Interplay of Photochemistry and Beer: How Lightstruck Flavor Is Formed and How It Can Be Prevented

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Beer goes “skunky” when exposed to light! What an ill-fated scenario: having a beer or two with friends, preferably in a sun-drenched spot, and photochemistry ruining your happy hour by dispersing a disagreeable smell and taste. Yet, beer consumers all over the world are faced to this susceptibility of beer to light. From a scientific viewpoint, it is remarkable how little we know about the phenomenon of the so-called “lightstruck flavor” of beer. This account aims at reporting on the current state-of-the-art, thereby highlighting seminal contributions from our laboratory.

Obviously, beer is a popular drink, as ca. 60 ml is consumed daily per world inhabitant, about as much as milk and fivefold more than wine. There are good reasons for this liking. Beer is an alcoholic beverage, but it is very diluted (90%+ water). It is a great socializing aid and it confers a feel of relaxation. Some prefer beer when they are thirsty, others appreciate the diversified palette of so many beer flavors and tastes. There is also growing evidence supporting the nutritional and health benefits of moderate beer consumption as part of a healthy lifestyle.

However, as the composition of beer is quite complex, chemical changes induced by the presence of oxygen, by temperature and, in particular, by light may affect beer flavor. Since all beers are colored—from very pale-yellow to almost black—a number of absorbers of visible and UV-A light are present and photochemistry must ensure on light exposure. The nature of most photochemical reactions is unknown, but it appears that they do not lead to readily observable organoleptic changes with one notable exception. The problem of a particular off-flavor in beer exposed to light was recognized as early as in 1875, and simple tests on the protective power of glass indicated that brown bottles were most effective. Gray et al. were first to show that thiols were involved in the development of an offending off-flavor. In the early sixties, Kuroiwa et al. used model systems to establish that a photochemical reaction in the wavelength range of 350-500 nm, involving a flavin such as riboflavin, beer bitter agents (isohumulones), and sulfur-containing compounds, led to the so-called “lightstruck flavor.” Other drinks including champagne, wine, and milk are also sensitive to light, however, none produces the unique “skunky” odor and taste of lightstruck beer.

Role of Hops

The hop plant (Humulus lupulus L.) is an essential ingredient for beer brewing, together with malted barley, water, and yeast. Hops are special to brewers, because they distinguish beer from all other alcoholic drinks. Hop can be considered as a beer herb, since only 1 or 2 grams are needed to brew 1 liter of beer, whereas malted barley is used in much larger quantities, typically 200-300 grams, thus serving as the “body” of beer and providing most of the proteins, polyphenols, dextrins and other substances. In the brewing process, hops are boiled for about 1.5 hours with wort, a sweet-tasting solution resulting from enzymic degradation of starch and proteins contained in malted barley. During the boiling in the brewing kettle, hop acids, called humulones (a mixture of 2 isomers and 1 homolog) are isomerized to cis- and trans-isohumulones in a ratio of approximately 7:3 (Scheme 1). It should be mentioned that the cis-trans notation refers to the tertiary hydroxyl group and the prenyl side chain at vicinal carbon atoms of the five-membered ring in isohumulones. These compounds are extremely bitter-tasting with a threshold value of ca. 5 milligrams per liter.

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From the Executive Director

D. C. Neckers, Executive Director, Center for Photochemical Sciences, Bowling Green State University

This month's feature article is an update by Denis De Keukeleire on the photochemistry of beer. In our 14 years of publishing The Spectrum, Denis' original article on the “Photochemistry of Beer” set the record for requests for additional copies, attention from graduate students, and general interest. In fact, we do not even have an issue left in our archives! Apparently, thinking great thoughts about, as the Dutch would say, licht doet bier slecht smaken (exposure to light ruins the taste of the beer) because the beer is contained in clear glass bottles plays well among our readers.

Tom Brady, President and CEO of Plastics Technologies, Inc. located in Toledo, was one of the leaders in the development of PET bottles for soda and water. Who would have thought that the largest selling soft drink (guess that’s what it is called) in 2001 would be water bottled in clear plastic? We don’t often see beer in those same PET bottles although Miller beer is starting to appear that way now. Those same crystalline polymers that are impervious to carbon dioxide are more sensitive to oxygen, and it doesn't take much oxygen seeping into beer to greatly change its smak. Somewhere between 1 and 2 ppm will do the dirty deed. That’s not much air sneaking through those little holes in the plastic.

It always amazes me at how much there is still to learn about even the most routine things. Until I met Brady I’d never thought about beer in plastic bottles. For that matter I was quite happy with those old glass bottles for Coca Cola. I don’t really know why all those polymer chemists had to change that. Of course, I still don’t get all this diet pop and lite beer stuff either! Some diet colas look and taste like dirty water. It’s no wonder careful students prefer cold, clear mountain spring water—even if it does come from some inner city tap.

On our campus old friend Chris Dalton has caused a huge furor because he signed an exclusive contract with Pepsi Cola to be Bowling Green’s soda supplier. All Chris wanted to do was to make a few extra bucks for the University. It turns out by signing an exclusive contract Pepsi would rebate some of the profits “to help keep the cost of tuition down, and faculty salaries up”. The Faculty Senate had Chris on their special hot seat reserved for really dastardly deed doers for that indiscretion. I bet Chris never knew Coca Cola had so many friends. I hate to think what my colleagues would have done were they living in Japan. Former postdoctorals tell me that the new 2000 Yen notes are particularly beautiful, but they haven’t figured out how to use them to get Asahi out of vending machines yet. The poor Japanese are having to carry their six packs home from the store for the first time in years.

Denis De Keukeleire got me thinking about all this again. When I heard him lecture a decade or more ago that licht doet bier slecht smaken met vriendelijke groet, I learned another Dutch phrase. Somehow that’s a much more colorful way to say leaving a clear bottle of beer in the sunlight makes it taste bad, even when it is said with a Flemish accent.

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(comparable to quinine). In this article, the term “isohumulones” refers to the group of 6 isomers and homologs including cis-trans epimers (the isohumulones with a 3-methylbutanoyl side chain constitute > 80% of the mixture, the iso-iso-humulones with a 2-methylbutanoyl side chain are very minor constituents). The total concentration of isohumulones in beer varies between 10 and 100 milligrams per liter. Subsequent to the boiling step, the bitter-sweet hopped wort is transferred to fermenting vessels and yeast converts sugars to ethanol and carbon dioxide within about one week. Young beer needs a maturation period at low temperature during several weeks prior to packaging.

Although less than 100 milligrams of hop-derived compounds remain in 1 liter of beer, they exhibit various important functions. Hop acts as a natural preservative in beer, partly due to its bacteriocidal activity, partly because of its high content in antioxidative flavonoids. Isohumulones are essential to stabilize bubbles in beer foam and they account for most of the bitterness, particularly in pale beers including lager or pilsner-type beers. Unfortunately, isohumulones decompose rapidly either by direct exposure to UV-A light, or by indirect processes involving degradation of a “photosensitizer” by visible light.

