METHODS IN
CELL BIOLOGY

VOLUME 30

FLUORESCENCE MICROSCOPY OF LIVING CELLS IN CULTURE
PART B

EDITED BY

D. LANSING TAYLOR
YU-LI WANG

Prepared under the Auspices of the American Society for Cell Biology

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VOLUME 30

Fluorescence Microscopy of Living Cells in Culture
Part B. Quantitative Fluorescence
Microscopy—Imaging and Spectroscopy

Series Editor

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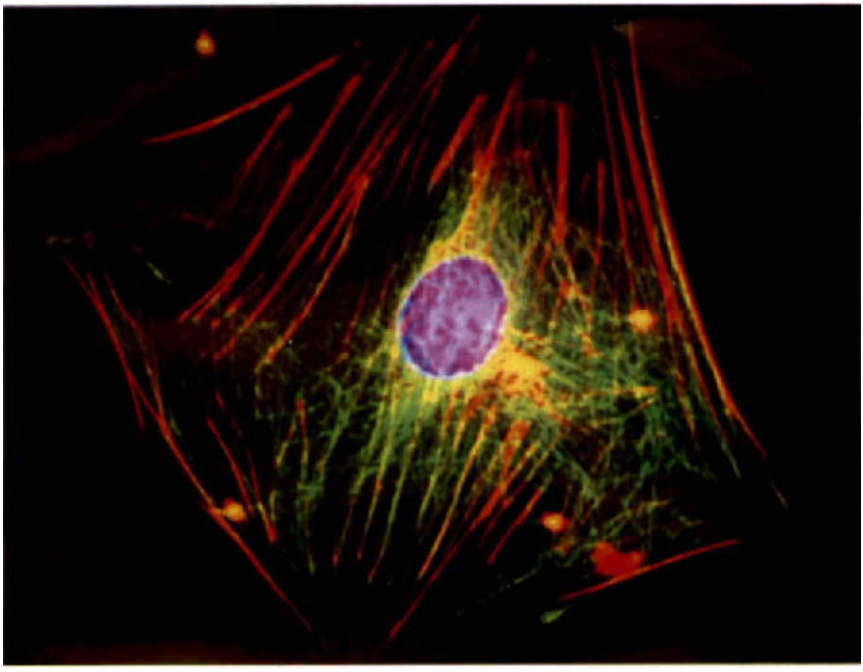

Multiple spectral parameter mapping image created by the simultaneous display of three individual images (See Chapter 17).

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Fluorescence Microscopy of Living Cells in Culture
Part B. Quantitative Fluorescence Microscopy—Imaging and Spectroscopy

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PREFACE

Fluorescence techniques are uniquely suitable for probing living cells because of their sensitivity and specificity. Since fluorescence from a single cell can be detected with a microscope both as an image and as a photometric signal, fluorescence microscopy has great potential for qualitative and quantitative studies on the structure and function of cells. However, owing to previous technical limitations, fluorescence has been used primarily for staining fixed cells for many years. It has not been until recently that the true power of the techniques has evolved for use with single living cells. The most important advances that have made this possible include the development of (1) probes for specific structures or environmental parameters; (2) methods for delivering fluorescent probes into living cells; (3) methods for detecting weak fluorescence signals from living cells; and (4) methods for acquiring, processing, and analyzing fluorescence signals with microscopes.

The primary purpose of this and the accompanying volume of *Methods in Cell Biology* is to provide readers with detailed descriptions of methods in these four areas. While techniques for flourescence spectroscopy in solution are described in various sources, there has been no convenient source for the methods specifically applied to living cells. Even with an extensive literature search, one often finds crucial technical details, including instrumentation, sample handling, and precautions, left out in many research articles. It is our hope that these volumes will provide enough detail to make the new developments approachable by most investigators. Although some biological perspectives are provided in many chapters, the main emphasis of the volumes is practical laboratory methods; the job of biological reasoning and experimental design is left to individual investigators. The books are thus targeted primarily at experienced cell biologists who wish to apply modern fluorescence techniques. However, they should also be of great interest to biochemists and molecular biologists who attempt to correlate results in test tubes with activities in living cells. In addition, many chapters should be valuable to those specializing in instrumentation, including microscopy, electronic imaging, and digital image processing.

