fluorescent red when examined with any of the three excitation wavelengths described in Table I.

The apparent broad excitation spectrum of the phytin/dye complex demonstrates a significant advantage of fluorescence analysis. For example, short wavelength excitation (FC I) permits simultaneous demonstration of autofluorescent protein bodies (yellow-orange) and cell wall ferulic acid residues (blue) as well as the stained phytin deposits (red) as shown in Fig. 4. At longer excitation wavelengths (green, FC III), only the phytin crystals are visible, and in very high contrast. Thus, fluorescence filter systems can be manipulated considerably to provide additional information. The chemical basis of the phytin/Acriflavine HCl interaction has not yet been determined.

(ii) Aromatic Amines and Niacin

It is a feature of fluorescence microscopy that a variety of fluorochromes or procedures may be available for detecting a particular compound or functional group. Therefore, a reagent may be selected to maximize color contrast with background autofluorescence, or to confirm the identity of specific components. For example, acidic dimethylaminobenzaldehyde (Ehrlich's reagent, DAB) was initially used to locate aromatic amines in cereal aleurone layers (Fulcher *et al* 1981). The method is sensitive, but suffers from the disadvantage that the induced fluorescent reaction product is similar in its yellow color to occasional background protein autofluorescence. Substitution of a similar reagent (*p*-dimethylaminocinnamaldehyde, DAC) in the original procedure (see Fulcher *et al* 1981 for details) produces a bright red fluorescent reaction product which is quite distinct from background fluorescence (Fig. 5). Cereal bran contains significant concentrations of *o*-aminophenol and its glycosides (Mason and Kodicek 1973; Fulcher *et al* 1981) and the fluorescence colors of authentic *o*-aminophenol when reacted with DAB (yellow) or DAC (red) are visually indistinguishable from the colors induced in cereal aleurone layers by the same reagents. These reactions support the suggestion (Fulcher *et al* 1981) that aminophenol is concentrated in the aleurone cells. Reaction products with DAB or DAC are not found in the scutellum.

Similarily, niacin residues can be demonstrated using a variety of methods based on detection of glutaconic aldehyde groups following cyanogen bromide (CNBr) cleavage of the niacin pyridine ring (Feigl 1979). Following CNBr treatment, a yellow reaction product may be induced by reaction with *p*-aminobenzoic acid or an orange-red product (Fig. 6) with barbituric acid (Fulcher *et al* 1981). The latter is preferable for distinguishing the niacin reserves from autofluorescent substances. The reaction is sensitive and readily demonstrates that niacin reserves are concentrated in specific sub-units of aleurone layer protein bodies (Fig. 6).

(iii) Storage Lipids

Triglycerides are stored in plants in spherosomes (oil droplets) bounded by a half-unit or monolayer membrane of proteins and diacylphospholipids (Yatsu 1972, Jelsema *et al* 1977, Wanner and Theimer 1978). The aleurone layer and scutellum of most cereals are packed with lipid droplets and lower concentrations may also occur in the starchy endosperm. Nile Blue A is an excellent fluorochrome for demonstrating droplets of neutral lipid and has been employed together with chemical analysis of hand-dissected grain fractions) in the first demonstration of spherosomes in the starchy endosperm of wheat (Hargin *et al* 1980). The dye can be applied to cereal flours, hand-cut sections (Fig. 7) or thin sections of modified GMA-embedded material.

In visible light, Nile Blue A is a deep blue, water-soluble oxazine dye containing small concentrations (<3%) of a red, oil-soluble oxazone derivative (Smith 1908, Thorpe 1907). However, in the presence of neutral lipids and short wavelength illumination, the red component becomes intensely fluorescent yellow and provides an excellent microscopic marker for cereal lipid reserves which are typically 60-70% triglyceride and other non-polar lipids (Sahasrabudhe 1979).

Complete details of the staining procedure have been described recently (Hargin *et al* 1980). Briefly, hand-cut or modified GMA-embedded sections are stained for 30-60 seconds using 0.01% (w/v) aqueous Nile Blue A. The sections are washed briefly, mounted in water and viewed immediately using fluorescence filter system

FC II. Lipid reserves become intensely fluorescent yellow using this simple procedure and individual spherosomes can be resolved with little difficulty. Extraction of sections with hexane before or after staining confirms the identity of stainable reserves as neutral lipids. Figure 7 illustrates the distribution of neutral lipid reserves in a hand-cut section of an oat kernel. Lipid droplets can also be detected readily in flours by mixing a few drops of dye with a small amount of flour and viewing immediately.