From Excited Isohumulones to the Lightsstruck Flavor

A formal mechanism for formation of beer lightsstruck flavor in model systems, composed of isohumulones, riboflavin, and cysteine, on exposure to visible light, has been suggested by Kuroiwa et al. already in 1963. Photoexcited riboflavin induces cleavage of isohumulones to a 4-methylpent-3-enoyl radical, which undergoes decarbonylation to a 3-methylbut-2-ene-1-thiol radical. Trapping of this stabilized allyl radical by a thiol radical derived from cysteine leads to formation of 3-methylbut-2-ene-1-thiol (MBT) (Scheme 2). This mechanism has been frequently referred to in articles dealing with light-induced off-flavors in beer and, in fact, all relevant data collected to date do not contradict the Kuroiwa-premise.

In particular, MBT has garnered a status as “skunky thiol”. MBT, together with other sulfur-containing constituents, has been identified in malodorous secretions of the anal glands of skunks (Mustela vison L.). Its formation on exposure of beer to light has been confirmed, while a range of techniques have been reported to quantify MBT. MBT is one of the most powerful taste- and flavor-active compounds known, while concentrations around 1 nanogram per liter (ca. 9.8 pM) can make (pale) beer unpalatable. Therefore, even very small photochemical conversion rates of isohumulones (0.28-2.8 mM in beer) can produce this effect. Recent work has pointed out that the “skunky flavor” is composed of a rather intricate mixture of sulfur-containing compounds. Nevertheless, MBT remains largely responsible for the offending flavor and odor (Figure 1).
Although the key components were identified by Kuroiwa, details of the mechanism have not been clarified. Some time ago, we showed that trans-isoalcohol was photolyzed to dehydrohumulinic acid on irradiation at 300 nm in methanol as indirect proof of the involvement of a Norrish Type I α-cleavage in the formation of the lightstruck flavor (Scheme 3). We examined—in collaboration with Prof. Dr. Malcolm Forbes, Department of Chemistry, University of North Carolina, Chapel Hill, NC, USA—the primary photochemical events using time-resolved (continuous wave) electron paramagnetic resonance (TREPR) spectroscopy and computer simulations of the spectra to identify the free radicals produced. The TREPR experiment has superior time response (ca. 60 ns) over steady-state EPR methods, yet retains high structural resolution needed to measure hyperfine interactions used for assignment of the signal carriers. Due to the presence of chemically-induced electron spin polarization (CIDEP) in the TREPR signals, we have also obtained important new insight into the photophysics of the excited states leading to the observed radicals.

Figure 2 shows the X-band (9.5 GHz) TREPR spectrum obtained at a delay time of 0.3 μs after photolysis at 308 nm (excimer laser) of a toluene/methylenechlorohexane solution (1:1 ratio) of a mixture of trans-isorumulones (trans-isoalcohol, trans-isocalcohol, and trans-isoalcoholone), while a computer simulation using appropriate magnetic parameters is displayed also. The strong TREPR signal proves that free radicals are indeed produced, when UV-light strikes isoromulones. The high intensity in the center of the spectrum and weak signals at the edges indicate the presence of at least two different radical species with significant electron-nuclear hyperfine interactions. We have strong arguments to conclude that the five-membered ring radical, derived from trans-isorumulones, accounts for the intense signal (18 closely spaced lines), while the 3-methylbut-2-enyl radical has its intensity distributed over a very large number of hyperfine lines (128) that cover more than 100 Gauss. The net emission is generated via the triplet mechanism of CIDEP.

The TREPR data allowed us to propose a detailed mechanism for formation of the lightstruck flavor (Scheme 4). UV-light is absorbed by the β-tricarbonyl chromophore of the isoromulones and, after intersystem crossing, triplet energy transfer occurs on the submicrosecond time scale to the α-hydroxyketone, resulting from very strong through-bond coupling of both chromophores and relatively long electron spin-lattice relaxation times of the triplets involved in the energy transfer process. Subsequent photochemistry furnishes free radicals via a Norrish Type I α-cleavage. The events can be monitored by TREPR, because of strongly emissive triplet-mechanism CIDEP, which is produced initially and then propagated throughout the various photophysical and photochemical pathways. Simulation of the TREPR spectra provides unambiguous assignment of the signal carriers to the radicals proposed in the mechanism. Future work will include attempts to obtain kinetic studies on the intersystem crossing, spin relaxation, and
From Excited Riboflavin and Isohumulones to the Lightstruck Flavor

The lightstruck flavor is most likely formed on exposure of beer to visible light, the most damaging wavelengths being in the range of 350-500 nm. Since the isohumulones as key constituents do not absorb in this wavelength region, the intervention of a suitable “photosensitizer” is necessary. Riboflavin (RF) or vitamin B2 is present in beer in concentrations around 1 milligram per liter and other flavins have been detected as well. In model systems, as applied by Kuroiwa, RF absorbs visible light and interaction with isohumulones leads to degradation products and radicals that eventually give rise to formation of MBT.

We envisaged to exploit TREPR to get insight into the mechanism for formation of the lightstruck flavor using RF or flavin mononucleotide (FMN) as a “photosensitizer” under visible light conditions. Since RF exhibits two broad absorption bands with maxima at 350 nm and 450 nm, the third harmonic of a pulsed YAG-laser (355 nm) was a most suitable
decarbonylation processes involved in this photochemistry. The primary photophysical and photochemical processes result in formation of a 3-methylbut-2-ene radical, which, subsequently, is trapped by a thiol radical derived from an as yet unidentified sulfur source to give MBT and, consequently, lightstruck flavor. We have probed this important reaction by a preliminary TREPR study on trapping of radicals, derived from trans-isohumulones, using n-butane. Very remarkably, we observed a time-dependent alteration of the reference TREPR pattern, which clearly indicates interaction in the time domain below 1 μs. Further studies are necessary to gain more insight into the role of the sulfur source with regard to formation of the lightstruck flavor.

Product analysis of the reaction mixture obtained after irradiation of trans-isohumulone at 254 nm in methanol confirms the proposed mechanism, as typical photoreaction products are derived from recombination of the 3-methylbut-2-ene radical with the incipient five-membered ring radical following Norrish Type I α-cleavage (Scheme 4). The major photodecomposition product of trans-isohumulone is decarbonylated dehydrated isohumulone (Figure 3, peak 9) and the immediate precursors, decarbonylated cis-isohumulone and decarbonylated trans-isohumulone (peaks 5 and 7), are present as well. Other peaks are due to interfering photochemical reactions including retro-oxa-di-α-methane rearrangement to humulone and photoenolization of the β,γ-enone chromophore in the side chain of trans-isohumulone sequenced by further dark reactions. It can readily be understood that, in the presence of a suitable sulfur source, the 3-methylbut-2-ene radical should be converted to MBT rather than recombine with other isohumulone-derived radicals, thus leading to the lightstruck flavor.