The two volumes represent a collective effort of many investigators. The chapters were assembled by specific areas which, in our view, were important or held great promise in the future. We then invited those researchers with extensive experience in the particular area to make contributions. There was a certain degree of subjectiveness in choosing the topics. On the one hand, we have included topics crucial to, but not specific for, fluorescence microscopy of living cells, including microscopy cell culture, microinjection, microscopy

photometry, and low light level imaging. On the other hand, we decided to sacrifice several useful topics that were either not in a mature stage of development or where we were unable to obtain a commitment from an authority.

The first volume (Volume 29) deals with the preparation, delivery, and detection of fluorescent probes. The first half is focused on the preparation of specific structural probes, including fluorescent analogs that can be utilized by living cells in structural assembly, fluorescent molecules that bind to specific cellular components, and probes that can be used to label particular cellular compartments. There are special challenges in the preparation of each class of probes, including proteins, small peptides, heterocyclic compounds, lipids, and polysaccharides. Subsequent chapters discuss factors that determine the destination of probes and methods for delivering probes to specific sites in living cells. The second half of the first volume discusses the detection of fluorescent probes in living cells, including issues related to sample physiology (microscopy cell culture), optics (basic fluorescence microscopy), and signal detection (electronic photometry and imaging, immunoelectron microscopic detection of fluorophores). The last few chapters introduce modern techniques in image detection and provide a continuity to quantitative analytical methods covered in Volume 30.

The second volume (Volume 30) explores a combination of the theoretical and technical issues related to the quantitation of fluorescence signals in the living cell with a light microscope. The first section explores the engineering principles required in the characterization of the performance of an imaging system. The use of system validation procedures and quantitative fluorescent standards are explored in detail. The remainder of Volume 30 is devoted to specific applications and optical methods. A mix of theoretical and practical issues is discussed, including the measurement of membrane potential, ionic concentrations, tracer diffusion coefficients, total internal reflection, fluorescence polarization, and three-dimensional reconstruction. Thus, the two-volume set defines a technical continuum from organic chemistry, through biochemistry, cell biology, physics, and engineering, to computer science. The present status of the field reflects the occurrence of a revolution in cell biological research.

We would like to thank all contributing authors for providing us with their extensive experience in various areas. Most of them have worked closely with us in planning their chapters and minimizing overlaps, then submitting excellent manuscripts in a timely fashion and answering questions which arose during editing.

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Chapter 1

Image Fidelity: Characterizing the Imaging Transfer Function

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METHODS IN CELL BIOLOGY, VOL. 30

I. Introduction

Fluorescence microscopy is today one of the most significant tools for the examination of cells and cellular constituents. Fluorescent probes and fluorescently marked immunological probes offer us the ability to visualize and quantify basic structures within the cell. The ability to perform quantitative measurements on fluorescence images, however, can be severely limited by the same instrument that provides us with the images—the microscope itself. When a fluorescence image is converted to a digital image for subsequent computer processing, the effect of the scanning instrument as well as the quantitization process can further compound the problem. It is the purpose of this chapter to study the various limitations and distortions inherent and introduced in quantitative fluorescence microscopy and to describe ways to compensate and/or eliminate them.

A. The Reality of Distortion

To begin, it is important to realize that distortion in fluorescence images—or, for that matter, any image—is unavoidable. Even if electrooptical sensors were linear and introduced no noise and microscope lenses had no geometrical aberrations, no chromatic aberrations, and no glare, images observed through a microscope and recorded through a sensor would still contain distortion. At the most basic level this is caused by the finite size of microscopes, their lenses, and, most importantly, their apertures. It is not necessary for us at this time to go through the theory that describes this result. Suffice it to say that the diffraction limits of light optics (dictated by the wave nature of light) do not permit us to produce arbitrarily sharp images.

This phenomenon is illustrated in Fig. 1. The cytoskeletal actin molecules in a fibroblast have been labeled with a fluorescently tagged

FIG. 1. Actin molecules in a fibroblast stained with a fluorescently tagged antibody.

antibody. The actin filaments, while they have lengths that can be measured in micrometers, have diameters (widths) that are measured in nanometers—two orders of magnitude smaller than the wavelength of visible light.

