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Perspective

Abraham Patchornik: The Contemporary Relevance of His Work for Chemistry and Biology

Kenneth A. Jacobson and Graham C. R. Ellis-Davies*

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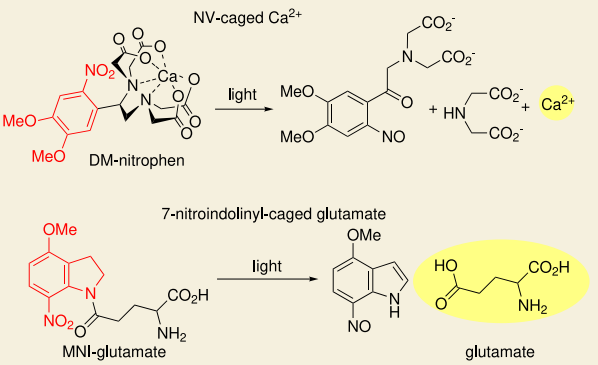
ABSTRACT: Abraham Patchornik was born in 1926 in Ness Ziona, a town in Palestine founded by his great-grandfather Reuben Lehrer in 1883. He started to study chemistry as an undergraduate at the Hebrew University. However, this was interrupted by the war, and he completed his studies in various locations in West Jerusalem. From 1952 to 1956 Patchornik completed his PhD at the (new) Weizmann Institute of Science with Ephraim Katchalski. After a postdoc at the NIH, he returned to the Weizmann in 1958, when he joined the Department of Biophysics. In 1972−1979, he became chairman of the new Department of Organic Chemistry at the Weizmann, and his own research was geared toward applying creative chemistry to solve biological problems. Patchornik passed away in his hometown of Ness Ziona in 2014. Patchornik was a conceptual leader in peptide and polymer chemistry. Given the importance of selective functional group protection for the construction of oligomeric molecules, he became interested in using "nonstandard", orthogonal chemistry for this purpose, i.e. photosensitive protecting groups (PPGs) in place of thermal reactions. It was R.B. Woodward who suggested this strategy to Patchornik in 1965, while Patchornik was on sabbatical leave at Harvard. However, it was not until Patchornik returned to the Weizmann that this idea of a versatile PPG to enable multistep synthesis was realized. Here, we provide an account of the early photosensitive protecting groups that Patchornik and co-workers developed, and the immense impact they have had on various fields. In particular, we survey the use of PPGs in live cell physiology (i.e., caged compounds), and the development of gene chips via light-directed solid-phase synthesis. Further, we highlight recent work applying new PPGs for "photochemical delivery" of drugs, otherwise termed photopharmacology. Finally, we discuss the relationship between caged compounds and how contemporary neuroscience uses genetically encoded chromophores to control cell function.



KEYWORDS: *Patchornik, Woodward, nitroveratryl, 7-nitroindolinyl, uncaging*

1. HISTORICAL INTRODUCTION

1.1. The Early Life of Abraham Patchornik

Patchornik's great grandfather was born in Poland in 1832. He moved to the Middle East in 1883, purchased land and built the settlement of "Nachalat Reuven" in Wadi Hanin, which later became the town of Ness Ziona. The family was involved in agriculture, growing citrus trees and specialized in modern beekeeping.

Patchornik was born in 1926, and, naturally, was involved in the family farm when he was young. He received secondary education at Gymnasia Re'alit in Rishon LeZion, where he began studying chemistry. But the scientific curriculum there was not yet recognized officially by Department of Education of the Jewish National Council, so Patchornik had to take additional exams while working on the family farm. This allowed him to enter the Hebrew University, but his studies were interrupted by the War of Independence in 1948. He completed his undergraduate studies at various locations after the war, and then in 1952, he started doctoral studies with Prof. Ephraim Katzir at the new Department of Biophysics at the Weizmann Institute of Science. His PhD thesis was titled "Polyamino Acids as a Model for Proteins" and was completed in 1956. Subsequently, as a Weizmann Professor, he took pride in the fact that at least nine of his students became faculty members at Israeli universities. From 1970 to 1973 Patchornik served as the first chief scientist of the Ministry of Development. From 1987−2007 he served as an advisor to Ministers of the Israel Police and to the Police Forensic

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Department. In 1988 he was awarded the Rothschild Prize in Chemical Sciences.

1.2. The Invention of Photosensitive Protecting Groups

1.2.1. John Barltrop and the Invention of Photosensitive Protecting Groups. Photosensitive protecting groups (PPGs) were self-consciously invented in 1962 by Barltrop and co-workers.[1] Like many good ideas, we now know other scientists had been thinking along the same lines. Eminent organist chemists such as Derek Barton[2] and John Sheehan[3] both followed up on Barltrop's initial report with their own approaches. And before these studies, the Dutch photochemist Egbert Havinga reported photosolvolysis of *meta*-nitrophenylphosphate.[4] However, with the perspective of history, we can say that it was Barltrop's 1966 report of *ortho*-nitrobenzyl (oNB, Figure 1a) protected acids that was the

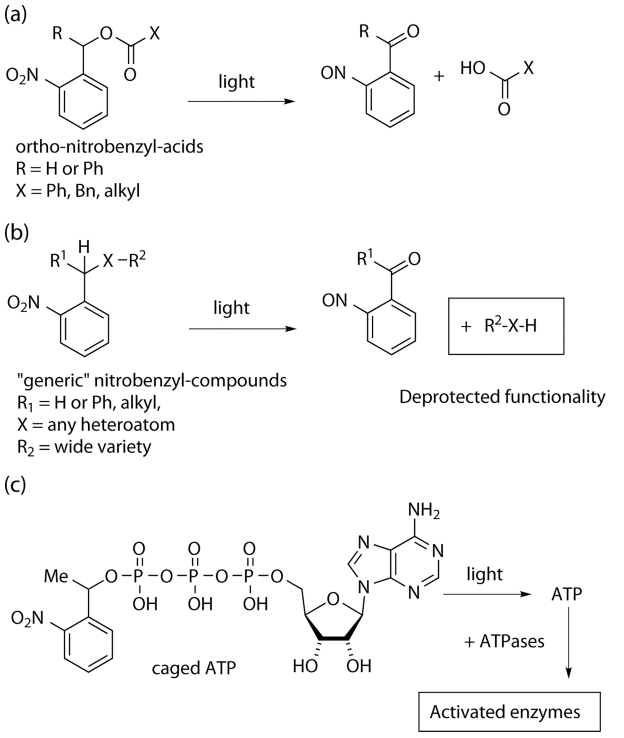

Figure 1. John Barltrop's *ortho*-nitrobenzyl (oNB) photosensitive protecting group (PPG). (a) Structure and photoproducts of carboxylates protected by the oNB chromophore.[5] (b) Presentation of the "generic idea" suggested by Barltrop's 1966 study. (c) Summary of the first physiological application of Barltrop's PPG by Kaplan et al. in 1978.[9]

most important contribution to this field.[5] We live in an era when almost every "short communication" has extensive supplemental data, it is noteworthy that this report is less than one page. But the intent of the work is stated clearly as follows: "We have attempted to design a photosensitive protecting group which uses in the key deprotection step the **general** photochemical oxygen-transfer reaction of aromatic nitro-compounds which have a C−H bond in the *ortho*-position" (emphasis ours, see Fig. 1b).