Scheme 4. Mechanism for formation of 3-methylbut-2-ene-1-thiol (lightstruck flavor) on direct irradiation of isohumulones and structures of the main photoreaction products on irradiation of trans-isohumulone (254 nm, methanol).
excitation source. When RF and isohumulones were irradiated, a broad emissive/absorptive EPR signal was detected with a maximum intensity at a delay time of ca. 750 ns. Similarly, a very strong predominantly emissive signal was observed on excitation of FMN in the presence of isohumulones, together with a superimposed emissive signal, which we were able to attribute to a 3-methylbut-2-enyl radical. Additional experiments and comparison with EDTA as an efficient electron donor led us to establish a reasonable mechanism for the initial photochemical events leading to the lightstruck flavor (Scheme 5).

First of all, it was demonstrated by Hastings et al. that the values of the triplet energies of RF (about 210 kJ/mol) and isohumulones (about 300 kJ/mol) exclude direct energy transfer.\(^\text{15}\) Hence, RF is incapable of generating excited-state isohumulones and, thus, RF-initiated photosensitization is not operative. Triplet-excited RF is a very strong electron acceptor\(^\text{16}\) and electron transfer from isohumulones affords an oxidized species. The electron may be released
from the β-tricarbonyl unit or from either oxygen of the α-hydroxyketone. No details are known yet, but for each of the possible radical cations, stabilization pathways can be envisaged that lead, via a formal Norrish Type I α-cleavage of the α-hydroxyketone, to a 3-methylbut-2-enyl radical en route to the lightstruck flavor. One clue regarding the nature of oxidized species, derived from isohumulones, has been the identification of oxydehydromulanic acid (Scheme 5) as a major photoprodut from the visible-light irradiation of trans-isohumulone in the presence of RF. The mechanism for formation of this oxidized derivative of trans-isohumulone remains elusive pending further research.

It should be clearly pointed out that RF does not function as a photosensitizer according to definition, since it is itself reduced in the process. Rather, RF or flavins are the photoreactive entities in model systems—and very likely also in beer—which induce decomposition of isohumulones. It is, furthermore, very remarkable that both direct and “photosensitized” excitation of isohumulones channel the breakdown to the pivotal 3-methylbut-2-enyl radical, although accesses are entirely different.

Prevention of Formation of the Lightstruck Flavor

Apart from storing beer in light-proof containers, such as dark glass, kegs or cans, or immediate consumption, the photosensitivity can be circumvented by quenching of the excited triplet state of the isohumulones and/or RF (not readily achievable) or by the use of chemically modified isohumulones, whereby formation of the lightstruck flavor is prohibited. This is currently achieved on an industrial scale in several hop processing plants around the world reflecting the state-of-the-art of hop technology aimed at controlling not only the light-stability of beer, but also bitterness levels and desired foam features. About half of the hops produced worldwide is extracted with liquid or supercritical carbon dioxide (hops ranks first in the application of this technology thereby preceding decaffeination of coffee). After removal of the hop essential oil, humulones are isomerized to isohumulones according to a very efficient procedure (more than 90% yield) developed some time ago in our laboratory; whereas conversion of humulones in the brewery is very low (less than 30%) when hop cones are used. The isohumulones, commercially available as standardized aqueous solutions, can be used to dose exactly the bitterness of beer. In a further manipulation, isohumulones are (quantitatively) reduced to dihydroisohumulones and tetrahydroisohumulones (Scheme 6; both are mixtures of isomers, homologs, and stereomers) by sodium borohydride reduction and catalytic hydrogenation, respectively. These so-called advanced hop products are now widely used in the brewing practice. Introduction of this advanced hop technology during the last decade has led to a much improved overall hop utilization.

Dihydroisohumulones, in which the photosensitive α-hydroxyketone is reduced to a photoinactive 1,2-diol entity, are fully light-resistant, since a Norrish Type I α-cleavage can not occur. Consequently, beers, bittled with dihydroisohumulones, can be kept in clear glass bottles without any harm being done by light. In contrast, tetrahydroisohumulones are as photoreactive as isohumulones, however, MBT cannot be formed subsequent to Norrish Type I α-cleavage, since the double bond in the photosensitive side chain is lacking. In principle, it may be feasible that some sort of a lightstruck flavor is developed from tetrahydroisohumulones, but until now a distinct flavor change in beers, bittled with tetrahydroisohumulones, has not been observed. In this respect, many consider tetrahydroisohumulones to be light-proof, which is obviously not correct. It may be added that tetrahydroisohumulones have gained fame, because they are twice as bitter as isohumulones and they have a strong impact on the stability of the head on a glass of beer and on the foam cling to the glass.

The light-associated characteristics of dihydroisohumulones and tetrahydroisohumulones have been nicely confirmed by our TREPR experiments. Photolysis of trans-tetrahydroisohumulones (trans-tetrahydroisohumulone, trans-tetrahydroisocohumulone, and trans-tetrahydroisoadhumulone) at 308 nm yielded the TREPR signal, displayed in Figure 4, which, again, has a simulation shown immediately below it. The shape is altered significantly with respect to that of trans-isohumulones (see Figure 2). It is noteworthy that the signal of the trans-tetrahydroisohumulones has
narrowed considerably, which is to be expected if, after Norrish Type I α-cleavage, decarboxylation to a stabilized allyl radical is no longer taking place on the μs-time scale and, consequently, the hyperfine structure due to a 3-methylbut-2-enyl radical disappears. There are two additional transitions seen on the perimeter of the spectrum, one emissive line on the low-field side and a weaker absorptive line on the high-field side. These transitions, marked with an asterisk (*), are due to an unknown radical, which could not be simulated. The signal may arise from a competing photochemical reaction or from secondary photochemical processes. The fact that this signal is more strongly spin-polarized by the radical pair mechanism of CIDEP indicates that it does not result from a primary process. It is more likely to have been generated at a later delay time, after the strong net emissive polarization from the parent ketone triplet has relaxed.

Photolysis of a solution of dihydroisohumulones at 308 nm does not lead to observable TREPR signals. This is the first direct spectroscopic evidence for the resistance of dihydroisohumulones to photolysis and it is the most conclusive proof to date that the photochemistry leading to the lightstruck flavor in beer requires activation of the α-hydroxyketone moiety.

To provide additional support for the mechanism, proposed in Scheme 4, the triplet spectrum of frozen dihydroisohumulones in methyliclohexane solution was generated and observed by TREPR (Figure 5A). This experiment should characterize the β-tricarbonyl triplet in the absence of energy transfer, as observed for isohumulones. The emissive half-field transitions, indicated by an asterisk, strongly suggest that the net emissive polarization, shown in Figure 2, originates from the β-tricarbonyl chromophore. A rough estimate of the dipolar interaction D in the triplet can be made by measurement of the separation (in Gauss) between the outermost Δm = 1 transitions, indicated in dashed vertical lines. This gives $D = 1100 \pm 100$ Gauss or approximately 0.1 cm⁻¹, which is very consistent with a delocalized triplet such as this β-tricarbonyl unit.

