CHAPTER

4

SPECTRAL BIO-IMAGING

YUVAL GARINI, NIR KATZIR, DARIO CABIB,
and ROBERT A. BUCKWALD

Applied Spectral Imaging Ltd.
Industrial Park
Migdal Haemek, Israel

DIRK G. SOENKSEN

Applied Spectral Imaging Inc.
Carlsbad, California 92009

ZVI MALIK

Life Sciences Department
Bar Ilan University
Ramat-Gan 52900, Israel

4.1. INTRODUCTION

Spectroscopy is a well-known analytical tool that has been used for decades to characterize the spectral signatures of chemical constituents. The physical basis of spectroscopy is the interaction of light with matter. Traditionally, spectroscopy is the measurement of the light intensity (I) that is emitted, transmitted, scattered, or reflected from a sample, as a function of wavelength, at high spectral resolution—but without any spatial information. Imaging, on the other hand, is primarily concerned with obtaining high spatial resolution information from a two-dimensional sample—but imaging usually provides only limited spectral information (e.g., by imaging with one or several discrete bandpass filters (1).

The work described here is based on the newly developed SpectraCube™ method of spectral bio-imaging, a combination of Fourier spectroscopy, charge-coupled device (CCD) imaging, light microscopy, and analysis soft-

Fluorescence Imaging Spectroscopy and Microscopy, edited by Xue Feng Wang and Brian Herman. Chemical Analysis Series, Vol. 137.
ISBN 0-471-01527-X © 1996 John Wiley & Sons, Inc.

Fluorescence Imaging Spectroscopy & Microscopy, edited by Xue Feng Wang and Brian Herman. © 1996 John Wiley & Sons, Inc.
Reprinted by permission of John Wiley & Sons, Inc.

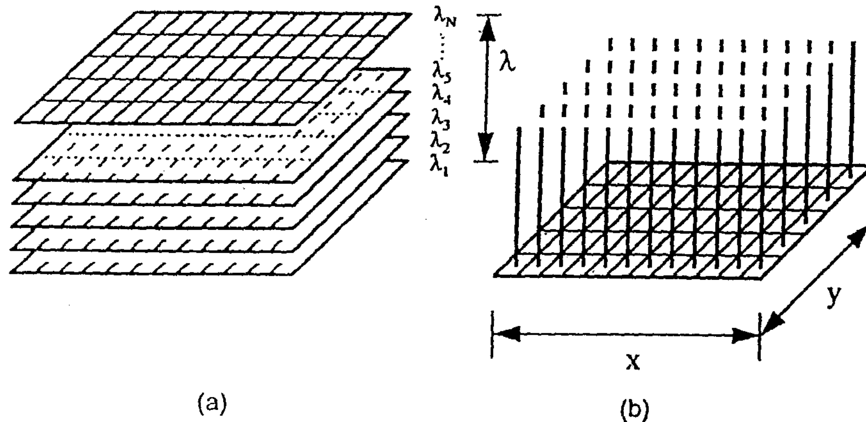


Figure 4.1. A spectral image can be viewed as a stack of contiguous images, each measured at a different wavelength, or as a two-dimensional array of vectors, with each vector representing the measured light spectrum at a given pixel.

ware for biological research applications. Spectral bio-imaging is a powerful method for measuring the spectrum of light at every picture element (pixel) of a two-dimensional image. The spectral images acquired by a system based on the SpectraCube™ method constitute a cube of information $I_{x,y}(\lambda)$, in which the full spectrum has been measured at each pixel position x, y. Since spectral images bring together the salient features of spectroscopy and imaging, new insights can be gained into the structure and dynamics of life by applying both spectral and image analysis algorithms to the same image data cube. Figure 4.1 is a schematic of a typical spectral image.

Combining spectroscopy with imaging is particularly useful in investigations with fluorescent probes. For example, spectral bio-imaging can be used to identify and map several fluorophores simultaneously in one measurement. In fact, the inherently high spectral resolution of spectral bio-imaging is ideally suited for sorting out fluorescent probes (or other chemical constituents) with overlapping spectra. Similarly, the imaging capabilities of spectral bio-imaging enable the detection, at any location in the image, of subtle spectral shifts (e.g., probes for pH). Spectral bio-imaging is a new modality of bio-imaging, a modality in which the high-resolution spectrum measured at each point in the image adds a new dimension, one that will enable the exploration of cells and tissues in new and exciting ways.

Spectral imaging has been, and continues to be, used successfully in the area of remote sensing to provide important insights into the study of planets, including Earth. By identifying characteristic spectral absorption features, it has been possible to study many aspects of Earth, including environmental

changes, important rock-forming minerals, the effects of soil composition on trees (i.e., by measuring shifts in the chlorophyll absorption band), forest fire damage, etc. However, the high cost, large size, and complexity of remote sensing spectral imaging systems (e.g., Landsat, AVIRIS) has limited their use to well-funded agencies (2–6). These systems are not practical for laboratory use with a microscope.

Performance improvements and the cost-effective availability of CCD imaging detectors, low-noise electronics, computers, and image analysis software have enabled the development of a commercially available spectral bio-imaging system for biological microscopy applications. In the following sections, the general structure, elements, and performance considerations of a spectral bio-imaging system based on the SpectraCube™ method will be described. Some potential fluorescence imaging applications, including examples of spectral images acquired with the SpectraCube™ method, will also be presented.

4.2. SPECTRAL BIO-IMAGING SYSTEMS

4.2.1. Overview

Conceptually, a spectral bio-imaging system consists of a measurement system and analysis software. The measurement system includes all of the optics, electronics, and software necessary to acquire a spectral image. The analysis includes all of the software and mathematical algorithms that are necessary to display and analyze spectral images acquired with the measurement system.

4.2.2. System Architecture

The system architecture of a typical spectral bio-imaging system is shown in Figure 4.2. The spectral bio-imaging system attaches, like a conventional CCD camera, to the video port of a microscope. Light is projected from the microscope port into the optical head, which consists of three main elements:

1. An inteferometer (or spectral dispersion element) that enables the separation of light into its spectral components, i.e., different wavelengths
2. A CCD array detector that collects, for all pixels in the array simultaneously, the light intensity required to measure (or calculate) the spectrum at each point in the image
3. Collection and imaging optics that produce a real image on the CCD array while enabling proper operation of the interferometer; in particu-