Quite how Barltrop became interested in photochemistry is unclear. It has been speculated that after he took a sabbatical with the great Melvin Calvin (UC Berkeley) in the mid-1950s, he could have returned to Oxford with the idea to use photochemistry for synthetic organic chemistry.[6] John Coyle was Barltrop's leading PhD student, going on to become a Professor at the Open University, and writing two books with Barltrop.[7,8] In 1982, Coyle was the external examiner for the Ph.D. of Ellis-Davies. Recent contact with Coyle revealed that by the time Coyle met Barltrop, the latter had moved on from the development of PPGs, so the exact origins are lost in the mists of time (Coyle personal communication to Ellis-Davies). However, the impact of the oNB PPG went far beyond what Barltrop envisaged in terms of synthetic organic chemistry, as it was the basis of Kaplan's caged ATP work (Figure 1c), published in 1978.[9] Like all great technologies, the first deployment is often not the best, and here we outline the contribution of the Israeli chemist, Abraham Patchornik, to this field, and the amazing impact this work has had on science.

1.2.2. 1970: Patchornik's Seminal Scientific Contribution—The Invention of the *ortho*-Nitroveratryl PPG, and the Role of R.B. Woodward in This Process. In 1970, Patchornik and co-workers published a study entitled: "Photosensitive Protecting Groups" in the *Journal of the American Chemical Society*.[10] When this seminal study is cited, Woodward is often given sole credit for this work. Phrases such as "Woodward's NV protecting group", or similar, are very common in the chemistry literature. The reason for this is obvious, he is the most famous organic chemist[11] of the 20th century. However, the presence of R.B. Woodward as an author has always seemed puzzling for the simple reason that Woodward was not a photochemist. So how did Woodward become an author of this important paper?

Around the time of Barltrop's 1966 publication, Patchornik, was on sabbatical leave (1965−6) in the laboratory of Woodward.[12] It was Woodward who suggested to Patchornik that he explore the use of the electron rich, dimethoxy version of oNB, namely the 4,5-dimethoxy-2-nitrobenzyl (DMNB, aka *ortho*-nitroveratryl, or 6-nitroveratryl, or "NV") chromophore, as photosensitive protecting group (see Figure 2). This is mentioned anecdotally by Patchornik proceedings from a conference in Milan in 1967 ("Photosensitive blocking groups may afford mild deblocking if the groups are sensitive at wavelengths at which proteins are stable. R.B. Woodward has suggested such a photosensitive blocking group, the 6-nitroveratryloxy (NV−O−) group, which can be removed by irradiation with wavelengths longer than 3200 Å. In collaboration with Woodward we have succeeded in showing that NV derivatives of amino acids release their amino peptides" ref.[13]). All the work was carried out by graduate student Boaz Amit at the Weizmann Institute in Israel,[13] leading to the 1970 JACS paper[10] (Figure 2a). In Figure 2b we show a comparison of the absorption spectra of oNB and NV, but note these data were not mentioned in the 1970 report.

Organic chemists in general, and peptide chemists in particular, are interested in protecting groups for obvious, practical reasons. Thus, we can imagine that a peptide chemist such as Patchornik would be attracted by the orthogonality inherent to photosensitive protecting groups, especially one like Barltrop's oNB chromophore. However, Patchornik has no publication incorporating photochemistry before his sabbatical with Woodward in 1965. Thus, just like Barltrop's sabbatical with Melvin Calvin in the 1950s, meeting Woodward became a crucial turning point for Patchornik.

1.2.3. 1972 Onward: Establishing Generality of *ortho*-Nitrobenzyl Photodeprotection. Barltrop asserted in his 1966 paper that he was seeking to develop a "general" PPG.[5] In fact, his testing was limited to various organic acids, or

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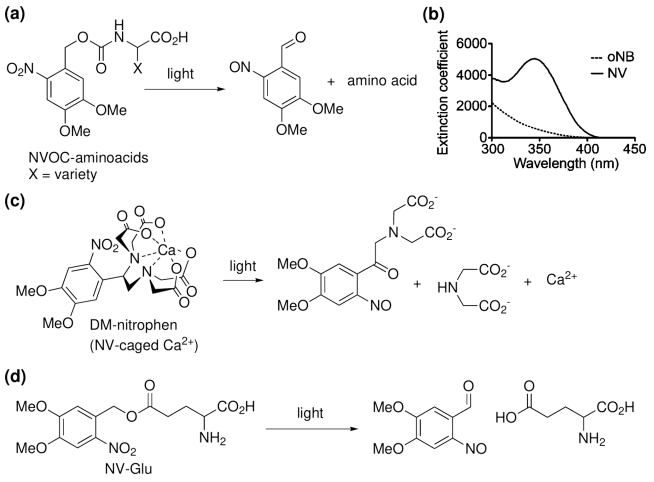

Figure 2. Structure and application of Abraham Patchornik's nitroveratryl (NV) chromophore. (a) Structure and photoreaction of the NV chromophore[10] used to protect various amino acids via an oxycarbonyl linker, hence "NVOC". (b) Comparison of the absorption spectra of the oNB and NV chromophores. Note the NV chromophore has absorption in the violet. (c) Application of the NV chromophore to make a caged Ca^{2+} probe (DM-nitrophen[24]). Ionized calcium is the most important second messenger inside cells. (d) Application of the NV chromophore to uncaging glutamate.[38] Glutamate is responsible for about 80% of neurotransmission in the central nervous system.

carbamates (Figure 1a). In his 1970[10] paper Patchornik essentially did the same thing. Subsequently, it was Patchornik and co-workers who started to establish that the oNB and NV groups fulfilled the generality goal. In 1972 they showed C−O bonds in ethers could be deprotected,[14,15] in 1975 phosphates,[16] and in 1977 phenols.[17] Further, two other groups showed in 1975 that C-amide bonds[18] and C-histidine bonds[19] could be deprotected.

1.2.4. 1973 Onward: Invention of a Second Nitroaromatic PPG. During the period that Patchornik and co-workers explored the use of the oNB or NV PPGs in peptide and carbohydrate chemistry, they developed a second set of PPGs using a very similar photochemistry to oNB, namely, that of *ortho*-nitro-phenacetyl[20,21] (Figure 3a). They explored open chain[20] and cyclic versions[21] of this chromophore. In contrast to oNB and NV PPGs, this type of chromophore could not become a generic PPG, as it uses an amide functionality to create an acid. However, we will see that this type of PPG became quite useful for specialized applications in physiology.