In addition to the marked sensitivity of this dye in the fluorescence mode, Nile Blue A offers an additional advantage over other conventional lipid stains. Unlike the common Sudan dyes and Oil Red O for example, Nile Blue A is water-soluble and can be applied to specimens directly with little danger of lipid extraction during staining (presumably, the fluorescent derivative in Nile Blue A preparations partitions immediately into oil-rich reserves). In contrast, the common lipid dyes are applied in organic solvent/water mixtures. This procedure may extract a portion of the cellular lipids and invariably produces non-specific precipitates of dye on sections.

(iv) Cell Wall Carbohydrates

Two fluorochromes, Calcofluor White M2R New, and Congo Red are excellent microscopic markers for cereal endosperm cell walls. Calcofluor White is well known as a general stain for plant cell walls (Hughes and McCully 1975), and recent results have suggested that the fluorochrome exhibits a marked affinity for oat and barley β-(1→4)(1→3)-D-glucans (Wood and Fulcher 1978). Congo Red also produces a fluorescent product with these carbohydrates and is particularly useful in detecting β-glucan residues in tissues where blue, autofluorescent phenolic compounds are indistinguishable from the fluorescence of Calcofluor-stained structures. Figure 8 illustrates Congo Red-stained components in the aleurone cell walls of oats (associated with blue, ferulic acid-rich areas), as well as in the thick sub-aleurone starchy endosperm cell walls. Calcofluor-stained material is shown in Figure 9.

Details of the application of these two fluorochromes have been described previously (Wood and Fulcher 1978; Fulcher and Wong 1980) and further discussion regarding the chemistry of their interaction with β-glucans and other polysaccharides is available (Wood, 1980). Similarly, the use of Aniline Blue as a potential marker for β-(1→3)-D-glucans has also been described (Fulcher *et al* 1977; Fulcher and Wong 1980). All of these dyes are simple to use and provide extremely high contrast for most applications.

Additional fluorescent markers are also available for other major cereal components and details of their use have been described earlier (Fulcher and Wong 1980). These include markers for protein such as 8-anilino-1-naphthalene sulfonic acid (ANS), Fluorescamine, Acid Fuchsin, and Orange G, and for starch, which is readily detected using the periodic acid/Schiff's (PAS) procedure. Other components for which fluorescent markers are now available include such diverse components as DNA, flavonoids, and lignin.

It is a feature of fluorescence microscopy that two or more fluorochromes may be applied to a single section or specimen to demonstrate the distribution of up to several substances simultaneously. Figure 9 for example, illustrates a section of oat kernel which has been stained sequentially with Calcofluor White M2R New (blue, for β-glucans), propidium iodide (red, for DNA), the PAS reaction (substituting Acriflavine HCl for Schiff's reagent to demonstrate starch grains, green), and ANS (blue-white, for protein bodies). Several other of the reagents described in this review can also be used simultaneously, or sequentially, provided that the staining reactions do not interfere. Because there are often two or more fluorochromes available to detect a particular cereal component, it is also possible to select dyes to provide maximum color contrast in sections stained with multiple fluorochromes (as in Fig. 9).

Concluding Remarks

The figures accompanying this review illustrate only a few of the many fluorescent markers which are available for cereal analysis. They have been selected to best demonstrate the advantages of the fluorescence microscope over conventional microscopic methods, namely improved sensitivity, contrast and, in most instances, chemical specificity. The methods are extremely simple, rapid and are readily applicable to many different types of preparations, especially whole grains and flours. It is anticipated that these developments should prove to be particularly adaptable for more precise evaluation of both the distribution and chemistry of seed components by image analysis and microspectrofluorimetry.

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Figure Captions

Unless otherwise stated, all fluorescence photographs illustrate GMA-embedded sections of cereal grain tissues. Scale bars in μm. Abbreviations: al = aleurone layer; end = starchy endosperm; p = pericarp. Fluorescence filter combinations indicated as FC I or FC II (see Table I).

Figure 1. A sample of oat grain trichomes mounted in immersion oil showing intense autofluorescence. FC I.

Figure 2. A section of outer grain tissues of wheat showing blue ferulic acid autofluorescence in the aleurone cell walls (arrows). FC I.