We further irradiated dihydroisohumulones in the presence of 2,2,6,6-tetramethylpiperidine-1-oxy (TEMPO), a stable free radical. In the TREPR spectrum an emissively polarized three-line spectrum of TEMPO is observed (Figure 5B). No TREPR spectrum is obtained with either dihydroisohumulones alone or TEMPO alone. The emissive polarization of the TEMPO free radical is due to the radical
triplet–pair mechanism of CIDEP,\(^\text{20}\) which results from the diffusive encounter and magnetic interaction between an excited triplet molecule, in this case that of the dihydroisohumulones, and a doublet-state free radical. The net emission must arise from the polarized triplet state of the dihydroisohumulones, as TEMPO does not absorb the light, nor does it have any spin polarization it can acquire on its own. Both spectra in Figure 5 allow us to conclude that the emissive polarization, observed in the TREPR spectra in Figure 2, is originally generated in the intersystem crossing process from \(S_\text{1}\) to \(T_\text{1}\) of the \(\beta\)-tricarbonyl moiety, the primary light–absorbing chromophore in the isohumulones.

Conclusions

Photochemistry undoubtedly deteriorates the quality of beer and protection against light is absolutely necessary. Isohumulones, the main beer bitter compounds derived from hops, undergo light-induced decomposition either on direct illumination with UV-A light, or via a photoredox reaction involving excitation of a visible-light absorber such as riboflavin. Both routes lead to formation of a 3-methylbut-2-enyl radical, which is trapped by a thiol radical originating from a suitable sulfur source thereby affording 3-methylbut-2-ene-1-thiol, known as “skunky thiol”. This lightstruck flavor, observable at a concentration of the thiol of around 1 nanogram per liter, is the most offending off-flavor known to occur in beer. Our studies of this phenomenon by means of time-resolved electron paramagnetic resonance, applied to the photochemistry of individual beer bitter compounds and model systems, in conjunction with identification of photoreaction products, have permitted to establish reliable reaction mechanisms for formation of the lightstruck flavor. The results obtained so far should form a fundamental basis for further complicated studies on beer itself.

Physical prevention against the lightstruck flavor calls on the use of dark glass bottles (preferably brown-colored) or any opaque package, but modern hop technology enables application of high-tech and efficient chemical protection. Dihydroisohumulones are perfectly light-stable and beers bittered with these advanced hop products can safely be packaged in clear glass bottles. Tetrahydroisohumulones are sensitive to light, but, since 3-methylbut-2-ene-1-thiol can not be formed, a possible flavor change does not compare with the lightstruck flavor. In the brewing practice, a hopping procedure using a combination of dihydroisohumulones (between 15 and 20 milligrams per liter) and tetrahydroisohumulones (few milligrams per liter) is a very attractive option as an alternative for hop cones. Indeed, dihydroisohumulones account for light-stability, while tetrahydroisohumulones accentuate bitterness and greatly improve the stability and cling of beer foam.

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1. The present article updates a previous paper by the same author on Photochemistry of Beer, which was published in The Spectrum \textit{1991}, 4(2), 1-7.

About the Author

Dr. Denis De Keukeleire received his Ph.D. in chemistry at the Ghent University, Belgium, in 1971. He worked as a NATO-postdoctoral fellow on the photochemistry of trichromophores with George Hammond at the California Institute of Technology, Pasadena, California, during 1971-1972. From 1972 until 1991 he was a senior research associate of the Belgian Fund for Scientific Research with a position as photochemist and phytochemist at the Laboratory of Organic Chemistry, Ghent University. In 1991, he joined the Laboratory of Plant Biochemistry and became full professor in 1992 at the Faculty of Pharmaceutical Sciences, Ghent University. His research interests are in photochemical synthesis and natural products, in particular hops. The expertise on beer bitter compounds derived from hops and on hop aroma is currently being complemented by focusing on health-beneficial and medicinal properties of hops, mainly on phytoestrogenicity. His address is Ghent University, Faculty of Pharmaceutical Sciences, Laboratory of Pharmacognosy and Phytochemistry, Harelbekestraat 72, B-9000 Ghent, Belgium (+3292648055); e-mail: Denis.DeKeukeleire@rug.ac.be; http://allserv.rug.ac.be/~ddkeukel.
A Tribute to George S. Hammond in Celebration of His 80th Birthday

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In seeking an introduction to a tribute to George S. Hammond in honor of his 80th birthday, it seemed fitting to start with a quotation about the joys and beauty of chemistry and photochemistry by Sir C. N. Hinshelwood, on the occasion of the 100th anniversary of the Chemical Society of London:

“...Chemistry, that most splendid child of intellect and art. Chemistry provides not only a mental discipline, but an adventure and an aesthetic experience. Its followers seek to know the hidden causes which underlie the transformations of our changing world, to learn the essence of the rose’s color, the lilac’s fragrance and the oak’s tenacity and to understand the secret paths by which the sunlight and the air create these wonders.

And to this knowledge they attach an absolute value, that of truth and beauty. The vision of Nature yields the secret for power and wealth, and for this may be sought by many. But it is most often revealed only to those who seek it for itself.”

This quote captures the love and excitement of chemistry, and especially photochemistry, that George Hammond instilled in his students, in his collaborators and colleagues and in those who followed his distinguished and remarkable career.

Hammond, Scientific Revolutions and Thermodynamics.

According to Kuhn’s “Structure of Scientific Revolutions”, a paradigm shift in science occurs when a scientist or group of scientists change the way that other scientists think. By this definition, George Hammond has been a paradigm shifter many times over! In particular, George’s research during the late 1950s, 1960s and 1970s caused paradigm shifts which created the platform for the way organic photochemistry is done today. Kuhn suggested that not only do revolutionary scientists change the way that other scientists think, but exceptional scientists provide splendid exemplars of their research that serve as models for emulation and rapid progress in the field. George’s research during the golden age of photochemistry at Caltech provided exemplar after exemplar for the community to study and to imitate.

There is an amusing analogy between the rules of thermodynamics and the impact that George Hammond has had on the field of photochemistry. Thermodynamics teaches that the entire universe evolves under the influence of two conflicting tendencies: the drive towards chaos (entropy) and the drive towards organization (enthalpy). It is this conflict and the resulting balance of these two driving forces of evolution that determine the richness of diversity we observe in the world around us. Students of science never fail to wonder at the art with which nature continuously interweaves these two great themes into an effective mode of action we chemists call free energy and which has led to the incredible rich array of animate and inanimate objects that constitute our Universe. When George entered the field of photochemistry, the system was entropy rich and in need of organizational enthalpy. George provided the intellectual and scientific leadership that gave structure to the field and yet allowed for the expansion of the discipline as complexity and new methods, ideas and structures emerged and evolved.

Hammond and Dynamics.