2. APPLICATIONS OF BARLTROP AND PATCHORNIK'S WORK

While Barltrop and Patchornik were not biologists, their background in synthetic organic chemistry gave both the understanding of an ideal for chemical protecting groups. The Nobel Prize winning chemist Bruce Merrifield expressed this eloquently in 1977: "An orthogonal system is defined as a set of completely independent classes of protecting groups. In a system of this kind, each class of groups can be removed in any order and in the presence of all other classes." However, while the oNB and NV chromophores were used somewhat in the 1970s for synthetic organic chemistry, on reflection these applications seem more like "proof of principle" experiments, as a thermal PG could have been used in such synthetic schemes. Since light can be "bio-orthogonal", the most

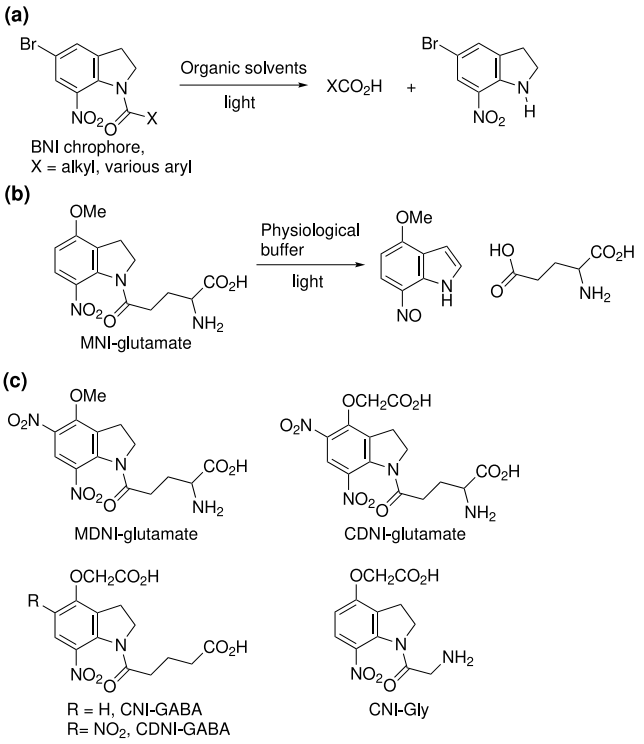

Figure 3. Structures and photoreactions of 7-nitroindolinyl-caged compounds. (a) Structure and photoproducts of carboxylates protected by the 5-bromo-7-nitroindolinyl (BNI) chromophore.[21] (b) Structure and photoreaction of 4-methoxy-7-nitroindolinyl-glutamate.[58,59] Note the aromatic side product in water is the nitrosoindole, whereas in organic solvents it is the nitroindoline. (c) Structures of various other 7-nitroindolinyl caged neurotransmitters made in the Ellis-Davies laboratory.[116−120]

important applications of PPGs have been in cell physiology, and microarrays. Here, photochemistry reaches a place thermal chemistry cannot, so PPGs make a unique contribution to science.

2.1.1. Caged ATP and Caged Calcium

In 1978, Kaplan and colleagues used Barltrop's PPG to block the hydrolysis of ATP by covalent attachment of the oNB chromophore to the *gamma* phosphate[9] (Figure 1c). They showed that this photochemical probe (christened "caged ATP") was biologically inert toward its target enzyme, the Na^+,K^+-ATPase. Not only did the PPG prevent hydrolysis by the enzyme, they demonstrated that it did not block ATP hydrolysis. (i.e., caged ATP was neither substrate or antagonist). Further, they showed caged ATP was sufficiently stable under physiological conditions, that it could be loaded into red blood cells, and irradiation released sufficient ATP to support active ion transport out of the cell. It is noteworthy that in the very first paper in this field Kaplan et al. did an experiment that is impossible with thermal chemistry, making use of a key quality of light, namely, that it passes through cells, providing access to the intracellular milieu. In contrast, even though Engels and Schlaeger synthesized a range of cAMP phosphate esters in 1977 (including oNB-cAMP), they did not report any photochemical release of cAMP from their oNB-cAMP inside cells.[22]

A second, desirable, feature of photochemical release is that it should be faster than many physiological processes, offering the possibility to be a "switch" to turn on a process. The first

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example of this came in 1982, with Kaplan's caged ATP, laser photolysis allowed detailed kinetic study of skeletal muscle relaxation kinetics, something not feasible with traditional rapid mixing techniques.[6] The success[6] of probes such as caged ATP, cAMP, cGMP, IP_3, etc. suggested that a caged Ca^{2+} probe could be very useful for cell physiology. However, since ionized calcium cannot form covalent bonds, and new strategy needed to be developed for this. Kaplan's idea was that one could use oNB photochemistry to cut high affinity, tetracarboxylic Ca^{2+} chelators in two, producing low affinity diacids (Figure 2c). In 1988 an oNB derivative of EGTA and a NV derivative of EDTA were made.[23] The latter allowed Ca^{2+} uncaging inside cells, and it was shown that this improved the kinetics analysis of muscle contraction compared to rapid mixing[24] (ca. 5x faster). Shortly after this there were several biology papers applying this new probe.[25−37]

2.1.2. Caged Neurotransmitters

With use of caged ATP, cGMP, cAMP, IP_3, Ca^{2+}, etc. in physiological experiments it was "inevitable" that the uncaging method would be applied to neurotransmitters. George Hess and co-workers pioneered the application of both the oNB and NV chromophores to the photorelease of neurotransmitters (glutamate,[38,39] GABA,[38,40] acetylcholine-receptor ligands,[41,42] etc.). Thus, the first caged neurotransmitter used in complex brain tissue[43] was NV-Glu[38] (Figure 2d). This probe released glutamate in the submillisecond range[38] (i.e., fast enough for cell physiology), and while this property is crucial, the fact that light can be targeted to any region of a cell, or collection of cells in an acutely isolated brain slice meant that functional mapping in complex biological tissue became practical for the first time.[43,44] Rapid, local perfusion is possible in such preparations, but to do so repeatedly over large volumes (i.e., connectivity mapping) is technically extremely challenging. In contrast, light is like a "magic wand", allowing facile, repetitive, stereotypical command of physiological function. Often one is told that the use of caged compounds provides "high-resolution spatial and temporal control" of X. However, we are not told "compared to what?" Such connectivity mapping is a such benchmark,[44] as such physiological studies would be impossible by traditional methods.

2.1.3. Photomicroarrays and the Human Genome Project

Microarrays are the most elegant and powerful application of caged compounds. Conceptualized by Pirrung,[45] the first publication applied to parallel synthesis of peptide and nucleoside microarrays appeared in Fodor at al., in 1991.[46] This work used Patchornik's NVOC chromophore as the PPG. A physical mask is used to define the location of photolysis, a technique developed previously for photoliography of electronic microcircuits, and liquid phase synthesis of requisitely protected peptides or deoxynucleosides allows construction of microarrays of defined sequences to be made.[47] These microarrays had immense impact on the biological sciences immediately after they were developed, including helping speed up the Human Genome Project. For a review of this field see.[48] A crucial feature of the use of nitrobenzyl photochemistry for this work is that it works in air; other PPGs require solvent to be effective (reviewed[47,49]).