A single labeled actin filament cannot be resolved. What we see in the image is a distortion of the light distribution along the length of the filament. However useful though the resulting image may be, it remains a distorted version of reality.

B. Models of a Fluorescence Imaging System

To understand the origin and the nature of distortions in a quantitative fluorescence microscope system, it is essential that we understand the various components that form such a system and how they work together to produce a digital image in a computer memory. We begin with a

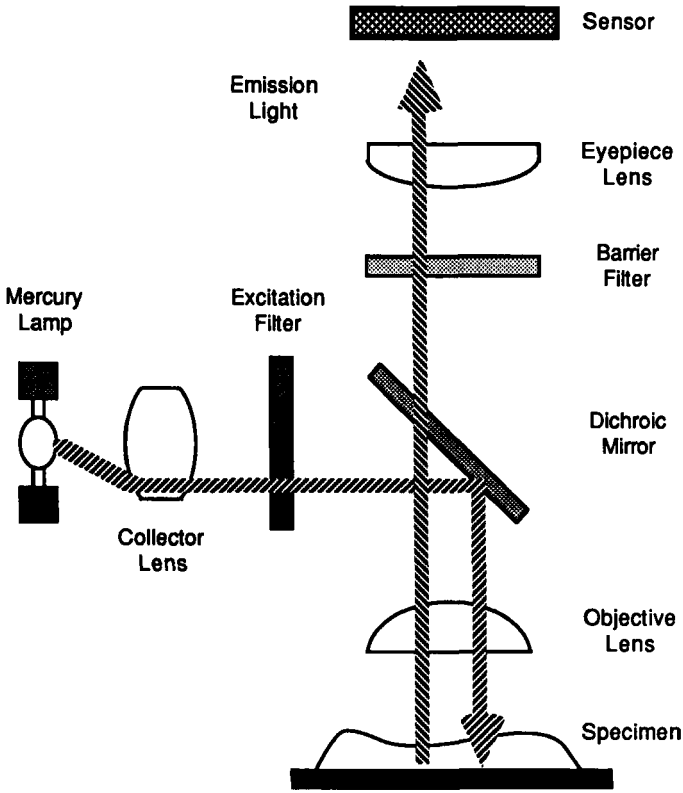

FIG. 2. Schematic diagram of a fluorescence imaging system.

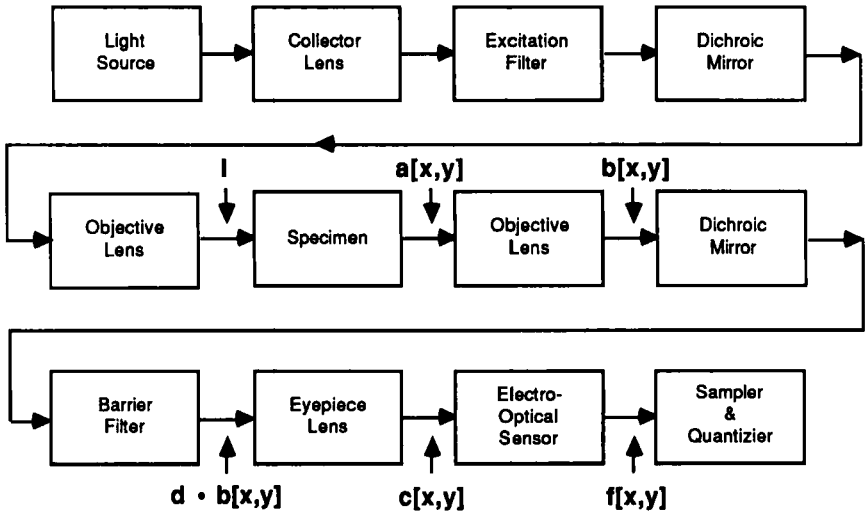

FIG. 3. System diagram of a fluorescence imaging system. The illumination I produces $a[x,y]$ from the specimen. Various lenses and filters interact to produce the final image $f[x,y]$.

schematic model of such a system as shown in Fig. 2. This model represents an epiillumination fluorescence imaging system [after Ploem, 1967]. Unless we indicate otherwise, we will always be referring to this epiillumination model.

