COUPLED BIOOLUMINESCENT ASSAYS

Methods, Evaluations, and Applications

MICHAEL J. COREY

A John Wiley & Sons, Inc., Publication
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“There are two ways of spreading light: to be the candle or the mirror that reflects it.”

–Edith Wharton
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Every scientist with a career of sufficient length has a favorite story of serendipity. In the 1990s, while studying acceleration of the complement system by antibodies directed against its regulators, I became dissatisfied with the available means of measuring cell death. Either I had to load up the cells with a radioactive label, hope it would not perturb the results, and then clean it up; or I had to wait a long time for a weak signal from lactate dehydrogenase released by the dying cells. It was then that my mentor Bob Kinders said, “Why don’t you come up with a luminescent assay?” Five minutes after Bob posed his question, I was racing upstairs to try the method that eventually became aCella-TOX™, a coupled bioluminescent (CB) general cytotoxicity assay. After much help from many people, I obtained my own patent (an experience every life scientist should have at least once), published, found partners, got it on the market, and finally wrote this book.

Today, it is not difficult to come up with a new CB assay, but in a sense, all of the developments derive to some degree either from Arne Lundin’s ideas as expressed in the late 1970s and 1980s regarding the many possible ways of expanding the use of luciferase in coupled assays or from the important work of Shimomura in isolating and characterizing the first photoproteins. Other academic workers and, notably, vendors such as Promega have driven the field far beyond the initial applications, to the point where there is probably a CB alternative for more than half of all biochemical measurements proposed. The activities of enzymes that are critical in today’s medicine, including kinases, phosphatases, proteases, acetylcholinesterase, nitric oxide synthase (NOS), and many others, may be measured rapidly and with extraordinary sensitivity by using this approach. In other cases, such as reporter assays, luciferase activity simply serves as a robust quantitative marker. The power of CB methods to provide information with extraordinary speed and sensitivity is growing
and expanding, yet apart from reporter assays, they are virtually unknown to many life scientists.

Because of the general lack of familiarity with CB technology, I begin the book with what I hope is a solid introduction to bioluminescence in general, including the tools available, as well as the biochemical and enzymatic principles that make these assays possible and useful. To those who have skipped or forgotten their thermodynamics, it may appear paradoxical that CB reactions (and other coupled enzymatic reaction series) can be run even if the reaction series is endothermic, that is, even if it absorbs energy at the level of the individual molecule. This is just one of a series of illuminating surprises that await the scientist who engages this field for the first time.

The reader will note the presence in this book of a great deal of material related to competing nonluminescent methods, especially in chapters dealing with major drug discovery targets, such as kinases and G-protein-coupled receptors (GPCRs). This is both essential and inevitable. The advent of CB assays for many applications is still upon us, or in the future, and the amount of available material relating to them is therefore limited, whereas we know a great deal about fluorometric assays, to give a prominent example. Therefore, it may be highly useful to the assay developer, as well as the scientist seeking to understand the alternatives, to have information about both the CB options and the other choices together in the same chapter. In some cases, the majority of space in the chapters is occupied by the other methods, to allow the developer to assess the full range of strategies he/she can employ. Thus, the intent is both to introduce and describe the CB possibilities and to enable readers to evaluate other methods if they are more suitable.

The book mostly focuses on assay target (kinases, proteases), but there is necessarily some overlap. Calcium, an essential cofactor in aequorin luminescence, is also a major player in the biochemistry of the GPCRs, which are currently the most important class of targets in drug development. Kinases and phosphatases also play roles in GPCR signal transduction, and so on. Finally, I have endeavored to suggest applications that are not directly related to the drug discovery juggernaut, including food safety testing and bioburden measurements, as well as environmental applications. These chapters are arranged differently.

Although every effort has been made to keep the information up-to-date, inevitably a number of methods will have been inadvertently omitted, while others are just appearing on the market as the book goes to press. There is no better evidence of the vitality of the field of CB assays than the range of new assay types now being marketed. Among those that have appeared too recently to treat in depth herein are several fascinating offerings from Promega, including P450-Glo™ for the cytochrome P450s so crucial in drug metabolism; MAO-Glo™ for the monoamine oxidases, which are increasingly important targets for psychoactive pharmaceutical development; GSH-Glo™ for glutathione quantification; Proteasome-Glo™; and Pgp-Glo™ for assessing the activity of the P-glycoprotein ATPase. These fields of study and their nexus with CB assay development are worthy of their own chapters in a future volume.