Figure 3. A section of the outer tissues of barley kernel showing green autofluorescence in the aleurone cell walls after exposure to ammonia vapour. FC II.

Figure 4. A section of wheat aleurone cells showing red fluorescence in phytin granules (arrows) after staining with Acriflavine HCl. Blue autofluorescence is also visible in the cell walls (*) and aleurone protein bodies are yellow. FC I.

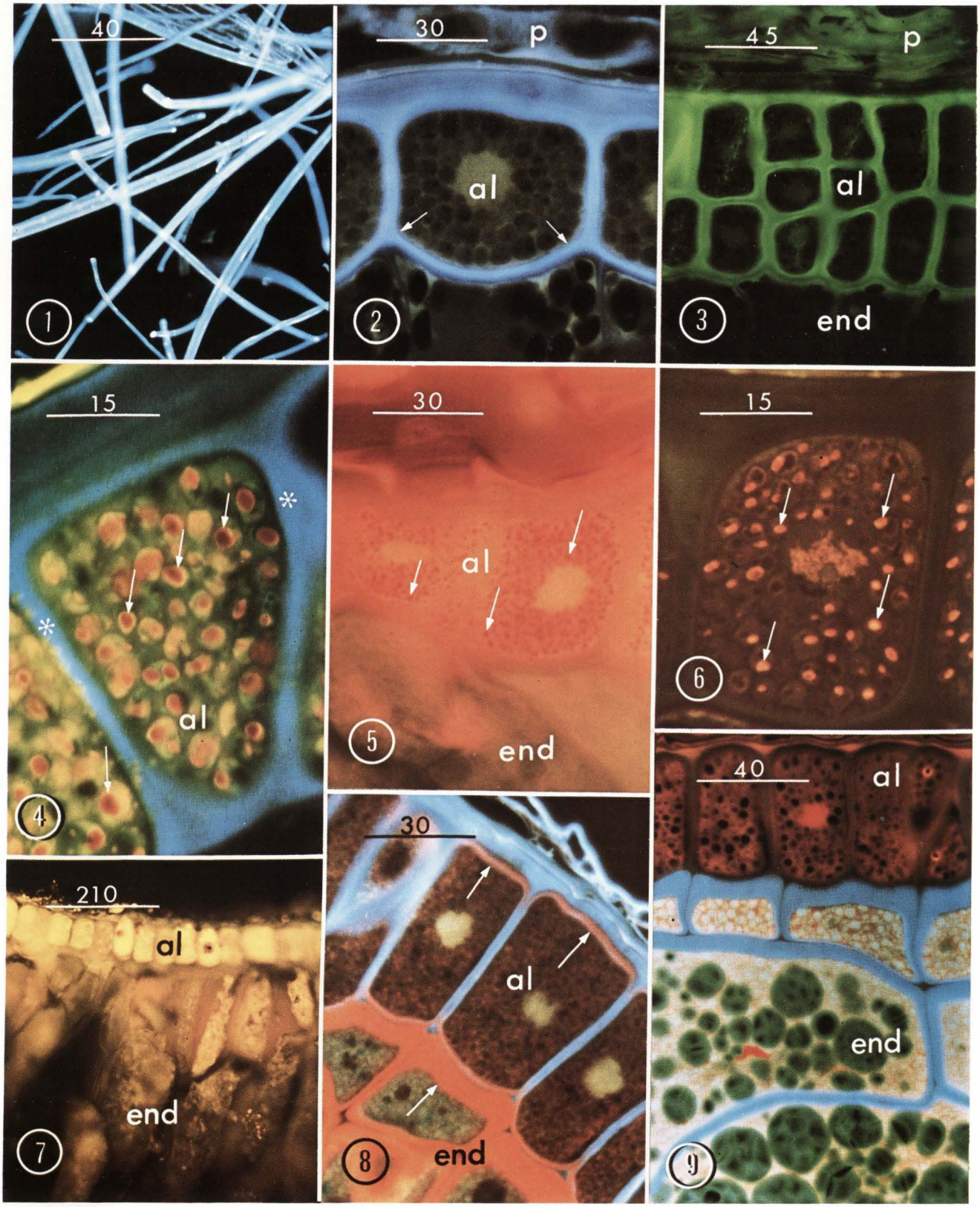
Figure 5. A hand-cut section of oat kernel stained with dimethylaminocinnamaldehyde (DAC) to show fluorescent red deposits of aromatic amines (arrows) in aleurone cells. FC II.