The model does not directly indicate how the various distortions are introduced nor how they can be described. It does, however, offer us a starting point for building an analytical description of the imaging process. This description, by its very nature, requires the use of a mathematical formalism, that is, a set of equations to describe the various components and their interrelationships.

While the model shown in Fig. 2 may indicate the physical layout of a quantitative fluorescence microscope system, it is not the best choice for representing the "flow of data" in such a system. The model shown in Fig. 3 is more appropriate and is usually referred to as a system diagram.

Each of the components is now a subsystem and the "flow of data" is more easily represented and described. Based upon this model, we are now in a position to introduce a major assumption concerning many of these subsystems. Specifically, we assume that the (optical) imaging components of this system form a linear, shift-invariant system. To understand the importance of this assumption as well as its consequences, it is necessary to define carefully each of these terms.

II. The Concept of a Linear, Shift-Invariant (LSI) System

A. What Is Linearity?

Informally, the concept of linearity is quite straightforward. Let us start with a distribution of fluorescently labeled objects that under a certain illumination produce a distribution of light $a[x,y]$. Through an imaging system this yields a distribution of light $b[x,y]$. Let us now alter the fluorescent dyes (fluorochromes) or the illumination such that $a[x,y]$ becomes $2a[x, y]$, that is, twice as much light is produced by the objects. If the imaging system is linear, then $b[x,y]$ will become $2b[x,y]$. In general, if $a[x, y] \rightarrow b[x,y]$ and the system is linear, then $\eta a[x,y] \rightarrow \eta b[x,y]$. (The symbol "$\rightarrow$" is to be read as "will give under the imaging operation.") In words, we say that, if the input image ($a[x,y]$) is multiplied by a scale factor (η) and the system is linear, then the resulting output image ($b[x, y]$) will be multiplied by the same scale factor. This, however, is only half of the definition of linearity. The remainder is as follows.

Consider now that a given combination of illumination and fluorochrome produce $a_1[x, y]$ which yields image $b_1[x, y]$. A second combination produces $a_2[x,y]$, which in turn yields image $b_2[x,y]$. We now construct a combination of illumination and fluorochromes such that fluorescently labeled objects produce a distribution of light $a_1[x,y] + a_2[x,y]$. If the imaging system is linear, then the resulting distribution of light will be $b_1[x,y] + b_2[x,y]$. If the input image is the sum of two images ($a_1[x,y]$, $a_2[x,y]$) and the system is linear, then the resulting output image will be the sum of the two output images ($b_1[x,y]$, $b_2[x,y]$).

These two conditions can be summarized in a single statement. Let $a_1[x,y] \rightarrow b_1[x,y]$, $a_2[x,y] \rightarrow b_2[x,y]$, and η_1 and η_2 be scale factors. If the imaging system is linear, then

$$\eta_1 a_1[x,y] + \eta_2 a_2[x,y] \rightarrow \eta_1 b_1[x,y] + \eta_2 b_2[x,y] \tag{1}$$

As can be seen from the previous discussion, this relation summarizes the necessary and sufficient conditions for a system to be considered linear.

One of the "by-products" of this definition should be clear: If we let η_1 and η_2 be zero in Eq. (1), then we have the result that, for a linear system, zero in gives zero out ($0 \rightarrow 0$). While this might seem like a trivial observation at this point, it will have important consequences when we come to the problem of shading correction in fluorescence imagery.

B. What Is Shift Invariance?

We are used to the idea that, if a cell is observed in the upper left portion of a microscope field of view or the lower right portion of that field of view, the result will be the same. We expect that the image produced by a microscope will be *invariant* to shifts. While this is the goal of all microscope designs, it is a goal that is only approached, never reached. Using the terminology introduced in the previous section, let us consider the input distribution of light $a[x, y]$ and the output distribution of light $b[x, y]$. Shift invariance means that if the input image is shifted from $a[x, y]$ to $a[x - x_0, y - y_0]$ then the output image will be shifted from $b[x, y]$ to $b[x - x_0, y - y_0]$.