How was it possible for mechanistic organic photochemistry to have developed so rapidly with George at the helm? Thermodynamics determines the evolution of chemical systems toward equilibrium, but is silent to the rates and kinetics of the achievement of equilibrium. What can be said about the incredibly rapid rate at which groundbreaking research and outstanding photochemists were produced under George’s guidance? Thermodynamics is
determined by average conditions. Kinetics are determined by fluctuations of energy and occasional dramatic departures from the average. These exceptional departures characterize the stuff of Kuhn’s revolutionary science and paradigm shifts. The presence of a great individual of action or thought determines the location and time of such revolutions. So it was with George and his group at Caltech.

George, we all thank you and love you for your mentoring, your insights, your dedications and your ability to imprint your scientific attributes on your students and collaborators.

**Peter Leermakers, the Caltech Recruiter.**

For the remainder of this report, I would like to present a brief set of recollections of my years with the Hammond group during 1960-1963.

I started my chemical career as an analytical chemist working summers in the Connecticut State Water Laboratory at Wesleyan University. It was there that I met Peter Leermakers who was a Wesleyan student two years my senior and who also worked in the water lab during the summers when he was an undergraduate. Peter left for graduate school at Caltech in the fall of 1958. During the summer before my senior year, Peter returned and informed me that Caltech was the place I had to go for my Ph.D. and furthermore, that I had to work for George Hammond! With typical respect for my elders, that’s exactly what I did. I turned out to be the second in a series of Wesleyan graduates who went west to work with George. Bill Herkstroeter, Don Valentine, and Dave Eaton were to follow.

Sandy and I drove across the country and landed in Pasadena at the end of August of 1960. Peter was there waiting for us and helped us settle into the >100° Southern California temperature by inviting us to his apartment which was equipped with a wonderful swimming pool and with margaritas, so powerful that your lips began to curl as the glass of tequila approached them. Peter’s apartment complex in northern Pasadena housed a number of Caltech graduate students in chemistry and in physics.

**The Caltech Years in the Hammond Group (1960-1963).**

**The Triplet State Raises Its Three-Pronged Head and Photochemistry Is On Its Way Off the Launching Pad.**

When I spoke to George Hammond about a research project, he mentioned that he was working on the “triplet state” (how to visualize the “three states” was a puzzle to me at the time and has remained a theme for our research group over the decades) and something about catalyzed formation of triplets by metal complexes. Three states sounded better than one to me, but I really had no idea of what this was all about and was afraid that it might be too physical chemical-ish for me. Nevertheless, I was very impressed with George and his wonderful reputation for working with students, and I was delighted that he accepted me into the group. I was also lucky to have some wonderful classmates such as Jack Saltiel and other lab mates such as Peter Leermakers, Bob Foss, Karl Kopecky, Jim Clovis, Fred Fischer and Lee Mahoney.

**Metal-Complex-Catalyzed-Decomposition of Alkyl Pyruvates**

My initial project was not photochemical, but was the investigation of a thermolysis that might involve a triplet state. The idea was to study the thermolysis of methyl pyruvate and see if it could be catalyzed by paramagnetic metal complexes. If the decomposition proceeded through a triplet and collisions with the paramagnetic species, it could lower the A factor and cause a catalyzed rate acceleration. There was some evidence in the literature from measurement of Arrhenius parameters that the cis-trans isomerization of ethylenes might go through a triplet state and that certain catalysts of the cis-trans isomerization might operate by “catalyzing” formation of the triplet.

My first lab experiment was to synthesize methyl pyruvate. Eagerly, I went to the library and found an Organic Synthesis prep involving the reaction of diazomethane with pyruvic acid. Unfortunately, the storeroom did not stock diazomethane so I had to make some. To my amazement, diazomethane was described in the literature as a yellow gas. That sounded neat. I went to chem stores and signed out the equipment required to prepare diazomethane. I forget the details, but I remember it required a decomposition that produced this remarkable yellow gas that could then readily be distilled and collected in a cold trap.

I set up the equipment, started the reaction according to the Organic Synthesis recipe and everything went like gangbusters! The yellow gas came bubbling out of the round bottom flask containing the reactants and condensed into a cooled ether solvent in the collection flask. I was so proud of all this that I rushed down the hall to see if there was someone from Robert’s group to whom I could show off the set-up. John Baldwin, a second year graduate student working for Jack Roberts, came and looked at the set-up, turned ashen white and raced out of the room screaming, “You should never use ground glass joints when distilling diazomethane in ether! The ground glass can cause the
diazomethane to decompose uncontrollably and set off an explosion!!!” John was a real scholar and knew all this stuff. Hey, I was just a rookie; how was I to know? Anyway, the reaction worked well as did the synthesis of methyl pyruvate, so now I could start decomposing it in the presence of the metal complexes.

Interestingly, at the same time that I was using metal complexes to decompose methyl pyruvate, Peter Leemakers was using the same metal complexes to quench benzophenone triplets and looking for a correlation with paramagnetic properties. As it turned out, the thermolysis experiments did not show a correlation with paramagnetic properties but were a lot easier to run than photochemical quenching experiments. It turned out that the metal complexes that “catalyzed” the decomposition of methyl pyruvate were also excellent quenchers of benzophenone triplets. Pretty soon I was predicting which metal complexes would quench and which would not, in the photochemical experiments and doing this without a photon in sight!

One of Those Serendipitous, Career-Defining Events. The Friday Night Lecture Demonstration at Caltech.

After about six months of research on the metal complex catalyzed decomposition of methyl pyruvate, a serendipitous twist made a critical impact on my career. As an undergraduate at Wesleyan, I had discovered the luminol chemiluminescence experiment and would show it off any opportunity I could get. One day I was showing it off in the lab. George Hammond was about to present a “Friday demonstration lecture” to the Pasadena community and I needed him about the demonstration that he might be presenting, assuming that there would be none. George nailed me with, “Nick, why don’t you show the luminol experiment. It’s sort of photochemical and that’s what I’m going to talk about.” With a combined feeling of anxiety and excitement, I accepted. As I expected, George gave a fabulous lecture on the research that Peter, Bob Foss, Bill Baker and others had been doing on the photochemical reactions of benzophenone and his beautiful method for demonstrating the involvement of triplets. What I didn’t expect is that when he introduced my demonstration he jokingly termed me an “anti-photochemist” because my results on the catalyzed decomposition of methyl pyruvate were contrary to current paradigms about the effect of paramagnetism on photoreactivity. The demonstration went well (I still remember someone in the audience saying, “Wow! This is just like Disneyland!!!”).

The Saturday Morning After Experiment. The Beginning of Triplet Sensitized Photoreactions in Solution. Triplet Energy Transfer Becomes a Household Word.