Figure 6. A section through wheat aleurone cells showing fluorescent orange niacin reserves (arrows) in the aleurone protein bodies after treatment with cyanogen bromide/barbituric acid. FC II.

Figure 7. A hand-cut section of oat grain demonstrating fluorescent yellow lipid reserves after staining with Nile Blue A. FC II.

Figure 8. A section of oat grain showing fluorescent red stained aleurone and endosperm cell wall components (arrows) after Congo Red staining. Ferulic acid residues are autofluorescent blue. FC I.

Figure 9. A section of oat grain stained using ANS, propidium iodide, Calcofluor White M2R New, and PAS treatments. Cell wall mixed-linkage β-glucans are blue, starch granules are green, nuclear DNA is red, and starchy endosperm protein bodies are blue-white. FC I.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Discussion with Reviewers

J.M. Faubion: What are the limitations of the fluorescent technique (both in application *and* interpretation)? The initiated scientist may be inclined to plow ahead with this obviously powerful technique without an understanding of what it can and can't do.
A. Bridges: These techniques appear quite straightforward. Are there any cautions or warnings we should heed if we apply them to other plants or food materials?
Author: The primary limitations are chemical. It is essential that chemical studies parallel microscopic tests to establish the identity of seed components which autofluoresce or interact with fluorochromes. Once reasonable confidence in the identity of the appropriate compounds has been established, microscopic analyses may be conducted rapidly to provide considerable chemical information. In most of the examples illustrated in this overview, extensive chemical characterizations have been conducted over several years and are described in detail in the cited references. Extrapolation of staining results to biological systems other than cereals should be made only after similar studies are completed.

J.M. Faubion: For Figure 9, does the order of fluorochrome application affect the image quality or information content? In fact, could you elaborate on fluorochrome compatibility?
Author: Certainly the order of fluorochrome application will influence both image quality and information content. For Figure 9, for example, the PAS reaction was conducted first because the periodic acid adversely affects any staining reactions applied prior to the periodate. In addition to pH, one must also consider staining times, solubility of fluorochrome, and stability of tissue when devising multiple staining procedures. In general, staining specificity becomes less reliable as more treatments are used. Most microscopists would have little requirement to employ more than two stains simultaneously, and Figure 9 is used only to demonstrate that more than one component can be visualized in one section. It should also be noted that non-fluorescent dyes can be used in sequence with fluorescence methods to quench non-specific or background fluorescence and thus increase contrast.

A. Bridges: In some methods for fluorophore induction further sensitivity is achieved by using gaseous reactions and controlling the humidity. Would you expect a similar advantage for any of the procedures you mention? If so, have you tried using gaseous reactions for any of the examples you give?
Author: Gaseous reagents, if available, are advantageous in preventing or minimizing migration or extraction of cellular components during fluorophore development. In fact, the test for niacin employs cyanogen bromide vapor for this reason and the method is described fully elsewhere (Fulcher and Wong 1980).

A.A. Urquhart: You state that Nile Blue A is a specific fluorochrome for non-polar lipids. Is there any evidence that Nile Blue A stains a particular component of the non-polar lipids?
Author: Chromatographic evidence indicates that the dye is intensely fluorescent in association with triglycerides and less so with mono- and diglycerides.

A.A. Urquhart: Are there any fluorescent stains for polar lipids?
Author: Chromatographic studies also indicate that an additional component in Nile Blue A provides a fluorescent product with fatty acids and other polar lipids. However, in the conditions of the present study this interaction has not been exploited and does not interfere with spherosome detection. Studies are in progress to establish the chemical basis of this interaction.

A.A. Urquhart: Is there any evidence to suggest that treatments during processing of cereal grains might alter the specificity of reactivity of a stain for a particular compound? In other words, if, during processing, staining for a particular compound is no longer evident, can you be sure that processing has extracted that compound, or is there a possibility that processing has modified the compound so that the specific compound-stain reaction is eliminated?
Author: This is a dilemma which sooner or later confronts every microscopist and often it is by no means certain that lack of staining indicates absence of stainable substrate. Thus it is essential that food microscopists be fully familiar with the physical and chemical processes by which products are manufactured, and it is equally important that methods selected for microscopic preparation be understood and devised to provide minimal modification of components under investigation. All manipulations of raw materials will produce some degree of alteration during industrial processing or preparation for microscopy.