C. Is a Fluorescence Imaging System LSI?

While the two potential properties *linearity* and *shift invariance* are interesting, they would not be worth pursuing if they did not represent a reasonable description of the imaging process in a fluorescence microscope. In the context of mathematical definitions no quantitative fluorescence microscope system will be LSI. To an excellent *approximation*, however, the system will be LSI and further, the insight that we gain by using this assumption will help us in the evaluation of the effects generated by nonlinearities as well as spatial variance.

D. Fluorescence Image as a Superposition Result—Convolution

Let us now develop a basic result that follows from our LSI assumption for a quantitative fluorescence microscope system. We start by defining a basic test object that has the property that it has a spatial position, a finite total brightness, but no spatial extent. This test object is called a unit impulse $\delta[x, y]$ and we may think of it as a pinpoint of light on a black background. Mathematically this impulse function has the properties that

(i) $$\delta[x, y] = 0 \quad \text{unless} \quad x = y = 0 \tag{2a}$$

(ii) $$\int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} \delta[x, y]\, dx\, dy = 1 \tag{2b}$$

Equation (2a) states that the position of the impulse is $(x = 0, y = 0)$ but that it has no spatial extent. Equation (2b) states that the total

brightness of the impulse is one. It is possible to show that *any* distribution of fluorescent light produced by illumination and fluorochromes can be represented by a weighted sum of these impulses. Formally, any distribution can be represented by:

$$a[x,y] \approx \sum_{u=-\infty}^{+\infty} \sum_{v=-\infty}^{+\infty} a[u,v] \cdots \delta[x-u, y-v]\, \Delta u\, \Delta v \qquad (3a)$$

This is a complicated equation but it says, essentially, that a collection of unit impulses $\{\delta[x,y]\}$ at different positions with weighting coefficients $\{a[u, v]\}$ can produce an arbitrary image $a[x,y]$. It is beyond the scope of this chapter to prove this statement. For further details the reader is referred to Oppenheim *et al.*, 1982. The standard form of this statement is to allow the sum of Eq. (3a) to pass to an integral giving

$$a[x,y] = \int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} a[u,v] \cdot \delta[x-u, y-v]\, du\, dv \qquad (3b)$$

The interpretation of Eq. (3b) is the same as that of Eq. (3a). We are now in a position to develop the consequences of this last equation as well as the assumption that the imaging system is LSI.

Consider a single term in Eq. (3a), $(a[u,v]\, \Delta u\, \Delta v) \cdot \delta[x-u, y-v]$. Let us name the output image that results from a single input impulse $\delta[x,y]$ as $h[x,y]$. As a result of the LSI assumption we have the following:

(i) $\delta[x,y]$	$\rightarrow h[x,y]$ *(by definition)*
(ii) $(a[u,v]\,\Delta u\,\Delta v)\,\delta[x,y]$	$\rightarrow (a[u,v]\Delta u\,\Delta v)\cdot h[x,y]$ *(by linearity)*
(iii) $\delta[x-y, y-v]$	$\rightarrow h[x-u, y-v]$ (by *shift invariance*)
(iv) $(a[u,v]\,\Delta u\,\Delta v)\cdot\delta[x-u, y-v]$	$\rightarrow (a[u,v]\,\Delta u\,\Delta v)\cdot h[x-u, y-v]$ *(by LSI)*
(v) $\Sigma\Sigma\, a[u,v]\cdot\delta[x-u, y-v]\Delta u\,\Delta v$	$\rightarrow \Sigma\Sigma\, a[u,v]\cdot h[x-u, y-v]\,\Delta u\,\Delta y$ *(by linearity)*
(vi) $\int\int a[u,v]\cdot\delta[x-u, y-v]\, du\, dv$	$\rightarrow \int\int a[u,v]\cdot h[x-u, y-v]\, du\, dv$ *(by linearity)*

The last line of this proof is a central result. In Eq. (3b) we indicated that an arbitrary input image can be represented as a collection of weighted impulses. We also stated earlier that *by definition* if the input image was $a[x,y]$ then the resulting output image was $b[x,y]$. In line (vi) above we show that the image $b[x,y]$ can be computed through knowledge of $h[x,y]$ and by application of the *convolution* equation:

$$b[x,y] = \int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} a[u,v] \cdot h[x-u, y-v]\, du\, dv \qquad (4)$$