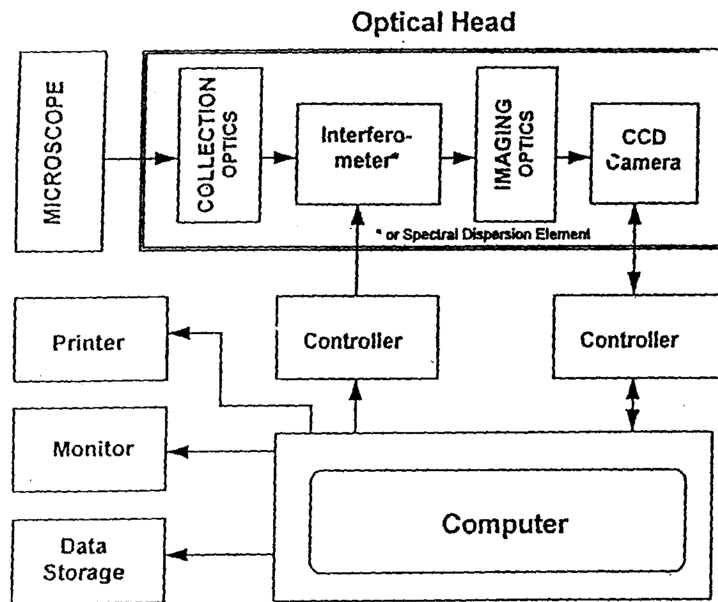


Figure 4.2. System architecture of a typical spectral bio-imaging system. The optical signal (image) originating in the microscope passes through the interferometer (or spectral dispersion element) and is focused on the CCD camera. Both the interferometer and the CCD have their own controllers, which in turn are controlled by the computer. The computer orchestrates the overall acquisition of spectral images and is used to display, analyze, and output spectral images.

lar, the collection optics should be such that all the light projected from the microscope will be collected by the optical head

Both the interferometer and the CCD camera usually require dedicated controllers. The coordination of the interferometer (or spectral dispersion element) with the CCD camera is orchestrated by a dedicated computer equipped with the appropriate image acquisition and processing hardware and software. Because spectral image analysis is one of the most important and challenging aspects of spectral imaging, this important subject will be discussed in Section 4.3.

4.2.3. Fourier Spectroscopy and Spectral Dispersion Methods

The heart of any spectral bio-imaging system is an interferometer (or spectral dispersion element) that enables the separation of the light entering the optical head into its spectral components. Interferometric spectroscopy (better known as Fourier spectroscopy) is one of three methods of separating light into its consituent spectral components. The other two methods, grating and spectral filters, are spectral dispersion methods that might also be considered for a spectral bio-imaging system.

In a grating (monochromator)-based system, only one axis of the CCD array detector (the spatial axis) provides real imagery data; the other (spectral) axis is used to measure the intensity of the light dispersed by the grating. An image can be obtained only after scanning the grating parallel to the spectral axis of the CCD. This inability to visualize the two-dimensional image makes it virtually impossible to choose, prior to making a measurement, a desired region of interest from within the field of view and to optimize the system focus or exposure time. Grating-based spectral imagers are popular for remote sensing applications because an airplane (or satellite) flying over the surface of the Earth provides a natural scanning mechanism. A list of spectral imaging systems based on a monochromatic grating can be found in Ref. 7.

Only the Fourier spectroscopy or filter approaches are able to provide a real two-dimensional image that can be observed, focused, and optimized prior to the measurement. These two methods acquire imagery data in both spatial axes (x and y) simultaneously, thus providing a complete two-dimensional image for every frame time of the CCD camera. A more detailed discussion of these two methods follows.

4.2.3.1. Fourier Spectroscopy

Fourier spectroscopy uses an interferometer and Fourier transform methods to derive the spectrum. The principles of this method are as follows: The interferometer divides the incoming beam (in this case, the light projected from the port of the microscope) into two coherent beams and creates a variable optical path difference (OPD) between them. The beams are then recombined to interfere with each other, and the resulting interference intensity is measured by the detector as a function of the OPD. This intensity vs. OPD function, called an interferogram, is then Fourier transformed to recover the spectrum. Fourier spectroscopy is a well-known technique that is widely used to make high-resolution, high-sensitivity spectral measurements for a variety of applications, mainly in the infrared spectral range (8, 9). When applied to an imaging system, the interferogram is measured individually at each pixel in the CCD array. Fourier transformation thus yields a distinct spectrum at each pixel. A more detailed explanation of Fourier spectroscopy is given in the appendix.

Fourier spectroscopy enjoys several important advantages as a method for measuring spectral data, especially when applied to fluorescence imaging microscopy. These advantages include:

1. High optical throughput, achieved by the efficient collection of the intensities at all wavelengths in the source spectrum during the *entire* acquisition time (unlike a filter-based system, which at any moment only measures the intensity in a single narrow spectral band)
2. High and variable spectral resolution
3. A wide spectral range that can also be extended to the ultraviolet (UV) and infrared (IR) spectral regions.

The high optical throughput advantage of Fourier spectroscopy results in measurements with higher signal-to-noise ratios (SNRs), relative to the SNR measured with a filter-based system, or enables faster measurements. Higher SNR or reduced acquisition times are important when low-light signals are measured in fluorescence microscopy. Furthermore, the advantage of high and variable spectral resolution allows the user to trade off sensitivity, spectral resolution, and acquisition time in a way that is not possible with any other method. Furthermore, interferometers do not polarize the incoming light, thus facilitating the direct measurement of polarization effects.

A disadvantage of interferometric spectroscopy is that the mathematical Fourier transform operation must be performed in order to observe the spectrum. In a filter-based system, on the other hand, the intensity of the spectrum can be measured directly (over a narrow spectral region).

4.2.3.2. *Filter-Based Spectral Dispersion Methods*

Filter-based dispersion methods can be further categorized into discrete filters and tunable filters. The use of discrete spectral filters typically requires inserting a few filters into a filter wheel, which is rotated, synchronous with the frame rate of the CCD camera. Tunable filters, such as acousto-optic tunable filters (AOTFs) and liquid-crystal tunable filters (LCTFs), have no moving parts and can be randomly tuned to any wavelength in the spectral range of the device. One advantage of using tunable filters as a dispersion method for spectral imaging is their random wavelength access, i.e., the ability to measure the intensity of an image at a number of wavelengths in any desired sequence. However, AOTFs and LCTFs have the disadvantages of (i) limited spectral range (typically $\lambda_{max} = 2\lambda_{min}$), and all radiation that falls outside of this spectral range *must* be blocked; (ii) temperature sensitivity; (iii) poor light throughput; (iv) polarization sensitivity; and (v) in the case of AOTFs, shifting of the image during wavelength scanning. Nevertheless, both LCTFs and AOTFs have been used successfully in spectral imaging systems, primarily for nonbiological applications. The interested reader is referred to Morris et al. (10) and Yu et al. (11) for further details.

4.2.4. Charge-Coupled Device Detectors

The CCD (camera) detector is one of the most critical elements of a spectral bio-imaging system. Although the CCD camera is located at the end of the light path, its performance largely determines the overall spectral imaging performance that can be achieved. For a detailed discussion of CCDs, refer to Chapter 21 in Mason (12). It is assumed that the reader has some familiarity with CCD detectors. A brief qualitative discussion of some of the more important parameters of CCDs and their relevance to spectral bio-imaging follows.