I owe thanks to many for help and support. Among them are my outstanding mentors Jack Kirsch, Bob Kinders, Bob Vessella, and Phil Maples; Helen Landicho,
who advised me on regulatory issues; technical associates Kathy Schaffer, Connie Ave-Teel, and Caroline Babcock; and all-around Document Wizard Anna Schneider. Tomas Corey and Eva Corey have offered excellent assistance in preparing figures. Lukas Corey assisted with indexing. Sumant Dhawan and Sanjeet Thadani of Cell Technology, Inc. have been invaluable allies in developing and marketing CB products, such as the first CB detection system for nerve gas.

Of the many joys of my scientific career, one of the high points was knowing the great Daniel E. Koshland, Jr., of the University of California, Berkeley. I wish to dedicate this book to his memory.

Michael J. Corey
April 8, 2008
PART I

BACKGROUND TO COUPLED BIOLUMINESCENT ASSAYS
1

INTRODUCTION

1.1 INTRODUCTION TO COUPLED BIOLUMINESCENT ASSAYS

The phenomenon of bioluminescence, the emission of internally generated light by living organisms, holds special fascination for the scientist. We are used to living things that use light: they may have light organs for detection and perception of their environment or nearby objects, light transducers that regulate circadian or seasonal rhythms, or membranes capable of harvesting light for conversion to chemical energy. But to the scientist who enters the field of bioluminescence with the usual requisite skepticism, the process whereby animals, fungi, and bacteria make their own light seems almost like a pointless biological extravagance that occurs merely for the benefit of our research. Despite all the sensible arguments about interorganism communication and photolocation (1, 2), the whole business has the flavor of an undeserved gift.

And a remarkable gift it is. Though the range of chemistries employed in light-producing reactions is limited, the applications span nearly the entire breadth of the life sciences. Hundreds of researchers who have probably never heard of “coupled bioluminescence” regularly perform reporter assays with recombinant luciferase genes. Thousands of workers in the food industry use a bioluminescent reaction to detect unwanted biological contamination. Scientists in fields as diverse as genetics, environmental monitoring, enzymology, biowarfare prevention and response, and especially drug discovery now have coupled bioluminescent alternatives to numerous slow, insensitive, and expensive procedures. In many cases, however, these scientists may not be aware of the possibilities; hence this book.
Our focus in this book is on the applications of coupled bioluminescence (CB). The discussion presented here of the physical phenomena of luminescence and bioluminescence is intended for the scientist with a general background, including knowledge of chemistry at some level, but no prior experience with luminescence or fluorescence. The material is necessarily limited in extent; for an in-depth introduction to the chemistry and biology of bioluminescence, the reader is encouraged to consult two classic and highly seminal works: *Bioluminescence: Chemical Principles and Methods*, by Shimomura, discoverer of aequorin (3); and *Bioluminescence*, by Harvey, who began working and publishing on the phenomenon of bioluminescence nearly four decades before he finally wrote this “bible” of the field (4). What follows is a survey of the kinds of bioluminescent reactions that can occur and how they may be harnessed to the service of coupled bioluminescent assays. The second half of the chapter is largely a review of the available molecular tools: the many interesting bioluminescent organisms and the relevant characteristics of the luciferases and photoproteins they offer. That section may be of special interest to the scientist considering cutting-edge development of novel coupled bioluminescent techniques. A thorough discussion of how to design, perform, and test these reactions appears in Chapter 2.

1.2 LUMINESCENT TECHNOLOGIES OF THE LIFE SCIENCES

We begin with a definition. *Luminescence* is a critical term for our purposes, and it is desirable to establish its precise meaning from the outset. The most accurate definition in general use is expressed with a sort of negative twist: luminescence is emission of light at temperatures below those that give rise to incandescence. In physical terms, luminescence consists of the emission of photons by electrons that have previously been excited to appropriate energy levels by any means *except* heating. Thus, fluorescence, phosphorescence, chemiluminescence, and bioluminescence all fall within this category.