This important result, sometimes written as $b[x,y] = a[x,y] * h[x,y]$, says that an output image formed by an LSI system can be described as the result of the convolution between $a[x,y]$ and the image $h[x,y]$ formed by a single input impulse $\delta[x,y]$. The image $h[x,y]$ contains all of the information necessary to describe the imaging system because, if we know $h[x,y]$, we can then compute the output $b[x,y]$ for any other $a[x,y]$. The response $h[x,y]$ to a single input impulse $\delta[x,y]$—a single *point* of light—is referred to in optics as the *point spread function* (PSF) and in system theory as the impulse response.

In summary, each arbitrary input image can be thought of as a weighted collection of impulses; each weighted impulse generates a weighted point spread function; the sum of the weighted point spread functions is the resulting output image.

III. Characterizing LSI Systems with Sinusoids

A. Sinusoids in/Sinusoids out

We are almost in a position to characterize the image fidelity of a quantitative fluorescence microscope system. What we will show in this section is that the key input image to observe, as it passes through a LSI optical system, is a sinusoidal signal. There are two reasons:

1. If the input signal to an LSI system is a sinusoid with frequency ω, then the output signal will also be a sinusoid *with precisely the same frequency* ω. The amplitude of the sinusoid may change, the phase of the sinusoid may change, but the frequency will be the same.
2. It is possible to represent virtually any input image as a weighted sum of sinusoids.

Using the property given in Eq. (1) together with the two statements above, it is possible for us to describe how sinusoidal terms in the input image will be altered as they pass through an LSI optical system.

B. The Complex Sinusoid and Convolution

There are a number of ways to represent a sinusoid. In this chapter we shall use the complex exponential form described by Euler's relation:

$$e^{j\omega x} = \cos(\omega x) + j \sin(\omega x) \qquad j = \sqrt{-1} \tag{5}$$

Using this formulation we can prove the first statement above. Consider a sinusoidal input image:

$$a[x, y] = \exp[j(\omega_x x + \omega_y y)] \tag{6}$$

Note that $a[x, y]$ has two distinct sinusoidal frequencies, ω_x and ω_y, one in the x direction and one in the y direction. We can now derive the result that the output image $b[x, y]$ has the same basic character as the input image and that only the complex *amplitude* of the sinusoidal term will be affected by the LSI system. Using Eq. (6) in Eq. (4) gives

$$b[x, y] = e^{j(\omega_x x + \omega_y y)} \left(\int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} h[u, v] \exp[-j(\omega_x u + \omega_y v)] \, du \, dv \right) \tag{7}$$

The first term in Eq. (7) is the sinusoidal term with the same frequencies, ω_x and ω_y, as in the input image. The term in parentheses represents the change in amplitude caused by the LSI system. This new amplitude will, of course, be dependent upon the specific values of ω_x and ω_y as well as the form of the PSF, $h[x, y]$. This dependency is usually summarized by:

$$b[x, y] = H(\omega_x, \omega_y) \exp[j(\omega_x x + \omega_y)] \tag{8a}$$

where

$$H(\omega_x, \omega_y) = \int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} h[x, y] \exp[-j(\omega_x x + \omega_y y)] \, dx \, dy \tag{8b}$$

In Eq. (8b) the variables x and y are dummy spatial variables of integration.

In Fig. 4 we see the effect of this phenomenon through the application of a PSF to four test images, each with a different value of ω_x. The effect of $h[x, y]$ in Fig. 4 is to change the complex amplitudes of the sinusoids from the initial values of one to the values shown. Test patterns, such as those used in Fig. 4, are sometimes referred to as sinusoidal gratings.

C. Description of an Image in Terms of Complex Sinusoids—the Fourier Representation

We stated previously that any input image could be represented as a weighted sum of sinusoids. This statement is essential if we are to use the results of Eq. (7) to describe how an image propagates through an LSI system. The foundation for this statement lies in the results of the nineteenth century French mathematician/physicist Jean Baptiste Joseph Fourier. In his work on the diffusion of heat, he showed how a very wide variety of physical signals, including those concerning us in this chapter,