2.2. Two-Photon Uncaging. Two-photon excitation is a nonlinear optical process that produces the first excited singlet (S_1) state by the near-simultaneous absorption of two photons of approximately half the energy that are required for normal, Franck−Condon excitation. Initial absorption of the first photon gives a virtual state which has a very short lifetime (ca. 10 fs), thus, a high flux density of photons is required to elevate the electron to the S_1 level before vibronic decay of the virtual state.[50] This means there is within the 2-photon method an inherent 3D localization of excitation, producing fine scaled uncaging in a volume of much less than 1 fL, if a lens with a high numeric aperture is used.[51,52] Since red and near-IR wavelengths are used for the method, this enables much deeper light penetration into biological tissue when compared with wavelengths in the 350−550 nm range.

2.2.1. Patchornik's "Second" PPG Applied to Contemporary Neuroscience

In his seminal 1990 *Science* paper on 2P microscopy,[51] Denk noted that "This technique also provides unprecedented capabilities for three-dimensional, spatially resolved photochemistry, particularly photolytic release of caged effector molecules". Thus, he went on to publish the first 2P uncaging paper in 1994.[52] However, he used a caged acetylcholine receptor agonist that Hess had developed for 1P uncaging[42] (α-carboxy-NB-choline). With the development of commercial, solid-state 2P lasers (Ti:sapphire), 2P microscopy became practical for normal biology laboratories. Thus, the next report of 2P uncaging came from a muscle physiologist, Ernst Niggli. Using the caged calcium called "DM-nitrophen" that was made with the NV chromophore,[23] he showed that 2P uncaging worked well with these new lasers.[53] This report inspired a new version of caged glutamate to be made, using a NV chromophore (α-carboxy-NV-Glu). First tests of this were done by Kasai in Japan. At the same time, a new caged calcium (called DMNPE-4[54]) was developed. Preliminary reports of these studies were made by Ellis-Davies at the Society of General Physiologists annual conference in 1999,[55] with Niggli's work being published in the same year.[56] However, the caged glutamate tested by Matsuzaki in Kasai's lab proved to be too hydrolytically unstable for long-term 2P studies.[6]

In 1973−6 Patchornik and co-workers published a series of studies that provided a solution to this problem.[20,21,57] In 1976, they stated: "Carboxyl functions are generally protected by conversion to esters. Their protection via their amides is rarely encountered since the amide is more resistant to group generally basic and acidic solvolysis than the ester group, and therefore rather vigorous conditions are needed to cleave it. On the other hand, the stability of the amide bond towards solvolytic conditions could be turned to an advantage, if a specific and mild method for its cleavage were available."[21] The best of these was the electron deficient 5-bromo-7-nitro-indolinyl (BNI) chromophore.[21]

In 2000 Ellis-Davies made an electron rich version of Patchornik's "second" PPG, effectively combining the absorption properties from NV with the hydrolytic stability of the 7- nitro-indolinyl chromophore. Instead of 5-bromo, a methoxy substitute was placed at the 4-position (giving "MNI-glutamate"). The first successful tests of MNI-glutamate were done in Japan in July 2000 (M. Matsuzaki, personal communication), and presented at the Society for Neuroscience (SfN) annual conference in October 2000.[58] After our original publication in 2001,[59] many groups used this probe to carry out 2P uncaging experiments.[60] Such use of MNI-glutamate required a parallel development of a 2P microscope to effect uncaging on visualized neurons. Such microscopes were homemade by Kasai, Svoboda and Sabatini; or were made commercially by Prairie Technologies. In the latter case,

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Ellis-Davies helped with this development. The 2004 Nature[61] paper with Kasai has been cited more than 1700 times. (In his Faculty of 1000 evaluation Tim Bliss wrote: One hundred years after the birth of DO Hebb, the Canadian psychologist who introduced the notion of the Hebb synapse to neuroscience, it remains uncertain how changes in efficacy are stored at individual synapses. In a technological tour de force, Matzusaki et al. have now shown that, after the induction of long-term potentiation (LTP) at single hippocampal synapses, the postsynaptic dendritic spine rapidly becomes bigger and more sensitive to glutamate. The authors used two photon excitation to release glutamate from an inactive 'caged' precursor over a volume small enough to activate a single visualized spine. When LTP was induced, either by high-frequency stimulation, or by the delivery of repeated pulses of uncaged glutamate (a non-Hebbian induction protocol), there was a rapid and persistent increase in the volume of the spine head. At the same time, the current induced by activation of this single spine by a standard pulse of uncaged glutamate was increased. These results reveal an immediate structural correlate of LTP, and demonstrate directly an increase in the response to glutamate at single potentiated spines. Significantly, there was no change in the volume of neighboring spines, or growth of new spines adjacent to the stimulated spine. (Tim Bliss: Faculty of 1000 Biology, 29 Jun 2004 https://archive.connect.h1.co/article/1019599. Bliss discovered LTP in 1973.)

The core technical achievement of this extension of Patchornik's second PPG was that 2P uncaging at single synapses could be tuned to match the physiological glutamatergic input perfectly.[59] Notably, not just one synapse could be stimulated, but multiple synapses could be targeted in rapid succession, allowing the spatial and temporal properties of excitation to be explored systematically for the first time.[62,63] Importantly, even to this day, optogenetic methods cannot match the capabilities arising from such uncaging techniques. It is fair to say Patchornik's "second PPG" transformed neuroscience.

2.2.2. Recent Advances beyond "Simple" Nitroaromatic Chromophores

Just as the NV chromophore has a small but useful improvement in the absorption properties of the oNB chromophore (Figure 2b), several groups have attempted even larger bathochromic shifts to violet, blue, or longer absorptions. Ludovic Jullien was the first to publish a systematic study of this in 2006.[64] Adding additional π-electrons at the *para* position produce substantial increases in the extinction coefficient (ca. 5x compared to NV), but small changes in the λ_{max} (350 to 375 nm), none showing substantial absorption in the blue range (i.e., 450−480 nm). Importantly, the quantum yield for photorelease seems to decrease with these changes in the NV chromophore. Thus, one might say the basic conclusion of this work is that is very difficult to make good, all-round improvements in the NV PPG. In the same year, another attempt to improve NV was published, with the development of a new PPG called NDBF.[65] This improved the quantum yield and extinction coefficient, but produced no red shift, with the absorption maximum remaining in the UV range.

In 2008, Goeldner's group published an important paper in which they attempted to use even more highly conjugated nitroaromatic chromophores.[66] In contrast to the work of Jullien, the best of these chromophores absorbed strongly around 400−415 nm (8−13x larger than NV). Further, these chromophores had large 2-photon cross sections, but the biology of their caged GABA seems inconsistent with such values. In 2015, the Ellis-Davies and Niggli laboratories introduced another extended π-electron system,[67] one based on a careful survey of the more than 2000 structures studied by many groups (reviews:[68−71]). It was concluded that a thiophene core was crucial to a significant bathochromic shift compared to NV. Thus, our caged compound (BIST-2EGTA) has an absorption maximum at 450 nm (with a value 13x NV than at 350 nm). Further, the 2-photon cross-section is about 5,800x that of NV, making Ca^{2+}-driven biology much more facile using 2-photon excitation. In 2019, BIST was applied to GABA, with this probe being, surprisingly, only sensitive to 2-photon excitation, as a result of the anionic dendrimer that was used to solubilize the probe.[72] The synthetic challenges of such extended π-electron systems are considerable, especially when compared to Patchornik's NV chromophore, so it seems that the field has moved away from such large nitro-aromatic compounds. Most work on coumarins, BODIPYs, or other chromophores, as they are much easy to tune chromatically.[73] These are discussed in section 3.4.