On the Saturday morning after the Friday demonstration lecture, I went into the lab and talked to Peter Leemakers about George’s comment the night before about my being an anti-photochemist. Peter had a great idea to get me into photochemistry: mix up ethyl pyruvate (my project) and benzophenone and irradiate (Peter’s project) the brew and see what happens! In fact, the experiment was set up by noon. We noticed that upon irradiation, bubbles of gas came streaming out of the photolysis setup. The bubbles stopped immediately when the lamp was off and started up again when the lamp was turned back on. This was so exciting that we called George at home, and in minutes he was in the lab to proclaim this was a sensational result. Neither Peter nor I had much of a clue why he was so excited, but it was the beginning of the use of triplet energy transfer to sensitize photoreactions, and George, with his typical insight, sensed where he could go with it. Little did Peter and I realize that this was the humble beginning of “triplet photosensitized photoreactions in solutions”. George had obviously grasped its importance immediately and within a few weeks half the group was working on some form of photosensitized reaction.

Peter and I then went to work immediately on trying to photosensitize the Diels-Alder reaction of a diene with maleic anhydride, a topic of considerable controversy at the time. We went to the storeroom to find an appropriate 1,3-diene to add to the maleic anhydride, which we already had in hand. The chemical storeroom at Caltech had a bottle labeled “purified 1,3-pentadiene”, affectionately known as piperylene. That sounded pretty good so we used piperylene as the diene in an attempt to photosensitize Diels-Alder. Checking the attempted photosensitized reaction by vpc indicated no reaction of the maleic anhydride, but the piperylene appeared to isomerize. We assumed that the “purified” piperylene was trans and that the isomerization was trans to cis. However, we discovered that the bottle actually was PURE cis-piperylene. Checking into the source of the cis-piperylene (remember, in 1960 there was no Aldrich and students often left chemicals in the chem stores after they finished their thesis work), we found that a student of a former Caltech faculty member had prepared it. The amazing thing was that the preparation involved taking a mixture of cis and trans-piperylene with maleic anhydride!! Obviously a scholar, the student, knew that the trans-piperylene reacted rapidly with maleic anhydride in a Diels-Alder reaction at room temperature, but cis-piperylene did not react at all due to simple steric hindrance considerations! Had Peter and I known that, we probably would not have used piperylene for our photosensitization experiments!!
Shortly after the discovery of triplet photosensitization, Karl Kopecky showed how to produce triplet carbenes from photosensitization of diazomethane decomposition. Jack Saltiel launched the photosensitized isomerization of stilbene, John Fox did the photosensitized decomposition of azo compounds, and Fred Fischer and I got involved in the norbornadiene to quadracyclic valence isomerization. Wow! This was a fabulous period of activity and excitement.

As a result of the prodigious research generated by George’s group, the period 1960-1963 was one of tremendous excitement in the field of photochemistry and scientifically as the field of mechanistic organic photochemistry was expanding in an exciting and explosive manner. What a group of colleagues! In addition to Peter, there was Angelo Lamola, Jack Saltiel, and Bob Liu who went on to blaze their own trails in photochemistry; Karl Kopecky, who synthesized and isolated the first 1,2-dioxetanes when he started his academic career at Alberta, was a labmate. Downstairs in Crellin Laboratory, Wilse Robinson was helping to put the ideas of molecular spectroscopy into photochemistry with postdocs like Mostafa El-Sayed.

**Fond Memories of Photochemistry at Caltech and Many Thanks.**

Everyone who was in George’s group during the golden years at Caltech recalls with fondness the incredible highs that were had as George led discussions in which new ideas crackled like sparks. It seems that after an hour’s discussion on the little blackboard near the entrance to the lab, within days new and exciting results were produced.

George showed us all how to share his love and excitement with his muse, chemistry. We all owe many thanks and enormous gratitude to George for creating so much new knowledge and for changing the way we think. Perhaps this feeling for George is captured in another quote from Hinshelwood:

“But of that most important kind of knowledge, that which does not seem to relate to any existing field, it is harder to speak on the basis of anything but faith. And yet in this knowledge lies the true seed of the future. It will come only from the least conforming of minds, and the discoveries of the greatest ultimate moment are the least likely to have been favored by official encouragement or support. They must be like the flowers of the poet

...daffodils,
That come before the swallow dares, and take
The winds of March with beauty.”

Such thoughts have united the Hammond clan for decades and will continue to do so for decades to come.

Respectfully submitted,

Nick Turro
Hammond group graduate and very proud of it!

**About the Author**

Nicholas Turro received his undergraduate degree from Wesleyan University (CT) in 1960 and his Ph.D. from Caltech in 1963 under the direction of George S. Hammond. After a year’s postdoc at Harvard with P. D. Bartlett, he began his career in 1964 at Columbia University where he is currently the William P. Schweitzer Professor of Chemistry, Professor of Chemical Engineering and Applied Chemistry, and Professor of Earth and Environmental Engineering. He is the author of 2 books and over 670 scientific papers. He is a member of the National Academy of Sciences and the American Academy of Arts and Sciences. Recent awards include the ACS Award in Colloids and Surface Chemistry and the J. Willard Gibbs Medal awarded by the Chicago Section of the ACS. He can be reached at Columbia University, Department of Chemistry, 3000 Broadway, MC 3119, New York, New York 10027, e-mail: njt3@columbia.edu.
Imaging the Dynamics of Gene Expression in Live Cells With Luciferase Bioluminescence

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Introduction

Many different organisms have evolved the ability to emit light from specialized chemical reactions through the process of bioluminescence. Although several intriguing functions have been attributed to bioluminescence such as luring prey and distracting predators, this discussion will instead focus on novel uses of bioluminescence to observe and analyze cellular processes. One current application of bioluminescence relies on the gene for the luciferase enzyme from fireflies that can be used to dynamically monitor gene expression in living cells. This luciferase reporter gene is typically introduced into cells along with a unique upstream flanking DNA sequence. When cell signaling events activate similar upstream regulatory regions of endogenous genes, they also activate the reporter gene. An increase in luciferase enzyme activity therefore reflects an increase in gene expression, as long as the reactants for bioluminescence are in abundance. Bioluminescence imaging techniques offer distinct advantages and disadvantages relative to comparable fluorescence based methods. Bioluminescence is particularly suited for long-term quantitative imaging of processes that last several days such as circadian rhythms, pulsatile hormone release, development, infection, or tumor growth.

The Firefly Luciferase Reaction

The commonly used luciferase from the firefly Photinus pyralis produces light by adenylating and then oxidizing the di-hydro form of d-luciferin (4,5-Dihydro-2-(6-hydroxy-2-benzothiazolyl)-4-thiazolecarboxylic acid) in the presence of ATP, oxygen, and Mg²⁺. Yellow-green light peaking at 560 nm in alkaline media is generated with a high quantum efficiency (0.88 Q.E.). Low turnover of luciferin due to the rate-limiting release of oxyluciferin from the enzyme enables long-term imaging but restricts photon emission rates. Although similar to other adenylating molecules, luciferase is a foreign protein that shows no toxic effects on the cell and does not appear to interfere with cell function. Furthermore, luciferin is very well tolerated by cells, whereas decanal, the substrate for bacterial luciferase from the reporter gene lux, is classified as an irritant of mammalian cells.