4.2.4.1. *Pixel Size*

The pixel size of a CCD array detector, typically between 6 and 25 μm along each dimension of the pixel, is important for several reasons. First, and most important, the pixel size defines the best achievable *spatial resolution* of the imaging system. Selecting a CCD array with smaller pixels improves the spatial resolution, allowing higher spatial frequency details to be sampled without aliasing. This reasoning would suggest that it is best to always select the CCD array with the smallest pixel size; however, this is not always the case because smaller pixels also have smaller well capacities for storing photoelectrons. Since the well capacity (and readout noise) define the dynamic range of the CCD detector (i.e., the ratio of the maximum to the minimum signal that can be measured simultaneously), pixel size is proportional to dynamic range. Thus CCD arrays with larger pixels have a proportionately higher dynamic range. In many spectroscopy applications, a large dynamic range is required in order to separate chemical constituents that exhibit subtle spectral differences; hence, spatial resolution and dynamic range must be traded off, depending on the specific requirements of an application.

4.2.4.2. *Spectral Response*

The spectral response of a CCD camera is primarily a function of the energy band-gap structure of the silicon material on which most CCDs arrays are based. In practice, the spectral range of a typical CCD detector extends from 400 to 1100 nm, with peak quantum efficiencies between 40 and 50%. By applying UV coatings to the surface of the CCD, the spectral response can be extended to the UV region. More expensive back-illuminated CCDs are available with good response in the UV and improved quantum efficiencies (as high as 85%).

4.2.4.3. *CCD Camera Noise*

There are three primary sources of noise in a typical CCD camera: shot noise, dark noise, and readout noise. Photon shot noise is the result of statistical

fluctuations in the signal; it is proportional to the square root of the signal intensity. Dark noise, on the other hand, is caused by electrons that are excited by the CCD's thermal energy. Dark noise is linearly dependent on the square root of the frame integration time of the CCD (typical units are electrons per second), where dark noise is the square root of the dark charge that accumulated in the CCD. Thus, in order to maintain low dark noise when long integration times are required (e.g., to measure low-light signals), it is frequently necessary to cool the CCD (e.g., thermoelectrically or with liquid nitrogen). Readout noise originates primarily in the output electronics of the CCD. The pixel readout noise is proportional to the data readout rate of the CCD camera. Thus, lower readout noise requires low pixel rates, while high pixel rates are usually accompanied by higher noise. Here too a trade-off must be made, depending on the application, between faster readout rate and reduced readout noise.

4.2.4.4. *Chip Architecture and Readout Modes*

The chip architecture of the CCD describes the structure of the CCD chip. A typical CCD chip employed in a high-performance camera is either a full-frame CCD or a frame-transfer CCD. In a full-frame CCD the active area encompasses the entire area of the chip. In a frame-transfer CCD, on the other hand, the CCD chip is divided into two areas: a sensing (active) area and a storage area. The photoelectrons that accumulate in the sensing area during a single frame integration time can be transferred to the storage area very quickly, allowing data in the storage area to be read while the sensing area is exposed to acquire the next image. Frame-transfer CCDs can accelerate the acquisition time of multiple images and are thus preferred for spectral bio-imaging. Another advantage of frame-transfer CCDs is that they can often be used effectively without a mechanical shutter, further facilitating higher effective CCD frame rates.

4.2.5. Performance of a Spectral Bio-Imaging System

Since a spectral bio-imaging system consists of several elements (e.g., an optical head, a CCD camera, and a computer), it is important to characterize its performance as a system. The performance parameters of a spectral bio-imaging system can be categorized into imaging, spectral, and overall performance parameters. A brief discussion of the most important parameters follows.

4.2.5.1. *Imaging Performance*

4.2.5.1.1. Spatial Resolution. The spatial resolution of a spectral bio-imaging system depends on two major parameters: the pixel size and the optical modulation transfer function (MTF) of the system. If the optics are well designed (usually a good assumption), the spatial resolution is essentially limited by the pixel size. In this case, the spatial resolution is approximately equal to $x = 2d/M$, where x is the smallest resolvable dimension in the sample, d is the pixel size, and M is the overall magnification of the optics between the sample and the detector, including the magnification of the microscope and any other magnification effects introduced by the system.

4.2.5.1.2. Field of View. The field of view, X (defined here as the total size of the sample that can be measured), is equal to Nd/M, where N is the number of pixels in a row (or column) of the CCD array and M and d are as defined above.

4.2.5.1.3. Sensitivity. Sensitivity (as defined here) is the lowest light level at which the system achieves reasonable performance. Because the illumination, the spectral response of the CCD, and the transmission of the optics are not uniform in a real system, the sensitivity is actually different for each wavelength. Sensitivity is usually specified in units of lux (lumes/m^2), a photopic unit that represents a "human eye" weighted average of the (spectral) sensitivity. When a high-quality cooled CCD is used, the sensitivity (to first order) improves linearly with the integration time; it is therefore important to specify the sensitivity at a given integration time. A parameter related to the sensitivity is the minimum detectable light level, i.e., the incident light level at which the SNR at the output of the detector is 1. The minimum detectable light level is always lower than the sensitivity.

4.2.5.1.4. Dynamic Range. The dynamic range of a spectral bio-imaging system defines the largest differences in the intensities of incident light that can be observed in one spectral image. The system dynamic range depends largely on the dynamic range of the CCD detector, i.e., the number of meaningful levels into which a full-scale optical signal incident on the CCD can be divided. The CCD dynamic range is typically given by the number of real bits of the CCD, n, and the corresponding number of separable levels is equal to 2^n. There are other factors that can affect the system dynamic range, and in some cases the actual system dynamic range can increase over that of the CCD detector.

4.2.5.2. *Spectral Performance*

4.2.5.2.1. Spectral Range. The spectral range defines the range of wavelengths over which the system can measure the spectrum. Because the response of a real system is not spectrally uniform, it is important to characterize the spectral response of the system (e.g., via a plot of relative response vs. wavelength). The

spectral range is usually given by the minimum and maximum wavelengths at which the spectral response exceeds some threshold (e.g., 5%).

4.2.5.2.2. Spectral Resolution. The spectral resolution defines the ability of a spectral bio-imaging system to distinguish (or spectrally resolve) two signals that are separated by a small spectral difference. Spectral resolution is typically specified by the full width at half-maximum (FWHM) criterion, a parameter that can be checked by measuring the "spread" in the spectrum of a very (spectrally) narrow source (e.g., a laser). Depending on the specific nature of the spectral peaks, the spectral resolution achievable in a real system is often smaller (i.e., better) than the FWHM value.

4.2.5.2.3. Polarization. Here polarization is defined as the degree of polarization of the system, i.e., the transmission of the system as a function of the polarization of the incident light.

4.2.5.3. General Performance

The measurement of a spectral image requires significantly more time than is required to acquire a single frame using a conventional (CCD) imaging system. The reason for this increase in the acquisition time is that the spectral image must be measured at different wavelengths (or different OPDs). The measurement time depends both on the effective frame time (i.e., the sum of integration time, data transfer time, and any additional overhead required to change the wavelength of the OPD) and on the total number of frames that should be measured. The time required to transfer the data from the CCD camera to the computer can impose a significant overhead on the effective frame rate; thus, selection of CCDs with fast readout rates is often desirable. For many high-performance CCD cameras, the readout rate can be increased by selecting a subset (i.e., a region of interest) of the total number of pixels in the CCD array.