Except phosphorescence, all of these kinds of luminescence are in widespread use throughout the life sciences; in fact, the only important category of quantitative liquid-phase assays that does not employ one of these kinds is the venerable and nearly obsolete method known as spectrophotometry (known in the trade as “UV/spec,” jargon referring to the fact that spectrophotometric measurements are frequently made in the ultraviolet range). However, these days even spectrophotometers may employ light sources other than incandescent lamps, rendering even this last bastion of the 1950s just another form of luminescence.

At this point, we introduce the two phenomena that are the simplest to explain, though not necessarily to work with, *chemiluminescence* and *bioluminescence*. Though distinct, these two processes have a critical point in common: in each case, light is generated within the sample by chemical reactions. Figure 1.1 illustrates the simple sequence of events underlying assays based on these principles: chemical energy transduced to light within the reaction vessel is radiated *anisotropically* (i.e., in all directions equally) and part of it is captured and measured by a detecting device (the nature of the device is discussed below).
FIGURE 1.1  Schematic representation of autoluminescence. Light (hν) is emitted equally in all directions by the luminous sample, impinging on one or more detectors, which may be photomultiplier tubes, charge-coupled devices, or other types. No lamp is required. (See the color version of this figure in the Color Plates section.)

This situation may be fruitfully contrasted with the situation depicted in Fig. 1.2, that of spectrophotometry, in which light from an instrument source, such as a lamp or light-emitting diode, is absorbed by molecules (or supermolecular aggregates, in the case of turbidity measurements) within the sample; the attenuated light intensity is then measured by a device that may be identical to that used for measuring chemiluminescence and bioluminescence. A critical feature of this system is that the intensity

FIGURE 1.2  Schematic representation of spectrophotometry. The lamp illuminates the sample, which absorbs a portion of the light, depending on the concentration of the absorbing species. The remaining light is transmitted through the sample vessel and impinges on the detector. The full intensity of the lamp is known separately from either careful calibration or beam splitting. The fraction of light remaining indicates the concentration to be determined, assuming the absorbance coefficient is known. Multiple concentrations may be measured simultaneously if all absorbance coefficients are known, data from multiple wavelengths are collected, and independent spectra of the absorbing substances are available. (See the color version of this figure in the Color Plates section.)
of the detected light can never exceed that of the incident light from the lamp. Perfect transmittance, which is equivalent to zero absorbance, therefore results in an intensity ratio of 1 in comparison with the blank reading.

The final case we consider here is that of fluorescence (or phosphorescence, which differs from fluorescence for practical purposes only in its timescale and is not treated further here). Fluorometry, or the controlled measurement of fluorescence, is one of the most useful techniques ever to appear in science. It partakes of both the physical complexity of chemiluminescence and bioluminescence and the conceptual complexity (at least) of spectrophotometry. Fluorescent phenomena require a light source, as does spectrophotometry, but, in sharp contrast to the latter method, fluorometry paradoxically requires that as few photons as possible from the source reach the detector. The reason for this can be inferred from Fig. 1.3. Only photons emitted by sample molecules are useful in fluorometry. These photons are not emitted as a direct result of chemical reactions or physical interactions of the molecules. Instead, they are the products of the decay of the electronic excitation energy imparted by the light source. In other words, the electrons within the sample are excited by the source, and the process whereby this excitation energy is released by the electrons in returning to the ground state produces the photons to be measured. The reader should note especially the 90° angle between the light path leading from the lamp to the sample and that leading from the sample to the detector. This arrangement is intended to minimize

![FIGURE 1.3 Schematic representation of fluorometry. Illumination by the lamp in a specific range of wavelengths is absorbed as electronic excitation by sample molecules and reemitted at longer wavelengths. Emission is in all directions, but to avoid receiving light from the lamp, the detector is mounted at an angle to the incident beam. The detector is also tuned by filters or a monochromator to accept only emitted wavelengths. (See the color version of this figure in the Color Plates section.)](image-url)
the degree of contamination of the sample fluorescence by light shed directly by the source. However, this is sometimes accomplished in other ways, such as by situating the detector proximate to the light source, thus requiring the emission to occur at a 180° from the incident light. The latter instrument geometry is common, for example, in fluorometric microplate assays, where the lamp must deliver light to a single tiny well and the detector must receive reemitted light exclusively from the same well to avoid cross talk.