3. CONTEMPORARY THINKING ABOUT PHOTOCHEMICAL TECHNOLOGIES

3.1. Has Optogenetics "Replaced" Uncaging?

The following quotation was ascribed[74] to the great Sydney Brenner: "Progress in science depends on new techniques, new discoveries and new ideas, probably in that order." The history of science is suffused with techniques that seem like "revolutionary moments". The use of GFP for genetically encoded labeling of cells is a recent example of this. However, that technology was built on other techniques such as cell-type specific promoters. By the time channelrhodopsin-2 (ChR2) was reported in 2003, by a German group (Nagel et al.[75]), molecular biology could provide a well-established set of tools that enabled a protein to be expressed in most cell types. This allowed expression of ChR2 in neurons at such a level that irradiation with blue light could fire action potentials *in vitro*[76] and *in vivo*.[77]

The term "optogenetics" was coined in 2006 by a journalist reporting[78] on a subgroup meeting at the Society for Neuroscience (SfN) annual meeting. Once the usefulness of genetically encoded photo-actuation became clear, the term became very popular, even though it is clearly a misnomer.[79] It was dubbed "Method of the Year"[80] in 2011 by the journal *Nature Methods*. In this period optogenetics was associated closely, even exclusively, with light-gated ion channels and pumps such as ChR2 and halorhodopsin. These are 7-transmembrane (7TM) photoreceptors that use a covalently linked retinal chromophore. Since 7TM involve a large family of G-protein coupled receptors, it was apparent that many of the latter could be made photoresponsive by genetic engineering.[81−83] The close relationship of such optogenetic tools and caged compounds is quite striking. Since the latter were used to control important cellular second messengers such as cAMP, cGMP, IP_3, nitric oxide, lipids, etc. it was natural that the former would seek to do the same. And so genetically encoded cAMP, cGMP, IP_3, nitric oxide, lipids, etc. were developed.[81−83]

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The success of these 7TM-based optogenetic proteins stimulated (pun intended) the search for other "photoproteins" that could be repurposed for photocontrol of cellular chemistry. For a recent excellent summary of the field see,[84] wherein we can see "optogenetics" has moved from genetically encoded control of simple physiological cell functions to include the enormous field of protein−protein interaction and enzyme action. While it might be possible to use a caged drug for such work,[85] the advantages of "modern optogenetics" come to the fore here.

So, *has optogenetics replaced caged compounds*? "Yes", seems like the simple answer, as optogenetics is used very widely. But, with a more careful consideration, we suggest that the answer is "No, it has added something new." The Table 1 we compare advantages and disadvantages of the two technologies.

Table 1. Comparison of the Uncaging and 7TM-Optogenetic Methods[a]

Property	Uncaging	Optogenetics
2-photon excitation	✓✓	✓/✗
Requires DNA	✗	✓
Exogenous co-factor	✓	✗
Non-natural products	✓	✗
Native receptors	✓	✓/✗
No fatigue	✗	✓✓
in vivo	✓	✓✓
Two color	✓	✗
Subcellular	✓✓	✓/✗
Wide range	✓✓	✗

✓✓: strong property; ✓: yes; ✗: no; ✓/✗: perhaps, but not a strong feature.

[a]It can be seen that these methods are quite complementary. For example, perhaps the biggest strength of optogenetics is that it uses DNA, as this allows cell-specific expression of ChR2. In contrast, uncaging does not use DNA, and this could also be a strength of this method, as this allows light itself to direct actuation. And along with the ability to uncage any molecule (i.e., "wide range"), it gives a certain freedom to the method not possible with optogenetics. Genetic encoding of actuation means that actuation with ChR2 is a method that does not experience fatigue, as ChR2 uses a photocycle that means the photoreceptor is not consumed. In contrast, uncaging is unidirectional, as actuation arises from bond breaking. Since optogenetics uses DNA, it makes the photoactuator readily used in living animals, especially mice, whereas uncaging is very limited in this regard. Uncaging works very well with 2-photon excitation enabling subcellular actuation, whereas these aspects are much less readily delivered with optogenetics.

3.2. Phasic versus Tonic Pharmacology—What Does Uncaging in Vivo Offer Today?

Pharmacology plays a vital role in our daily life and is essential for understanding the signaling cascades in cell biology and physiology. Drugs are applied tonically, to the entire subject, be it a cell or animal. And, as long as the drugs can enter the desired environment at an effective concentration, this simple, traditional means of application is very effective. Many caged drugs have been made, and these allow very rapid (or phasic) drug application when compared to tonic application of the drug. However, the real value of uncaging will be realized when the process under interrogation is itself rapid, or is in a space not accessible in a ready manner to perfusion. To help clarify these ideas, we suggest that multisite glutamate uncaging is a useful example of the former, and intracellular uncaging of Ca^{2+} a paradigmatic example of the latter. While ionotophoresis can mimic single location glutamate uncaging,[86] only uncaging can effect rapid, multisite stimulation.[62,63,87] In the case of intracellular uncaging, while the cell cytosol is readily accessible by whole-cell patch-clamp methods, exchange of the intracellular solution takes several minutes to be achieved by dialysis,[88] so is not useful for the study of events on the millisecond time scale.

So, what about drug uncaging? Are there comparable examples when the speed and/or location of drug application are vital for biological studies? Some elegant examples can be seen in the work of Jullien and co-workers[89−94] where they use a NV-caged tamoxifen analog to induce protein expression in selected cells in zebrafish embryos (review[95]). Another nitroaromatic caged drug was developed by Deiters and co-workers to control Rho kinase in zebrafish, to reveal left/right sidedness of Rho during development.[96] The work of Jullien and Deiters are excellent examples of the use of drug uncaging in complex biological system, giving rise to data that might not be obtainable with more traditional methods. However, with more reductionistic preparations, such as cultured cells (e.g., CHO, HeLa, HEK, etc.), we feel no clear advantage can be gained from drug uncaging versus perfusion.[97] Further, while Jullien developed a blue-light sensitive version of his caged drug,[94] it is interesting to note much of the real biology was done with the NV-caged drug,[93] belying the new dogma that UV light is too toxic for use with cells and animals. Furthermore, it is important to acknowledge that the majority of Erwin Neher's studies of the secretory mechanism (ca. 80 publications[98−103]) used UV light for Ca^{2+} uncaging, without any deleterious effects (see also[104−106]).