4.2.6. The SpectraCube™ Method

The SpectraCube™ method of spectral bio-imaging combines Fourier spectroscopy with a CCD array detector and powerful analysis methods. The interferometer is a special type of common path interferometer known as a Sagnac interferometer. A simplified optical diagram of the SpectraCube™ method is shown in Figure 4.3. In a Sagnac interferometer, the two beams (delayed by a known OPD) travel in the same (common) path, but in opposite directions, and are then recombined, enabling the measurement of interference intensity by the detector. The advantage of a common path interferometer is its intrinsic stability. Disturbances, such as a small shift of one of the optical elements, affect

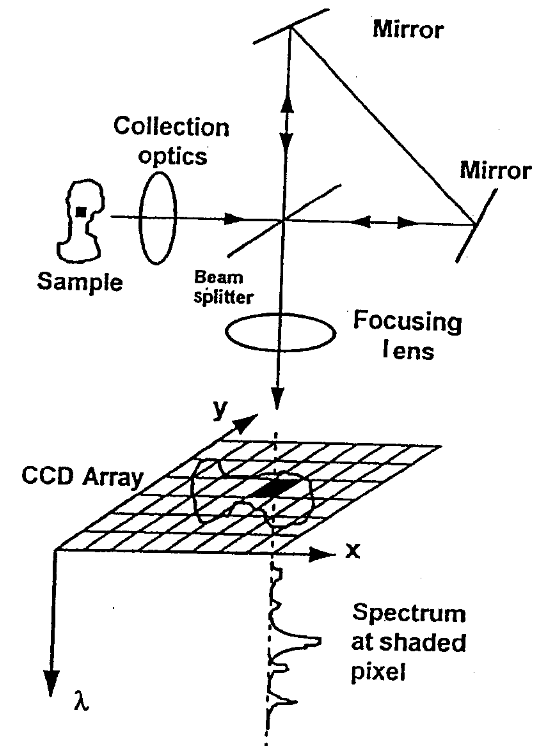


Figure 4.3. Simplified optical diagram of a spectral bio-imaging system based on the SpectraCube™ method. The light emitted by the sample is collected by the fore-optics and enters the Sagnac interferometer. The real image (plus interference fringes) is focused on the CCD array, yielding (on Fourier transformation) the spectrum at each pixel.

both beams the same way and hence have a minor effect on the measurement. In addition to being rugged, this type of interferometer is relatively compact. A more detailed discussion of the Sagnac interferometer is given in the appendix.

The SpectraCube™ interferometer is designed in such a way that a real image (on which the interference fringes generated by the Sagnac interferometer are superimposed) is projected on the CCD detector for any OPD. This image does not move when the OPD is changed. The ability to see a real image of the sample of interest facilitates definition of the region of interest (ROI) over which the spectral image will be measured, as well as optimization of measurement parameters such as focus, integration time, and so on.

During the measurement, the OPD is changed and many frames of data are acquired (and stored) at many different OPDs until the entire cube of inter-

ferometric data has been acquired. It is important to realize that each pixel acts very much like an independent Fourier spectrometer, and the acquisition of a spectral image is analogous to the simultaneous measurement of a complete interferogram, in parallel, by tens of thousands of point spectrometers, each focused on one small element of the sample. Fourier transformation of this interferogram data cube yields the complete spectrum at every pixel, i.e., the spectral image.

The spectral bio-imaging system described here, the SD200 (Applied Spectral Imaging Ltd., Migdal Haemek, Israel), is based on the SpectraCube™ method. The CCD camera employed by the SD200 is a 512 × 512 × 2 pixel, thermoelectrically cooled, frame-transfer CCD with 12 bits of dynamic range (Princeton Instruments, Trenton, New Jersey). The pixel size is 15 μm square, and the readout rate is 1 Mixpel per second. This system is based on a 486/66 personal computer. The SD200 achieves a spatial resolution equal to $30/M$ μm and a field of view equal to $8/M$ mm, where M is the effective microscope magnification. The sensitivity of the SD200 is approximately 20 millilux for 100 ms integration times, while the spectral range is 400–1000 nm. Spectral resolutions of 4 nm at 400 nm (14 nm at 800 nm) are achievable. The SD200 system easily attaches to any microscope with a C-mount or F-mount connector and can stand in any orientation during the measurement.

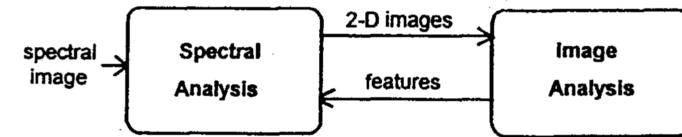
4.3. SPECTRAL IMAGE ANALYSIS

4.3.1. Overview

A spectral image is a three-dimensional array of data, $I(x, y, \lambda)$, that combines precise spectral informaton with spatial correlation. As such, a spectral image is a novel data base that enables the extraction of features and the evaluation of quantities that are difficult, and in some cases impossible, to obtain otherwise. Since both spectroscopy and digital image analysis are well-known fields (13) with an enormous literature, this discussion will focus primarily on the benefits of combining spectroscopic and imaging information in a single (spectral image) data base.

One simple approach to the analysis of a spectral image data base is to perform the analysis of spectral and spatial (image) data separately, i.e., to apply spectral algorithms to the spectral data and (two-dimensional) image processing algorithms to the spatial data. In this case, a two-dimensional monochrome image (or set of images) will be created as a result of spectral analyses that have been performed on each of the image pixels (point operations). These gray scale images can then be further analyzed using image processing and computer vision techniques (e.g., image enhancement, pattern recognition) to extract features and parameters of interest. This handshaking between the spectral- and

image-based algorithms can in general be iterated, as shown in the accompanying diagram.



Of course, it is also possible to apply spectral image algorithms based on nonseparable operations, i.e., algorithms that include both local spectral information and spatial correlation between adjacent pixels. The examples given in the following section will be limited to the simpler case where spectral and spatial algorithms are separable.

4.3.2. Displaying Spectral Images

One of the basic needs that arise naturally when dealing with any three-dimensional (3-D) data structure such as a spectral image, is visualizing that data structure in a meaningful way. Unlike other types of 3-D data such as tomographic data, $D(x, y, z)$, where each point represents, in general, a different location (x, y, z) in *space*, a spectral image is a sequence of images representing the intensity of the same two-dimensional *plane* (i.e., the sample) at different wavelengths. For this reason, the most intuitive way to view a spectral image is to view either the image plane (spatial data) or the spectral axis. In general, the image plane can be used to display either the intensity measured at any single wavelength or the gray scale image that results after applying a spectral analysis algorithm, over a desired spectral region, at every image pixel. The spectral axis can, in general, be used to present the resultant spectrum of some spatial operation performed in the vicinity of any desired pixel (e.g., averaging the spectrum from several adjacent pixels).