The fact that the emitted light comes from electronic decay events in the sample molecules has one very important consequence for the nature of the light: its wavelength is virtually always shifted from that of the incident light, and the shift is always in the direction of longer wavelengths (lower energy). The reason for this shift is that the process of capturing and reemitting light is not 100% efficient. Energy is lost to vibration and rotation, to collisions with other molecules, and to the shifting charge dipoles of the excited electron’s environment. Fortunately, the amount of change of the wavelength, known as the Stokes shift, is highly consistent and provides a probe of the electronic state and surroundings that is nearly as useful as the fluorescence intensity. The Stokes shift itself may be perturbed by pH, ligand association, and many other phenomena.

At this point, fluorometric assays are the main competitor of CB methods. The astonishing utility and versatility of fluorometry derives from the fact that both excitation and emission characteristics of fluorophores depend strictly on both the chemistry and the environment of the specific molecule. Fluorophores may behave differently depending on diverse phenomena such as pH, ionic strength, hydrophobicity, temperature, spatial separation from other fluorophores, delays between excitation and detection, and many aspects of molecular chemistry. Substrates may be designed specifically to yield fluorophores in response to the activity of enzymes. Fluorophores may be introduced into biological membranes to study their motions. Although fluorometric techniques have a number of inherent drawbacks, including the need for complex collimation and a costly source of illumination, the necessity of irradiating potentially labile samples, and the requirement for precise methods of selecting both excitation and emission wavelengths, it must be emphasized that the technique, or rather a set of techniques, is extraordinarily powerful, and it would be imprudent to assign limits to what fluorometry can do and will do.

Fluorescence may be defined in very different ways elsewhere in the literature; for example, some consider photon liberation in an electronic decay from a singlet state to be fluorescence if spin is preserved (5).

1.3 VARIETIES OF FLUOROMETRIC ASSAYS

Modern fluorometry has advanced far beyond the simple process depicted in Fig. 1.3 of shining light on a sample and measuring fluorescence at an angle. The temporal and spatial dependence of the intensity of emitted light can be exploited in a surprising variety of ways. Here, we describe three fluorometric technologies, all of which have their origins in prior decades but are still yielding new and useful methods:
time-resolved fluorescence (TRF), fluorescence resonance energy transfer (FRET), and fluorescence polarization (FP).

1.3.1 Time-Resolved Fluorescence

All fluorescence is time dependent, in the sense that unless continuous illumination is provided, the electronic excitation energy that gives rise to fluorescence will soon be exhausted, generally within nanoseconds. Moreover, even if the lamp never stops shining, photobleaching (oxidation of the sample induced by the radiant energy) will eventually attenuate the signal and alter the sample itself. However, the time dependence of certain fluorescent phenomena can be made to yield valuable information about the state of the fluorophore. In particular, fluorescence due to the label can be readily distinguished from autofluorescence or other adventitiously fluorescent molecules within the sample, since these sources of fluorescence decay much more rapidly than the emission of a fluorophore tuned for TRF through clever chemistry. Thus, TRF is made possible by the very long fluorescence lifetimes of the species employed for the purpose.

The lanthanide metals have so far proven to be by far the most useful fluorophores for TRF in the life sciences, with fluorescence lifetimes in the hundreds of microseconds, compared to roughly 1–100 ns for most fluorophores. By waiting to measure the fluorescence signal until light emission by the ordinary fluorophores has decayed, therefore, one can obtain a readout from the label that is very nearly quantitatively pure. Among lanthanides, the Eu$^{3+}$ (europium) ion is most commonly used, but Tb$^{3+}$ (terbium), Sm$^{3+}$ (samarium), and Dy$^{3+}$ (dysprosium) also show the phenomenon and, fortunately, exhibit well-separated emission wavelengths, allowing multiplexing. In other words, separate molecules within the sample can be labeled with each of these lanthanides, which can then be quantified via fluorescence at different wavelengths.