The low activity of luciferase (0.1 U/mg) requires sensors that can quantify individual photons and efforts must be made to minimize sensor and system noise. Of course, imaging is performed in complete darkness. Devices and optics suitable for bioluminescence imaging are readily available.

Important Properties of Bioluminescence

Although both fluorescence and bioluminescence imaging require very sensitive cameras, the light emission from luciferase is usually considerably weaker than fluorescence and often requires longer exposures for each image. In particular, the dark current of these cameras is nearly eliminated by cooling the sensor to around −90 °C with liquid nitrogen. More convenient cameras that use thermoelectronics to provide comparable sensor cooling have been introduced recently. Dark current is due to thermal noise accumulating in the sensor’s pixel wells as charge, which is roughly halved with each 7 °C drop in temperature. Because exposures of 10 to 60 minutes are often needed, high dark current would rapidly saturate the sensor or mask the image if not for cooling.

Unlike bioluminescence, fluorescence images can be brightened and sharpened by applying more excitation light. This need to excite can, however, be a disadvantage because of the cell damage caused by light-generated reactive oxygen species and fluorophore bleaching. These toxic and signal-degrading effects are not present with bioluminescence. Furthermore, bioluminescence may be suitable for use in light-sensitive structures such as the retina because of its very dim signal.

Along with phototoxicity, fluorescence excitation light generates autofluorescence that can substantially degrade the signal-to-noise ratio (S/N) of images. Special filters are often used to isolate the emitted light from this background noise, but these also lower signal strength. Bioluminescence provides remarkable sensitivity, as low as
femtomolar levels in vitro, partially because it is not limited by autofluorescence. An ultraweak luminescence due to metabolic activity and lipid oxidation has been reported in many cell types, but it is usually well below the luciferase signal intensity.

Luciferase imaging does have other limitations that have not yet been fully explored. For example, when cells within tissues are imaged, the light-generating substrate luciferin must be able to pass through membranes to the cells of interest. Some researchers use a surfactant such as Triton X-100 to improve loading of luciferin into cells. One study estimated about a 20- to 40-fold gradient of luciferin across the cell membrane and suggested that luciferin is distributed according to the Nernst potential because of its negative charge. Nevertheless, 0.1 mM luciferin applied outside is very likely saturating for luciferase activity generated inside cells by reporter genes.

Although luciferase has been used for many years in assays of ATP in vitro, it is far less responsive to fluctuations in the normal high (millimolar) levels within cells because of its low Km (63 µM). There is, of course, a dramatic decline in luciferase signals at cell death as ATP is depleted. Similarly, oxygen can be limiting in some in vitro and in vivo luciferase reactions. Brain slices sectioned at 200 µm and maintained on porous membranes appear to provide adequate oxygenation of the tissue during luciferase imaging.

One remaining noteworthy distinction is that fluorescence can be generated primarily within a single focal plane or spot, whereas luminescence is generated continuously throughout the specimen, thereby adding additional scattered light. Deconvolution and deblurring algorithms need to be developed to help remove this background noise.

**Sensors for Photometry and Imaging**

Photomultiplier tubes (PMTs) are extremely sensitive and cost-effective devices for recording bioluminescence and are even more useful when cooled to remove spontaneous dark noise. Signal-to-noise can be quite high because all of the light that would be used to form a camera image is instead directed to a single PMT. A similar increase in signal strength is achieved with cameras by grouping (binning) the signal detected at adjacent pixels of the sensor. PMTs are particularly useful for recording fast signal dynamics, although signal fluctuations due to gene activation are considerably slower and do not require this speed. The bi-alkali type PMT has the best spectral response for firefly luciferase. A solid-state alternative to PMTs is the avalanche-phodiode that also enables single-photon counting, although it has not been used extensively for live-cell luminescence measurements.

Luciferase images are usually collected with either a cooled charge-coupled device (CCD) or an intensified CCD (ICCD) camera. Essentially, the cooled CCD is a silicon chip sensor with high Q.E., whereas the ICCD uses a twodimensional array of narrow PMTs called microchannels to collect the image and pass it to a phosphor screen that is imaged by a standard CCD. Some of the image is, however, lost because photons strike the area between microchannels. Although ICCDs are noisier, more expensive, and have lower Q.E. than cooled CCDs, they can be more sensitive and photon-counting software is available to reduce dark noise that appears as false photon counts. Also, ICCD cameras show images in real-time as their pixels accumulate brightness, whereas images are read out of cooled-CCD cameras after the exposure.

**Signal-to-Noise and Imaging Concerns**

The pixels, or wells, of a cooled-CCD camera are areas of the sensor designed to accumulate charge (electrons) until they can be read out. Amplifiers that read the charge are the limiting source of noise in very low-light bioluminescence applications. This “read noise” creates a predictable uncertainty in the image and increases when the sensor is read out at a higher rate. Thus, many cooled-CCD cameras require several seconds to pass the image data to the computer for display and processing. At higher light intensities, “shot noise”, uncertainty in the photon flux, becomes the limiting noise in the image, while read noise has only a negligible effect on the total system noise. Shot noise follows a Poisson distribution and can be calculated as the square root of the signal intensity. Dark current noise is calculated as the square root of the dark current caused by thermal noise. These three major sources of noise are converted to electrons, which is how they occur in the sensor, and added in quadrature to calculate total system noise.

\[
\text{Total System Noise} = \sqrt{\text{SN}^2 + \text{RN}^2 + \text{DN}^2}
\]

Where SN = shot noise per pixel estimated as the square root of the signal, RN = read noise (e/ pixel), DN = dark current noise (e/pixel·sec times the exposure in seconds).
The CCD gain is described in terms of e/ADU where an ADU is the analog-to-digital unit characteristic of the final image, which is also called a “gray level”. For example, a 16-bit camera handles $2^4$ or 65,536 ADUs. The gain can be varied and is optimal where read noise still exceeds gain. A typical read noise is 8 e/ pixel while gain might be 1.5 electrons/ADU. Although the CCD can be digitized to any number of bits, the true dynamic range of the camera is found by dividing its full-well capacity, the maximum number of electrons a pixel can store during an exposure, by the read noise. Typical full-well capacity for a 16-bit camera is 330,000 e/ pixel. The inverse of the S/N can be used to indicate the reliability of pixel values in an image. The 95% confidence limit for a given pixel is considered to be twice the inverse of the S/N (two standard deviations) times 100.6

Radiation events often called “cosmic rays” are apparent in CCD images when exposure times are longer than about 10 minutes. These excessively bright areas, typically consisting of only one or two adjacent pixels, are generated by external radiation effects in the atmosphere or events within the silicon. This noise can be removed by taking the lowest pixel values from two sequential images captured using the same exposure and gain settings. Alternatively, a second image can be used to reconstruct lost pixels in an immediately earlier or later image by removing cosmic rays when they are clearly outside the intensity range or area of the object being imaged.