It is possible, for example, to display the spectral image as a gray scale image similar to the image that might be obtaind from a simple monochrome camera. Since such a camera simply integrates the optical signal over the spectral range of the CCD array, the equivalent monochrome CCD camera image can be computed from the 3-D spectral image data base by integrating along the spectral axis as follows:

$$\text{gray scale}\,(x, y) = \int_{\lambda_1}^{\lambda_2} w(\lambda) \cdot I(x, y, \lambda)\, d\lambda \tag{1}$$

Here $w(\lambda)$ is a general weighting response function that provides maximum flexibility in computing a variety of gray scale images, all based on the integration of an appropriately weighted spectral image over some spectral range. For

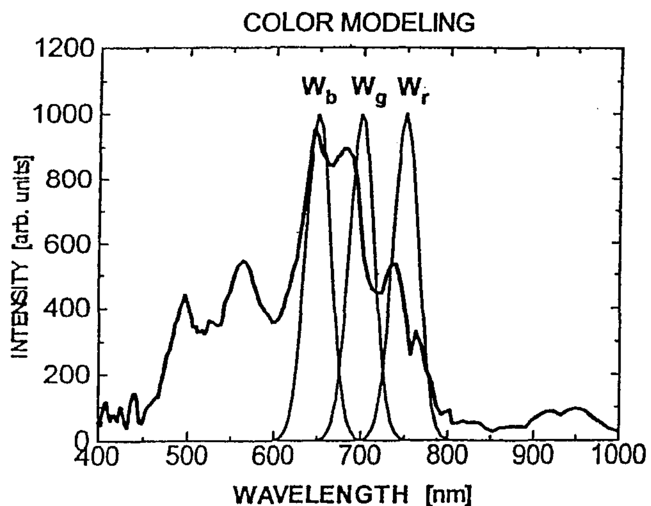


Figure 4.4. Definition of pseudo-RGB colors for emphasizing chosen spectral ranges. The intensity for each pseudocolor is calculated by integrating the area under the curve after multiplication by the appropriate pseudo-RGB spectral filter function.

example, by evaluating Eq. (1) with three different weighting functions, $\{w_r(\lambda), w_g(\lambda), w_b(\lambda)\}$, corresponding to the tri-stimulus response functions for red (R), green (G), and blue (B), respectively, it is possible to display a conventional RGB color image. It is also possible to display meaningful nonconventional (pseudo) color images. As an example of the power of this simple algorithm, consider choosing $\{w_r, w_g, w_b\}$ to be Gaussian functions distributed "inside" a spectrum of interest (Figure 4.4). The resulting pseudocolor image that is displayed in this case emphasizes only data in the spectral regions corresponding to the weighting functions, enabling spectral differences in these three regions to be detected more clearly.

4.3.3. Point Operations

Point operations are mathematical operations that can be performed on the image pixels of a variety of images. For example, in a traditional gray scale image, point operations map the intensity $v_1 \in [0, L]$ at a given image pixel into another intensity $v_2 \in [0, L]$ according to some transformation $v_2 = f(v_1)$. A simple example of a point operation applied to a gray scale image is the multiplication of the intensity at each pixel by some constant. The concept of point operations can also be extended to spectral images, in which each pixel constitutes a spectrum, i.e., an n-dimensional vector $_1(\lambda)$; $\lambda \in [\lambda_1, \lambda_n]$. A point operation applied to a spectral image would thus map the spectrum at any

image pixel into a scalar (i.e., an intensity value) according to the transformation

$$v_2 = g(V_1(\lambda)); \qquad \lambda \in [\lambda_1, \lambda_n]$$

The evaluation of the gray scale image in Eq. (1) is an example of this type of spectral image point operation. In the more general case, a point operation maps the spectrum (vector) at any image pixel into another vector in accordance with the transformation

$$_2(l) = g(V_1(\lambda)); \qquad l \in [1, N], \qquad \lambda \in [\lambda_1, \lambda_n]$$

where $N \leq n$.

If one now extends the definition of point operations to include operations between corresponding pixels of *different* spectral images, it is possible to categorize the entire field of spectroscopic calculations as point operations. Furthermore, since the spectrum at every pixel (x_0, y_0) is given by

$$s(x_0, y_0) = I(x_0, y_0, \lambda); \qquad \lambda \in [\lambda_1, \lambda_n]$$

the extraction of spectroscopic information from a spectral image (e.g., determination of absorption wavelengths and ratiometric computations) can be done separately on any pixel of the image. An important example of this type of point spectral arithmetic is the optical density function defined by

$$OD(x, y, \lambda) = -\log \frac{I_s(x, y, \lambda)}{I_b(x, y, \lambda)} \qquad (2)$$

where I_s is the (normal) transmission spectral image through the sample, and I_b is the spectral image measured in the absence of the sample (but under otherwise identical conditions). Note that the optical density is invariant both to the spectral response of the measuring system and to the nonuniformity of the CCD detector.

4.3.4. Spatial-Spectral Operations

In all of the spectral image analysis methods mentioned above, algorithms were applied to the spectral data. The value added by also displaying the spectrally processed data as an image is mostly qualitative, providing the user with the aesthetically pleasing picture. It is also possible, however, to use the available imaging data in much more meaningful ways by applying algorithms that utilize the spatial-spectral correlation inherent in a spectral image. Spatial-spectral operations represent the most powerful types of spectral image analysis algorithms. As an example, consider the following problem:

ains k cell types stained with k different fluorophores. Each fluorophore has orescence emission spectrum and binds to only one of the k cell types. It is find the average fluorescence intensity per cell for each of the k cell types. task involves the following procedure (subtasks 1–3):

y each pixel in the image as belonging to one of $k+1$ classes (k cell types plus ground) due to its spectrum.

nt the image into the various cell types, and count the number of cells of each

e fluorescence energy contributed by each class and divide it by the total r of cells from the corresponding class.

lure makes use of both spectral and spatial data. The relevant a take the form of characteristic cell spectra (i.e., spectral "signa- e the spatial data consist of data about various types of cells (i.e., cell y of which appear similar to the eye. The solution to this problem pectral image (data base). Since the (spectral) λ axis is intrinsically m the spatial axes (x, y), an intuitively simple analysis approach in ectral and spatial features are separable will be considered.
bove problem, cells can be differentiated by their characteristic nature. Hence, a suitable point operation will be performed to ynthetic image in which each pixel is assigned one of $k+1$ values. at the fluorescence emission spectra of the different cell types are $s_i(\lambda)$; $i=1,2,\ldots,k$, $\lambda\in[\lambda_1,\lambda_n]$ and the measured spectrum at $x,y)$ is $s_{x,y}(\lambda)$, $\lambda\in[\lambda_1,\lambda_n]$, the following algorithm is an optional he classification problem (subtask 1):

error between the measured spectrum and the known spectrum of the ttached to cell type i. Then, adopting the least-squares criterion, one can

$$e_i^2=\sum_{\lambda\in R_\lambda}(s(\lambda)-s_i(\lambda))^2, \tag{3}$$

spectral region of interest. Each point [pixel (x, y)] in the image can then to one of the $k+1$ classes using the following criterion:

$$)\in\begin{cases}\text{class } k+1 & \text{if } e_i^2<\text{threshold, for all } i\in[1,k]\\ \text{class } \rho & \text{otherwise, if } e_\rho^2<e_i^2\text{, for all } i\in[1,k] \text{ excluding } \rho\end{cases} \tag{4}$$

nd 3 (image segmentation and calculation of average fluorescence now straightforward, using standard computer vision operations etic image created in accordance with the algorithm described in 4).