Among a number of web-based resources further explicating the phenomenon of TRF, the excellent PerkinElmer material at the following URL is strongly recommended to the reader: http://las.perkinelmer.com/content/relatedmaterials/brochures/bro_delfiaresearchreagents.pdf.

1.3.2 Fluorescence Resonance Energy Transfer

Fluorescence resonance energy transfer, also known as Förster resonance energy transfer, is a process wherein one donor fluorophore in a sample emits a photon that, rather than proceeding out of the sample solution, excites an electron in a nearby acceptor molecule. The acceptor may or may not reemit the photon at a still higher wavelength; when it does not, the acceptor is known as a quencher. Because FRET very strongly depends on the separation distance between the fluorophores (in fact, it depends directly on the orbital overlap integral, which falls off with the sixth power of the separation), it can be used as a means of measuring intramolecular or intracomplex distances, although it is only sensitive within a small range that depends on the particular pair of fluorophores being used. Figure 1.4 presents a schematic diagram of the FRET process.
Over the years, FRET has enjoyed a productive career as a means of measuring separations of amino acid residues within proteins (6), assessing protease activity (e.g., with the EnzChek® Peptidase/Protease Assay Kit from Invitrogen), characterizing conformational changes in biomolecules (7), elucidating membrane dynamics (8), and studying protein–protein interactions (9). This is only a partial list of the assay methods that are possible with this versatile technique.

1.3.3 Fluorescence Polarization

As FRET is a means of obtaining information about molecular separations, so fluorescence polarization (formerly known as fluorescence anisotropy) is a way of measuring molecular size. This is accomplished by attaching a fluorophore to (or allowing it to associate with) the molecule or multimolecular complex under study. The sample solution is then irradiated with polarized light, the molecule or complex rotates, and the excitation energy is reemitted in a different direction (see Fig. 1.5). Because the average rate of molecular rotation depends inversely on the square of the size, it is precisely the time dependence of the emission that is informative as to the size of the fluorescent species. A large molecule rotates slowly (if a few dozen nanoseconds can be considered slow), yielding an emission signal later than a smaller fluorophore. It will occur to the reader that if the fluorophore is very small and can be made to associate with a large molecule or complex, the difference between the two FP signals will be correspondingly great. That is in fact the case, although FP can generally be
FIGURE 1.5 Schematic representation of fluorescence polarization. The unbound fluorophore of panel (a) is excited by polarized light from the lamp only if its excitation dipole is properly aligned. Other fluorophores are not excited. (b) The excited fluorophore rotates rapidly because of its small size, emitting light that reaches the detector, which is mounted at a 90° angle from the lamp beam. (c) The protein-bound fluorophore is also excited by the polarized lamp beam only if its dipole is properly aligned. The protein itself and other fluorophores are unaffected. (d) Because the rotation of the complex is slow, the fluorescence decays before the fluorophores dipole is aligned with the detector. Little or no fluorescence is seen. Thus the fluorescence reading depends on the size of the fluorescing complex. (See the color version of this figure in the Color Plates section.)

used to obtain binding information about two molecules of roughly the same size as well.

FP has been utilized in numerous recent studies of intermolecular associations and membrane phenomena, of which only a small number can be cited here (10–12). The method is highly versatile and often appears in surprising variations, such as a widely used kit available from Abbott Laboratories for assessment of homocysteine in plasma (13).

1.4 CHEMILUMINESCENCE AND BIOLUMINESCENCE

We return to our main focus to address these two forms of autoluminescence and explain the distinctions between them. The most prominent point is that while both fluorometric and spectrophotometric measurements rely on a lamp as the source of
energy, luminescent reactions by definition supply their own energy in chemical form. The nature of the energy-containing molecule(s) and the means by which the energy is transduced to light are of high interest to workers in the field and have profound implications for the utility of the various light-generating reactions in life science research.

It is possible to be fairly specific about the varieties of chemistries generally seen in these reactions. Both chemiluminescence and bioluminescence involve the emission of a photon accompanying the decay of a hyperoxidized intermediate. The difference is in how the intermediate is generated: in chemiluminescence it is the product of an oxidative chemical reaction, without obligatory enzymatic involvement, while in bioluminescence an enzyme (or photoprotein, such as aequorin) plays a critical role in forming the intermediate. Each of these processes is described in more detail below.