Binning, of course, increases the likelihood of losing data to a cosmic ray event because several pixels can be ruined at once. Nevertheless, binning increases the S/N by producing only one unit of read noise for all of the pixels within a bin rather than one unit for each pixel. For this reason, binning improves image quality at very low light intensities when pixel values are often limited by read noise.

**Use of Luc in Cell Lines and Transgenic mice**

Numerous studies have used firefly luciferase as a reporter gene by lysing cells and analyzing the light emitted by a solution made from the cells. Typically, this preparation contains luciferin, ATP-Mg$_2^+$, and ingredients such as Coenzyme A that sustain enzyme activity to facilitate measurement with a luminometer.6 Far fewer studies have followed gene expression in live, intact cells or animals containing luciferase reporter genes. Some recent studies have revealed aspects of gene expression that can be imaged in single cells in culture using stable transfected cell lines containing a strong promoter upstream from lac,-21 In particular, the gonadotropin releasing hormone promoter generated a surprising oscillating luciferase signal in single cells suggesting a novel rapid switching of gene expression.15 The prolactin promoter upstream from lac showed that individual cells within a cell culture respond in unique ways to the same stimulus, providing information not available at the level of cell population measurements.19

Other than transfecting cells with DNA, lac reporter genes can instead be injected into the pronuclei of oocytes before these eggs are implanted into a recipient female who produces a founder line for subsequent breeding. Typically, these transgenic mice can be crossed with other genetically modified lines and knock-out mice. Transgenic rats expressing lac have also been successful22 and enable studies of a larger animal with well described pharmacological, behavioral, and neural responses.

Two transgenic mouse lines containing immediate-early gene (IEG) promoters upstream from lac show rapid changes in gene expression in response to cell stimulation.25 One line contains the promoter of the human c-fos IEG, which serves as a general marker of neural activity, and the other has the cytomegalovirus immediate-early gene 1 (CMV IE-1) promoter upstream from lac. These mice provide an opportunity to image gene expression in all cell type of the animal. Images of brain slices showed that, as in cell lines, individual cells can be imaged and followed for days in culture.25 Early studies demonstrated that signal strength from the CMV IE-1 promoter is more than adequate for single-cell imaging.24
 Imaging dispersed hypothalamic cells from the CMV::luc transgenic mouse shows that luciferase is distributed throughout the cytosol and is for the most part excluded from the nucleus (Figure 1). These results confirm that the signal reflects the physiological state of the cell environment and that the enzyme is not compartmentalized into organelles. Also, the signal is not from luciferase leaking from damaged cells. Furthermore, the enzyme spreads to some extent into axons and other cell processes that could produce a sizable signal in nerves.

 Imaging Live Tissue

One noteworthy property of bioluminescence imaging is that signal strength increases as magnification decreases because more light is concentrated within each pixel of the sensor. Consequently, faint signals from cells within a large area of tissue can be detected at magnifications that do not resolve single cells. These images are often collected with lenses designed for 35-mm film cameras with an f value near 1. Shown in Figure 2 are signals from the area around the lateral ventricles of fos::luc brain slices imaged with a Nikon f/1.1 lens (Nikon) and a photon-counting system based on two stacked microchannel plate image intensifiers (VIM system, Hamamatsu).

At slightly higher magnification, for example 10x, cell bodies and immediately larger structures such as blood capillaries can be imaged in brain slices from CMV::luc mice maintained in culture. Actual cell sizes are, however, not easily measured unless the cells are in optimal focus. Otherwise, the point spread function of the lens broadens the cell image. Nevertheless, the dynamics of gene expression can be followed by integrating total cell emission.

 Imaging Luc Bioluminescence in Intact Animals

A major advantage of bioluminescence imaging is the potential to track gene expression and other cellular processes in intact living animals. This ability has not been fully realized although relatively low-resolution images have been made of the progress of infection and tumor growth by introducing into rats various cells containing luciferase reporter genes. In these studies, images collected through the skin depict tumor cells and internal organs such as infected kidneys.

To image firefly luciferase in vivo, luciferin must be administered either during or prior to imaging. To test the signal strength of luciferase signals from live brain tissue, a fos::luc mouse was given a lethal overdose of sodium pentobarbital and an approximately 1 cm² area of the parietal skull was removed. Luciferin (10 mM) was applied to this area at the time shown in Figure 3. A 1 mm diameter optic fiber was pressed against the dura covering the brain with the other end connected to a photon-counting module that is based on a low dark count bi-alkali photomultiplier tube (HC-135, Hamamatsu Bridgewater, NJ). The photon counts increased rapidly but then decayed following loss of cell respiration. This result demonstrates the feasibility of monitoring luciferase signals from brain in vivo and indicates that imaging should be possible from the brain of
Concluding Remarks

Technological improvements in photonics-based assays of live cells are progressing rapidly. With these advances, many practical advantages are being realized in research and medical laboratories. Fewer samples, cells, or organisms are needed because each preparation can provide its own baseline for comparing changes in signal strength over time. This conservation of experimental subjects should also reduce uncertainty of results caused by inter-individual variation and should be particularly valuable when organisms are scarce, in high demand, or otherwise limited for research purposes.

The lack of need for isotopes in these assays is of obvious importance in helping to reduce costs, eliminating hazards to personnel, and simplifying disposal. Bioluminescence provides additional advantages in the ability to couple enzyme reactions to luciferase, thereby opening a path of potentially limitless numbers of tests based on such hybrid techniques. In these assays, luciferase is held in a caged, or inactive, state until it is freed for reaction with luciferin following a particular enzymatic step. Unique biosensors of this kind are possible, but current uses are often limited to cell-free systems with luciferase bound to a surface.

Advances in cellular bioluminescence imaging will likely follow improvements in bioengineered molecules and imaging capabilities. Several luminescent marine organisms have been described and are just beginning to be exploited including the bivalve Pholas dactylus,14 the coelenterate Obelia,15 and the barnacle Vargula hilgendorfii that produces a secreted luciferase.16 These species and others offer particular advantages in respect to emission spectra, Ca²⁺ sensitivity, pH optimum, stability versus lability, etc. making each valuable in unique applications. Sensor designs will likely proceed to larger pixel format, as is occurring in astronomy, and simpler thermoelectric methods are replacing LN camera cooling. Computer-controlled robotic microscopes could provide tracking of cell movements during imaging, rapid focus, and automatic viewing of cells at multiple locations in cultures, thereby increasing data throughput to levels approaching that of multiwell luminometers.

References


**About the Authors**

Michael Geusz received a B.A. in zoology from the University of North Carolina at Chapel Hill in 1983 and a Ph.D. in biology from Vanderbilt University in 1989. He completed a postdoctoral fellowship at the University of Virginia in biology and joined the faculty of the Department of Biological Sciences at Bowling Green State University in 1997. His main research interests are in neuroscience and the cellular basis of circadian rhythms. His address is Department of Biological Sciences, Bowling Green State University, Bowling Green, Ohio 43403; e-mail mgeusz@bgnet.bgsu.edu.

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