Another approach to solving this problem is to express the measured spectrum $s_{x,y}(\lambda)$ at each pixel as a linear combination of the k known fluorescence spectra $s_i(\lambda)$; $i=1,2,\ldots,k$. In this case one would find the coefficient vector $C=[c_1,c_2,\ldots,c_k]$ that solves

$$F=\min\sum_{\lambda\in R_\lambda}(s(\lambda)-\hat{s}(\lambda))^2$$

where

$$\hat{s}(\lambda)=\sum_{i=1}^{k}c_i\cdot s_i(\lambda) \tag{5}$$

Solving $\partial F/\partial c_i=0$; for $i=1,2,\ldots,k$ (i.e., find values of c_i that minimize F) yields the matrix equation $C=A^{-1}B$, where A is a square matrix of dimension k with elements

$$a_{m,n}=\left[\sum_{\lambda\in R_\lambda}s_m(\lambda)\cdot s_n(\lambda)\right]$$

and B is a vector defined as

$$b_m=\left[\sum_{\lambda\in R_\lambda}s_m(\lambda)\cdot s(\lambda)\right],\qquad m,n=1,2,\ldots,k$$

4.3.5. Similarity Mapping Algorithms

Spectral images allow the comparison of any spectrum selected from within the spectral image to any of the other (tens of thousands of) spectra in the image. The degree of similarity between the spectrum at each pixel and some chosen (reference) spectrum can thus be calculated mathematically (i.e., it is a quantitative result). This procedure, called *similarity mapping*, creates a (two-dimensional) gray level image from the original (three-dimensional) spectral image. In principle, a similarity map algorithm highlights all the pixels in the image that have spectral characteristics similar to those of the selected reference pixel. The similarity map thus enhances the ability of the human eye to differentiate fine details in the stained cell.

There are many algorithms that could be envisioned for generating similarity map images. One form that has been used successfully is given by Eq. (6):

$$G_{x,y}=\frac{1}{\int_{\lambda_1}^{\lambda_2}|I_{x,y}(\lambda)-R(\lambda)|\,d\lambda} \tag{6}$$

In the equation $I_{x,y}(\lambda)$ is the measured spectrum at pixel x, y and $R(\lambda)$ is the (user-defined) reference spectrum. $G_{x,y}$ is the (scalar) result of the similarity algorithm at pixel (x, y). This algorithm calculates, for each pixel, the absolute difference in the area bounded by the measured spectrum and the reference spectrum over the spectral range $\lambda_1 - \lambda_2$. This "area difference" is computed for every pixel, which is then assigned a gray level that is inversely proportional to the area difference. It should be noted that while the reference spectrum in this example was selected directly from a pixel in the *measured* spectral image, it is also possible to perform similarity mapping using reference spectra that have been stored (e.g., in a library of reference spectra). This approach is useful for automating the analysis of spectral images, in particular, when the location of a chemical constituent with a known spectral signature is of interest.

4.3.6. Practical Considerations

It should be clear that the methods discussed above represent only a small fraction of the possible spectral image analysis methods. Customized algorithms will continue to be developed to support specific spectral bio-imaging methods and applications.

4.4. MEASUREMENT METHODS AND APPLICATIONS

4.4.1. Overview

Spectral bio-imaging systems are potentially useful in all applications in which subtle spectral differences exist between chemical constituents within an image. The measurement can be carried out using virtually any optical system—for example, an upright microscope, an inverted microscope, a dissecting microscope, or even a macro lens. Any standard experimental method can be used, including transmission (bright-field) imaging microscopy and fluorescence imaging microscopy.

Fluorescence measurements can be made with any standard filter cube (consisting of a barrier filter, an excitation filter, and a dichroic mirror) or any customized filter cube for special applications, provided that the emission spectra fall within the spectral range of the system. Spectral bio-imaging can also be used in conjunction with any standard spatial filtering method such as dark field, phase contrast, and even polarized light microscopy. The effects on spectral information when using such methods must, of course, be understood to correctly interpret the measured spectral images. [Refer to Kam (14) for a comprehensive discussion of microscopic techniques.]

There are many experimental methods and specific applications for spectral bio-imaging systems in transmission and fluorescence microscopy. These methods and applications include the following:

Fluorescence Microscopy

- Spectral identification of multiple fluorophores
- Detection of microenvironmental changes in subcellular compartments (e.g., pH) and dyes characterization
- Measurement of fluorescence from natural pigments (e.g., chlorophyll)
- Fluorescence resonance energy transfer (FRET)

Transmission Microscopy

- Measurements of stained histological samples

Other possible applications, which will not be discussed in detail here, include (i) time-resolved spectral imaging (by utilizing the pump and probe techniques with an appropriate external trigger) and (ii) Raman scattering measurements. A more detailed discussion, including examples of spectral images acquired using the SpectraCube™ method and the SD200 spectral bio-imaging system, follows.

4.4.2. Fluorescence Microscopy

4.4.2.1. Advantages of Spectral Bio-Imaging

It is generally agreed that the use of dyes as markers, particularly the use of multiple dyes (15), is one of the most powerful tools for analyzing organic tissues. Fluorescence microscopy is therefore one of the most important experimental methods used in light microscopy (16). The power of fluorescent probes is mainly due to the great variety of biological structures to which specific dyes can be bound (17). For a detailed review of fluorescence probes, see Mason (12) and Ploem (18). The rapid development of new and more sophisticated multicolor fluorescent dye molecules will continue to create the need for more advanced fluorescence imaging techniques that can utilize the full potential of these dyes. For a discussion of the revolutionary impact fluorescent dyes have had, and will continue to have, on the way research is conducted today, see Taylor et al. (19).

Spectral bio-imaging provides several important advantages for fluorescence imaging applications over more traditional filter-based approaches. These advantages include the following:

- Measurement of the complete spectrum, providing much more (quantitative) insight into the actual behavior of dye molecules in the sample of interest
- Simplification of fluorescence image acquisition, enabling, in a single measurement, the separation and mapping of many spectrally overlapping fluorescent probes
- Ability to overcome many of the traditional problems arising from undesirable background luminescence

In fact, by applying sophisticated data analysis algorithms such as multivariate analysis and principal component regression (20), it is possible to analyze many spectrally related parameters simultaneously.