In terms of thinking about treating human pathologies, pharmacology has some well known, inherent risks, in general involving several types of drug side effects. Consequently, few drugs are true "magic bullets". Thus, one idea that has been presented in the photochemistry community is to modify the "prodrug concept",[107] with the use of PPGs.[108−110] In general, prodrugs are formulations wherein the activity of the drug is masked in some way with a thermally or enzymatically labile functionality.[107]

A beautiful example of a "thermal prodrug" is omeprazole, the proton pump inhibitor used to treat gastric ulcers. Omeprazole is unreactive at neutral pH and undergoes a rearrangement to its reactive form at low pH, conditions found in the gastric mucosa, where it reacts covalently with the proton pump, inhibiting its catalytic cycle. While there are proton pumps in other plasma membranes, they are all situated in environments with normal pH, so the potential side effects

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Table 2. Summary of the Properties of Nitroaromatic and Other PPGs[a]

PPG	ε (/M/cm) λ_{max} (nm)	Advantages	Disadvantages	Principal[b] caged probes	Functionality
oNB	500 at 350 nm	Generic PPG. Nonfluorescent and compatible with standard fluorophores. Fragments ether and tertiary amines. Synthetically flexible. Very water-soluble.	Small ε. Very low 2PCS.	NPE-ATP, NPE-IP_3, CNB-Glu, CNB-GABA, NP-EGTA.	RNH_2, RR'NH, ROH, RCO_2H, $ROPO_2H$, RSH, PhOH. $RCONH_2$, $ROCONH_2$.
NV	4−5000 (350)	As oNB, plus larger ε than oNB, and useful 2PCS.		DM-nitrophen, nitr-5, NV-IP_3, NV-ATP,	As oNB.
DEAC	14−18000 (385)	Fluorescent, but compatible with green and red fluorophores. Useful for acidic functionalities.	Fluorescent. Not generic.	cAMP, cGMP, capsaicin, lipids,	RCO_2H, $ROPO_2H$, RSH, PhOH.
Bhc	17500 (368)	Good 2PCS.	Fluorescent. Not generic PPG. Poor water solubility.	mRNA	As DEAC.
RuBi	5600 (450)	Absorbs blue. Useful for primary and secondary amines. Nonfluorescent. Two attachment sites allow circularization. Very water-soluble.	Not generic.	GABA, Glu	RNH_2, RR'NH,
Nitro-indolinyls	4300−6400 (330)	Useful for acids. Nonfluorescent. Moderate 2PCS.	Not generic.	Glu, GABA	RCO_2H.
BODIPYs	40−100000 (510−570)	Useful for acids. Facile spectral tuning.	Not generic. Green-red fluorescence. Synthetically inflexible. Lipophilic.	Proof of Principle	RCO_2H, $ROPO_2H$, RSH.
Cy7	55000 (675)	Red light photolysis.	Not generic. Lipophilic.	Cy-Pan-C4	As BODIPY.

[a]Abbreviation: 2PCS, 2-photon cross-section. [b]a representative list probes used in several reports, not just proof of principle experiments.

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from omeprazole are relatively small. Clearly, a full discussion of the concepts being used by the pharmaceutically industry in the development of prodrugs in well beyond the scope of this review (see[107]), but it is easy to appreciate that using "caged drugs" as prodrugs is a conceptually appealing idea. If the caged drug was *fully* inert, then there would be no initial side effects, photolysis in the appropriate area would target the pathology alone. An excellent example of this strategy is discussed below in the cyanine PPG section.

One huge challenge for this approach, sometimes called "photopharmacology", is that the pharmacokinetic (PK) profile of the prodrug, and PPG side products must be characterized. Many "photo drugs" have been developed,[110] but in none of these studies is a PK profile presented or discussed in any way. A second huge hurdle to overcome is the "light delivery problem".[109] Model studies in rodents have presented two quite different solutions to this problem. First, the use of red light is touted as a solution,[110] as light in the 650−850 nm range is absorbed weakly in biological tissue. However, even red light only penetrates effectively ca. 5−8 mm, so as a means to deliver drugs in humans this seems limited to superficial layers of the periphery.[110] The implantation of an optical light guide is a standard technique in neurobiology,[81,83] so use of near-UV or blue light is made facile with this technique. Several studies have appeared with this method, some using Patchcornik's PPGs.[111−114] However, it is unclear what decisive advantage phasic application confers within these studies, as the drug are required to be applied (uncaged) locally for many tens of minutes. In other words, *such photopharmacology enables local tonic drug application*. A locally implanted canula could do the same thing. We would note that implanting light guides is a much more reliable technique than traditional canulas, as the latter block easily. However, head-mounted radio-controlled tonic drug delivery systems have been developed,[115] and are commercially available. A potential use of uncaging *in vivo* would be to control some activity or behavior that is governed by neuronal activity, as it is inherently phasic. However, genetically encoded actuation using ChR2 does this very efficiently, so the advantages of a caged drug are unclear. Some distinct potential could be imagined for a caged antagonist, especially if the drug is receptor subtype specific. In conclusion of this section, we feel that while there have been many experiments performed with caged drugs in living animals, the best of these have been in zebrafish embyos,[95] suggesting there is much work to be done to push this method into mammals to yield more than proof of principle studies. Further, the bar seems even higher for real phototherapeutic applications in humans.

3.3. Recent Applications of Patchornik's PPGs

We have highlighted above the use of the NV chromophore by Jullien and co-workers for drug uncaging in zebrafish embryos. In this section we present several other examples of the contemporary relevance of Patchornik's work for biology.

3.3.1. CNI-glycine. In 2001, the 7-methoxy analogue of Patchornik's BNI chromophore was used for 2P uncaging at single dendritic spines[59] In 2005, the QY of release of MNI-glutamate was improved approximately 5-fold by introducing a second nitro group at the 5-position, to give MDNI-glutamate[116] (Figure 3c, which was renamed DNI-glutamate by Femtonics). In 2007, the solubility of this probe was improved by attachment with a pendant carboxylate on the methoxy group with CDNI-glutamate[117] (Figure 3c). In 2008−10, the GABA analog of this probe was made, CDNI-GABA,[118] along with the mononitro analog, CNI-GABA[119] (Figure 3c). Thus, only glycine remained among all the rapid acting neurotransmitters that had not succumbed to 2-photon uncaging. In 2022. Ellis-Davies made CNI-glycine to show that excitatory glycine receptors were even distributed across the surface of pyramidal neurons.[120] This should be contrasted with the distribution of glutamate receptors on CA1 neurons, which are punctate.

3.3.2. Cloaked MNI-glutamate. A chronic problem for all caged glutamate and GABA probes is that they are antagonists of GABA-A receptors[121,122] Notably, RuBi-GABA is a very strong antagonist, having an IC_{50} of 4 μM.[72,122] Since MNI-glutamate is not an antagonist of its target receptors,[59] it was a surprise for us to find it had off-target antagonism to GABA-A receptors[123] (IC_{50} = 110 μM). In 2020, an analog of MNI-glutamate was made that showed no antagonism up to a concentration of 1 mM *in vitro*.[124] This allowed Mourot and Ellis-Davies to use this probe (G5-MNI-Glu) to activate glutamate receptors on dopamine neurons in the VTA in a phasic manner *in vivo*, without any concomitant side effects toward GABA-A receptors. Using "optofluidic uncaging" we could induce Pavlovian conditioning in freely moving mice.[124]

3.3.3. NV-caged Drugs. In 2023 Banghart and co-workers synthesized NV-caged versions of two drugs that are used for pharmacology of mu-opioid receptors.[114] The caged version of oxymorpholone was used to interrogate the effects of chronic morphine addiction of the release of dopamine from VTA neurons, with addiction significantly decreasing dopamine secretion the NAc brain region. Remarkably, a single pulse of UV light for 0.2 s elicited sustained dopamine transients of more than 10 min.