### 1.4.1 Chemiluminescence

Chemiluminescence has been well described, sometimes in concert with bioluminescence, from the points of view of both chemistry and applications in several useful volumes (14–16). Like bioluminescence, chemiluminescence is currently enjoying a rapid expansion of the range of its possible applications, but in the case of chemiluminescence, this is due almost exclusively to the progress in the relevant chemistries, while in the bioluminescence field, molecular biological tricks play an active role. It has become a routine to expect that if a fluorogenic substrate is on the market for assays of a particular enzyme, a chemiluminescent substrate is or will soon become available.

Among the simplest chemiluminescent reactions from a conceptual point of view is the spontaneous interaction of luminol with hydrogen peroxide (H$_2$O$_2$) in the presence of a second oxidant (such as iron or copper) to produce 3-aminophthalate (3-APA) and an impressive amount of bluish light, according to the mechanism shown in Fig. 1.6.

This reaction is exploited in a number of important coupled chemiluminescent reaction series, including the one involving horseradish peroxidase (HRP). The action of HRP greatly increases the luminescence of the reaction. The detection method involves covalent conjugation of the HRP enzyme to a ligand of interest. Typically, the ligand associates with a protein or other species adsorbed or covalently linked to an insoluble matrix (e.g., a Western blot membrane). Free ligand is washed away, an HRP-conjugated antibody directed to the ligand is added, more washing occurs, and finally the substrates are added to yield the luminescent signal. Although the components of the luminol/hydrogen peroxide reaction are easy to list, the role of the second oxidant and the process whereby HRP enhances the luminescence are not yet clear.

The luminol/hydrogen peroxide reaction is useful with certain categories of defined reagents, but it does not constitute a general system for chemiluminescent detection of enzyme activity. The major trend of this kind is toward molecules that incorporate the simple structure depicted in Fig. 1.7. The first stable dioxetane was synthesized in 1968, and the molecule was found to luminesce upon thermal decomposition (17).
INTRODUCTION

Luminol Azasemiquinone Aminophthalate

\[
\text{ONH}_2 \text{O}_2 \text{C} \text{N} \text{N} \text{C} \text{O} \rightarrow \text{ONH}_2 \text{C} \text{O} - \text{O} - \text{C} \text{O} \cdot \text{N} \text{H}_2 + \text{N}_2,
\]

\[
\text{e}^- + \text{H}^+ \rightarrow \text{light} + \text{N}_2.
\]

**FIGURE 1.6** Luminol/peroxide light-generating reaction series. Luminol undergoes spontaneous reorganization to the azasemiquinone radical. The superoxide anion also generated by spontaneous processes from \( \text{H}_2\text{O}_2 \) attacks the electrophilic carbons to form a dicarboxylic acid, releasing \( \text{N}_2 \) and generating a photon of visible light. Reproduced from [http://www.liv.ac.uk/Electrochemiluminescence/lusemiq.htm](http://www.liv.ac.uk/Electrochemiluminescence/lusemiq.htm) by permission of Dr. Robert Wilson.

Subsequent work on less stable 1,2-dioxetanes led to the issuance of a broad patent in 1994 covering “Enzymatically cleavable chemiluminescent 1,2-dioxetane compounds capable of producing light energy when decomposed” (18). One of the major advances of this work was the provision of water-soluble 1,2-dioxetane substrates. Other advantages of the new admantylidene dioxetane compounds, including a more rapid approach to steady-state light emission, were claimed. Alkaline phosphatase, \( \beta \)-galactosidase, and (by implication) esterases, acyl transferases, glycosidases, amidases, and a wide variety of other hydrolases were among the enzyme groups named as actual or potential assay targets for these chemiluminescent substrates.

**FIGURE 1.7** Common structure of the 1,2-dioxetanes, the predominant luminescent moieties of both synthetic and natural luminescent molecules. The light-generating process involves collapse of the dioxetane ring with liberation of \( \text{CO}_2 \).