4.4.2.1.1. Measurement of the Complete Spectrum. Measuring the complete spectrum at every image pixel can be an important advantage over filter-based fluorescence techniques. Knowledge of the complete spectrum provides more insight into the bahavior of fluorescent probes (and their binding sites). For example, undesirable or unpredictable spectral shifts that occur in the emission spectrum of a fluorescent probe, due to its microenvironment (e.g., temperature), will not cause errors in determining the concentration of the probe. When the fluorescence intensity is measured with only a bandpass filter, however, such spectral shifts would likely go undetected and might cause significant errors in determining the probe concentration.

4.4.2.1.2. Simplified Image Acquisition. The acquisition of multicolor fluorescence images can be greatly simplified when the power of spectral bio-imaging is combined with the appropriate fluorescent probes. Although a computerized system based on dual filter wheels and dual excitation sources has been shown to be effective in acquiring and registering the emission intensities of five spectrally distinct fluorescent probes (15), a fair degree of sophistication is required to control separately (for each probe) the excitation and emission filters, select the appropriate filter cube, and optimize the focus and exposure. Moreover, the resulting images must usually be registered prior to analysis, which can rarely be done with sufficient satisfaction. Recently, multispectral interference filters have also been used to enable imaging multiple fluorophores (21).

By enabling the simultaneous measurement of the emission spectrum of many fluorescent dyes (including dyes whose emission spectra overlap), spectral bio-imaging overcomes one of the fundamental limitations imposed by filter-based approaches: the need to acquire sequential images of the emissions of multiple fluorescent probes. The advantage of using a spectral bio-imaging

system is greatest when all of the fluorescent probes can be excited by a common excitation source. In this case, a single spectral image acquisition can capture the fluorescence emission of an almost unlimited number of dyes, and the need to select nonoverlapping dyes, change filter cubes, change excitation or emission filters, optimize the focus and/or exposure time, or register the images is eliminated.

4.4.2.1.3. Elimination of Undesirable Background Luminescence. Spectral bio-imaging also provides a means for eliminating problems associated with undesirable background luminescence. Fluorescence imaging microscopy is typically performed by using a (fluorescence) filter cube, which ensures that the sample is excited by the desired short wavelengths while allowing only wavelengths in a limited spectral band corresponding to the fluorescence emission of the probe to reach the detector (e.g., the eye or a camera) (12). Since fluorescence intensities are usually several orders of magnitude below the intensity of the excitation source, such background luminescence can never be eliminated perfectly (22). The three primary sources for undesirable background luminescence are as follows:

1. Radiation from the excitation source that is not completely blocked by the dichroic mirror coating and/or the barrier filter
2. Autofluorescence of the sample, and sometimes also from the optical elements, which can contribute significantly to the background fluorescence: the effects of sample autofluorescence can usually be reduced by selecting fluorescent probes whose absorption and emission bands do not overlap with those of the sample being measured; similarly, by choosing optical elements that are appropriately coated to reduce autofluorescence, the effects of this latter type of autofluorescence can also be minimized
3. Selection of an inappropriate (or suboptimal) combination of excitation filter, dichroic mirror, and barrier filters

In spite of the best filtering methods available, undesirable background luminescence often makes it difficult, and sometimes impossible, to bring out the relevant fluorescence signal from its background (noise). A spectral bio-imaging system has the advantage of being able to use differences between (i) the shape and spectral range of the fluorescent dye and (ii) the shape and spectral range of the background luminescence (including autofluorescence) to eliminate the effects of undesirable background luminescence. Thus, by applying the appropriate spectral image analysis methods to the emission spectra of fluorescent probes, it is possible to improve the SNR, and hence the accuracy, of fluorescence imaging measurements. For example, linear combinations and

principal component algorithms can be used to extract, at every pixel, the exact spectral contribution of background components. The desired dominant background spectrum to be extracted can be determined from a previous calibration measurement or by using statistical methods applied to the entire image. This advantage of a spectral bio-imaging approach is of particular importance for ratio imaging when quantitation of the results is desired. In addition, a spectral bio-imaging system can save time and effort that are otherwise expended in choosing the optimal filters for a specific filter-based measurement.

An example of how a spectral imaging can eliminate undesirable background is shown in Figure 4.5. (see Color Plates). Figure 4.5a shows the fluorescence spectral image of a cell stained with propidium iodide. This image was acquired using the SD200 SpectraCube™-based spectral bio-imaging system attached to an Olympus inverted microscope (IMT2). The sample was illuminated by a mercury source, and the fluorescence intensity was imaged through a DMG filter cube (DM580 dichroic mirror, D590 excitation filter, and BP545 barrier filter). Figure 4.5b is a plot of the fluorescence emission spectrum from three image pixels. Note that each spectrum has two peaks. The peak at 623 nm is due to the actual fluorescence emission spectrum of propidium iodide, while the second peak, at 775 nm, is just a residual of the excitation light that was not completely eliminated by the excitation or barrier filter. This undesirable background luminescence could be eliminated by adding another excitation filter (Olympus PB460) or an appropriate barrier filter. However, if this same measurement had been made with a nonspectral imaging system (without the PB460 filter), the intensity measured at each pixel would be proportional to the integral of the spectra shown in Figure 4.5b, *including* the contribution of the undesirable signal in the 775 nm peak. Correction algorithms exist that could be applied to the traditional CCD image, albeit at the expense of noise, to help this situation (22). However, with a spectral bio-imaging system that measures fluorescence intensity at every pixel as a function of wavelength, it is easy to confine the analysis to those wavelengths that correspond to the emission of the fluorescent dye of interest (i.e., the 623 nm peak).

4.4.2.2. *Spectral Identification of Multiple Fluorophores*

The use of spectral bio-imaging enables the *simultaneous* measurement of *many* dyes in one step. There is no restriction on the type of dye; even dyes that overlap spectrally (e.g., rhodamine and Texas Red) can be identified and their occurrence mapped in an image. If many dyes are to be used simultaneously, careful consideration should be given to their excitation wavelength, fluorescence intensity, and emission spectrum. When this is done properly, the results can be analyzed quantitatively as well. For example, the relative concentration of several proteins can be found in a single measurement. By using standard calibrated dyes, the absolute concentrations can also be determined. One important case where the detection of multiple fluorescent probes can be a significant advantage is fluorescence in situ hybridization (FISH) (23), which is used to analyze gene codes and find possible defects. Malik et al. (24) summarizes experimental results utilizing the SpectraCube™ methods of spectral bio-imaging to measure multicolor fluorescence.

4.4.2.2.1. Detecting Microenvironmental Changes. The use of fluorescent dyes (e.g., when attached to an antibody) is not limited to identifying the existence of certain chemical compounds. Some dyes can also be used to probe actual chemical and physical parameters. For example, a dye whose spectrum changes when it gains or loses a hydrogen atom can serve as a pH indicator. Dyes exhibiting spectral changes due to local electrical potential, pH level, and intracellular ion concentration [e.g., sodium, magnesium, zinc, or free Ca^{2+} (25)] are currently used in a variety of applications.