3.3.4. MNI-caged Auxins. Synthetic auxins are very powerful herbicides for wheat cultivation. A real challenge for their bioavailability is that most of these drugs have a carboxylate functionality, meaning entry through the complex membrane structure of planta is challenging. In 2015 Hiyashi and co-workers synthesized some MNI-caged auxins, wherein they used the PPG to mask the negative charge, this makes the prodrug much more lipophilic allowing it to enter the interior of planta.[125] This offers the potential to dose plants before sunrise, with the near-UV and violet part of sunlight uncaging the drug in the appropriate compartment.

3.4. Comparison of Patchornik's PPGs with Contemporary PPGs—The Strengths and Weaknesses of Each

Since the introduction of nitro-aromatic PPGs by Barltrop and Patchornik, many other researchers have proposed alternatives and potential improvements to these PPGs. In Table 2 we list of some of these chromophores. Given the size of the PPG literature (see[73,126] to get an idea of this), any prescriptive summary could be considered challenging, or, even, controversial. So, keeping these caveats in mind, we have, in Table 2, decided to "step into the lion's den", with a streamlined comparison of nitroaromatic PPGs with contemporary PPGs. Our selection includes some PPGs widely used in biology, and others that are of intense current interest to the photochemistry community, and so is chosen to give the general reader a feel for the field.

3.4.1. 7-Diethyl-aminocoumarin (DEAC). Coumarins have a long history as important fluorescent dyes,[127] often used in lasers. In 2001, Hagen and co-workers were the first[128] to make DEAC-caged biomolecules (cAMP, cGMP and Br-

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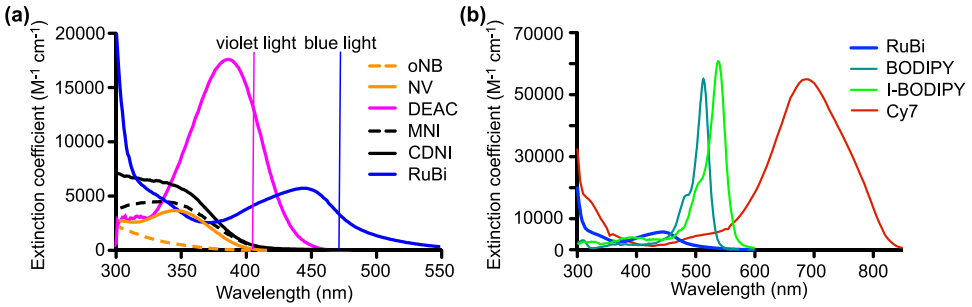

Figure 4. Absorption spectra of representative PPGs. (a) Absorption spectra of PPGs that are most widely used in cell physiology. Note DEAC has significant bathochromic and hyperchromic shifts when compared to nitro-aromatic PPGs, and absorbs violet light strongly. RuBi absorbs at even longer wavelengths, and has good blue light absoprtion. Violet light is often called blue in the caged compound literature. Spectra taken in HEPES at pH 7.4. (b) Absorption spectra of PPGs that have been developed recently. Spectra used by permission of Petr Klan (BODIPY) and Martin Schnermann (Cy7).

cGMP). Hagen proposed that the larger ε of DEAC compared to the oNB and NV chromophores would produce new probes that could be photolyzed more efficiently. The experiments in this first study are exemplary, with the type of side-by-side comparisons in cuvette and in HEK cells that should be carried in any study where new PPGs are being introduced. Importantly, they did the first tests of intracellular uncaging using a violet laser and showed that this wavelength did not uncage NV-cAMP very well inside cells. To date, we estimate that at least 500 publications use the DEAC chromophore as a PPG; compared to more than 3,000 for nitro-aromatic chromophores (i.e., ortho-nitrobenzyl, nitroveratryl and nitro-piperonyl).

Beyond the caged nucleotides and nucleosides made by Hagen, the most extensive biological application of DEAC has been by Carsten Schultz and colleagues, with the development of many caged lipids.[129−132] Recently, Schultz revealed[133] why they chose DEAC as opposed to NV: "Our group preferred the coumarin over the nitrobenzyl groups for various reasons. We wanted to reduce the impact of short wavelength light on cells, and DEAC allowed us to use 405 nm blue light for uncaging. (Note, calling 405 nm light "blue" is a common misnomer in the chemical literature. Obviously, it is violet, as there is no such thing as "ultra-blue" light.) Most importantly, DEAC is fluorescent and therefore cell entry and location of the lipid derivative can be followed by using very little excitation light." Note this quotation brings to the fore a real advantage of DEAC for use in cell biology, namely that it absorbs violet light very well. (Since, all confocal microscopes have a violet 405 nm laser, this makes the photolysis of DEAC-caged compounds within the purview of every laboratory, and probably explains the extensive use of DEAC, as noted above.) Schultz goes on to note that "The intrinsic fluorescence is not always beneficial, especially when fluorescent sensors are used that are also excited by blue light (again, he means violet light). Therefore, in some cases, we used NV cages to release lipids such as diacylglycerols." In Table 2 we suggest that the inherent fluorescence of DEAC is a disadvantage when compared to oNB and NV, which are nonfluorescent. However, the work of Schultz very cleverly turns this "disadvantage" into a real advantage.[129] In reality, most fluorescent probes used in cell biology occupy green and red channels, so the strong blue *fluorescence* of DEAC is not much of a problem for live cell applications of DEAC-caged compounds. We note that many groups have engaged in chromatic tuning of the DEAC chromophore to make PPGs that are responsive to longer wavelengths.[134−137] An attractive feature of some of these derivatives is that they offer the possibility of 2-color uncaging. This is because some of these PPGs have absorption minima in a region where nitroaromatic PPGs absorb efficiently.[87,134] A comprehensive review of this work is beyond the scope of this Perspective, and readers are referred to.[87,138]

In terms of the functionality that may be protected, DEAC is somewhat limited compared to the oNB and NV chromophores. For *direct* protection DEAC requires an acidic leaving group to be effective (i.e., carboxylate, phosphate, or phenol functionalities). It does not work with alcohols or amines. In contrast, NV-OR (ethers) and NV-NH_2, NV-NRH, NV-NRR' (amines) can be deprotected using nitro-aromatic photochemistry because of the internal photoredox reaction mechanism. To use DEAC for the release of alcohols and amines, one must resort to indirect protection via carbonates and carbamates, respectively. The former are quite unstable at physiological pH, whereas the latter show good stability. An unique feature of oNB and NV chromophores is that both may be used for photorelease of secondary amines from tertiary amines. This feature has been used to great advantage in several caged calcium probes as tertiary amines are a required featured of the high affinity Ca^{2+} chelators EDTA and EGTA.[23,24,54,65,67,139] Such probes have been important for many physiological experiments involving single-cell patch-clamp studies.[98] To get a feeling for this, Ellis-Davies has made about 30 g of these probes, which is the equivalent of about 1,200,000 attempted patch-clamp experiments (assuming each pipette had 5 μL of 10 mM probe).