The use of environmentally sensitive dyes is closely related to ratio imaging, a common analysis method (26). For some of the dyes in use today, environmentally related spectral changes occur in only part of the spectral range. By measuring the ratio of fluorescence emission in two spectral ranges, one can thus study environmental effects, independent of sample thickness, illumination nonuniformity, etc.

The ability of a spectral bio-imaging system to measure the complete spectrum, rather than using current techniques to measure the fluorescence at two (or three) discrete wavelengths, has several advantages for ratio imaging applications. Measurement of the complete spectrum significantly improves the accuracy and sensitivity of ratio imaging. For example, one can use the complete spectrum to measure the ratio of two integrated intensities in two different spectral ranges, and at the same time eliminate all the background intensity by integrating only over the relevant spectral range. It is also possible to use more sophisticated algorithms to analyze the spectral data, such as determining the environmental parameters of interest by fitting the measured spectral data to reference spectra stored in a library. Because spectral analysis is performed at every point in the image, a full morphological analysis of the sample is possible. Spectral bio-imaging thus provides the capability to map (i.e., display as an image) physical and chemical parameters of interest, potentially enabling more innovative and effective research.

By using spectral bio-imaging techniques, it may also be possible to use environmentally sensitive dyes that do not lend themselves to analysis using ratio methods. The detection of dyes sensitive to several environmental parameters (e.g., voltage and pH) could also be achieved by using appropriate analysis algorithms.

4.4.2.2.2. Measurement of Fluorescence from Natural Pigments. Chlorophyll is a natural pigment exhibiting fluorescence (27). The fluorescence spectrum of chlorophyll has been studied extensively, in part because it is more complicated than the sectrum from a typical fluorescent dye (28). For example, the measurement of chlorophyll fluorescence can be used to probe cell metabolism and to track photosynthesis. Fluorescence also occurs for porphyrins, native cytoplasmic proteins, and other compounds. The ability of a spectral bio-imaging system to measure spectral differences in different parts of a cell provides important insights into the functions of the organelles in a living cell.

An example of a chlorophyll fluorescence spectral imaging is shown in Figure 4.6 (see Color Plates). The algae *Oedogonium* was examined by fluorescence microscopy. Excitation was in the green spectral range (540 nm), and the emission in the red spectral band was measured by the SD100 SpectraCube™-based spectral bio-imaging system. Figure 4.6A reveals the total emitted fluorescence from the specimen. Both the red chlorophyll fluorescence and the reflected green excitation light from cell debris in the specimen can be clearly seen. The individual algal cells exhibiting red fluorescence are only partially visible. The fluorescence spectra from four different pixels of the specimen are shown in Figure 4.7. Specimen A, with an emission peak at 685 nm, was measured from a point in the central algae in which the red fluorescence dominates. Spectrum B, on the other hand, corresponds to a point of cell debris exhibiting green emission, with a peak at 542 nm. Spectra C and D correspond to two other pixels in the algae. By using spectrum A as a reference spectrum for a similarity map, it is possible to precisely locate and determine the relative concentration of chlorophyll in the algae, including portions of the algae obscured by debris (see Figure 4.6B). This (pseudo-colored) figure shows that the chlorophyll, although dispersed, is located in the center of the cell. Figure 4.6C is a similarity map using as a reference spectrum a point in the image at which the red fluorescence is faint. This similarity map demonstrates the existence of different chlorophyll intensities at different cell sites; for example, some of the fluorescence occurs at the periphery of the cell membrane. Thus, by using the similarity mapping function, it is possible to visualize the characteristic fluorescence emitted from specific subcellular points in the cell. Figure 4.6D highlights the spongy structure of the cell debris when a similarity map using the green spectrum from the cell debris (spectrum B) is applied. This last similarity map also reveals the algal cell wall, which reflects the green emitted light. In addition, this similarity shows the location of unicellular algae.

The study of autofluorescence has many other important applications. For example, studies of natural skin fluorescence have identified "spectral fingerprints" corresponding to different tissue constituents and histological organization of the tissue. The use of tissue spectral fingerprints to identify normal from abnormal tissue (29–31) has also been studied extensively. The potential clinical utility of spectral bio-imaging in such medical diagnostic applications appears to be significant.

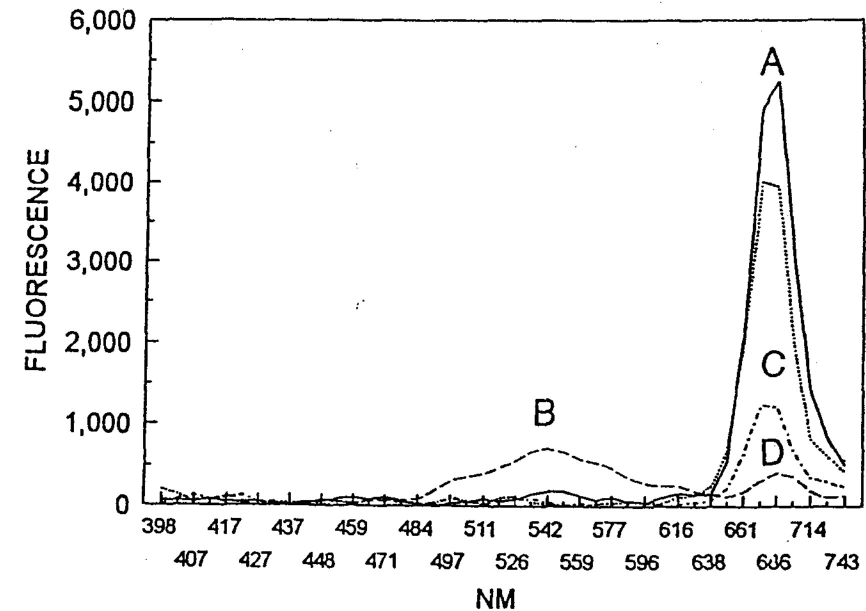


Figure 4.7. Fluorescence spectra from four different pixels (A–D) of the specimen shown in Figure 4.6 (see Color Plates).

4.4.2.2.3. Fluorescence Resonance Energy Transfer (FRET). FRET is a fluorescence method that enables the determination of the spatial separation between two fluorophores. In FRET, two different fluorophores, designated the donor and acceptor, are used. This pair of fluorophores is chosen carefully so that when the donor is excited, it can either fluoresce or transfer the energy being absorbed by it to the second fluorophore (the acceptor), causing it to fluoresce. Thus, it is possible to distinguish the donor from the acceptor because of differences in the emission spectrum.

The physical separation between the donor and acceptor is determined from the fact that the efficiency of the energy transfer strongly depends on the distance between the two fluorophores (typically, the efficiency is proportional to the inverse of the sixth power of the separation distance). When these two fluorophores are attached to two different types of molecules or to the same molecule in two different states, measuring FRET by using a spectral bio-