3.4.2. 6-Bromo-7-hydroxycoumarin (Bhc). Before Hagen applied DEAC to uncaging, Tsien and co-workers made a new coumarin-based PPG called "Bhc".[140] The motivation for this work was stated as "Unfortunately, none of the commonly used photolabile protecting groups such as substituted 2-nitrobenzyls, 3-nitrophenyls, benzoins, and phenacyls have been reported to have sufficient photosensitivity for two-photon excitation without tissue damage." The first author of this work was the pioneer of biological application of coumarins. Toshiaki Furuta made 7-methoxycoumarin(MEOC)-cAMP six years before Hagen reported DEAC-cAMP.[141] The Bhc report was submitted several months after Niggli's seminal report on 2P uncaging of Ca^{2+} in the *Journal of Physiology*[53] was published, so their claim quoted above is not accurate. But, Bhc did improve upon the 2P cross section of NV, increasing it from about 0.01 to 1 GM.

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However, the quantum yield from Bhc was only about 10% of NV. Bhc, like DEAC, is quite fluorescent and so has the same type of balance of advantage/disadvantage noted above. Synthetically, we have found Bhc much more difficult to work with than DEAC, and the poor solubility of the chromophore can be challenging. Since Bhc uses the same mechanism as DEAC for release, it suffers from the same limitations in scope. Thus, on balance, it is not entirely clear what advantage it provides compared to DEAC. Finally, the Bhc-Glu that was tested on brain slices in 1999 used a carbamate linker for glutamate attachment, resulting in very slow release of glutamate. Certainly, the 1999 Bhc report was an important step in the development of caged compounds for effective 2P uncaging, but many of the lessons were examples of what needed to be improved.

A very nice feature of the Bhc PPG is that it can become "conditionally photosensitive" in that the 7-hydroxyl functionality must be a phenolate for Bhc to be photoreactive. Thus, Furuta and co-workers have exploited this feature to make a series of probes that only become active in cells in which an exogenous enzyme is targeted.[142]

3.4.3. Ruthenium Bipyridyl (RuBi). The RuBi chromophore was used as a PPG for the first time in 2003 by Etcherique and co-workers.[143] While the photochemical properties (ε and QY) are similar to NV, it is photolyzed by blue light (440−480 nm, see Figure 4a), and this is a distinct difference when compared to the NV chromophore. Further, since blue light does not photolyze NV or MNI (Figure 4a), RuBi offers 2-color uncaging when used with these nitro-aromatic chromophores.[144,145] Notably, RuBi-GABA has been used by many laboratories since it became commercially available, and this success is strong testimony to the usefulness of this caged compound (more than 100 studies have been published). Also, RuBi is essentially nonfluorescent, so it can be used with most fluorophores. However, it uses the GFP excitation channel for photolysis. Perhaps the one limitation of RuBi compared to NV to that it is not a generic PPG, its main photochemistry is limited to C−N bonds, but since primary and secondary amines are so important biologically, this is not an important limitation.

In Figure 4a we compare the absorption spectra of the most used nitro-aromatic PPGs with DEAC and RuBi. It can be seen that the former absorb best in the UV range, whereas both DEAC and RuBi absorb violet light (400−420 nm) very well. However, the small absorption of the MNI chromophore at 405 nm has proved to very useful in some studies.[146]

3.4.4. BODIPYs. These are fluorophores[147] which, like coumarins, have been repurposed as PPGs.[148,149] BODIPYs are inherently highly tunable over a wide spectral range.[147] But compared to the oNB and NV chromophores, they are a synthetic challenge to work with as PPGs. Further, the photochemical scope is limited to the same functionalities as coumarins. The quantum yield of release of most BODIPY-caged compounds is less than 0.01,[150] much lower than many nitro-aromatic probes, which are often in the 0.2−0.7 range.[151,152] However, such low quantum yields could be advantageous in some applications, as it makes caged drugs much easier to handle in ambient light.[152] The fact that BODIPY probes remain strongly fluorescent can bring the same advantage as that noted above for DEAC-based probes. But, unlike DEAC, their fluorescence occurs in the same optical channels required by standard green and red fluorophores (Figure 4b); this is an important limitation for applications in cell physiology. Currently, reports of biological applications of BODIPY-caged compounds seem to be limited to proof of principle tests.

3.4.5. Cyanines. Cyanines are the "work horse" fluorophores for DNA sequencing. They replaced previous xanthene-type dyes (fluorescein, etc.), as they are much more stable. Like, coumarins and BODIPYs they have been repurposed for photorelease for small molecules.[153] The big attraction is that Cy7 absorbs red light, which penetrates tissue better than UV light. In 2014/5 Schnermann and co-workers reported the photorelease of various phenols using irradiation at about 650 nm.[153] This ingenious work used the inherent photo-oxidation of the Cy7 chromophore to release the caged compounds. The photolysis of antibody-drug conjugates (ADC) in rodents provided compelling proof of principle data showing that red light could be used for the photorelease of an antitumor drug very effectively.[154] Use of cyanine dyes is just one of many approaches to the use of red light for uncaging in mammals. For an excellent comprehensive review see.[110] Synthetically, cyanines are quite flexible, being able to be used with a good range of reagents, certainly much more than BODIPYs, but much less than oNB and NV. In terms of the type of functional group that can be released they are like coumarins, working best with acidic groups.

The absorption spectra of representative BODIPY chromophores[150] and Schnermann's Cy7-ADC[154] are shown in Figure 4b. The large extinction coefficients of these fluorophores are striking when compared to the RuBi chromophore. This property is quite useful, as it can compensate somewhat for the poor quantum yield of uncaging. However, for drug uncaging *in vivo*, the low quantum yield might not be much of a limitation.[152,154]

There are other conceptually different approaches to uncaging that are not discussed here, but since this is not a comprehensive review, we have decided to limit this Perspective to core chromophores. Other approaches are reviewed elsewhere.[155,156]

4. SUMMARY

We started this review of the continuing impact of the work of Abraham Patchornik with a brief biographical introduction. In summary, we would like to return to Patchornik the man. Even in the 1980s, he would sometimes chop wood in his orchard in the early morning before arriving at the laboratory. His knowledge of honey production caught the attention of his PhD mentor to be, Prof. Ephraim Katzir (later, a President of Israel). Katzir wrote about Patchornik: "I accepted him as a research student at the Hebrew University because I was fascinated by his tales from the hive, the fact is that from the very beginning I was far more impressed by his abilities in chemistry... his capabilities were substantiated by his innovative methods...."[157] Further, Patchornik completed his undergraduate studies in a situation when the very fabric of society was in doubt, during the War of Independence. So, perhaps greatest lesson we can all learn from Patchornik's life and work is the value of curiosity-driven persistence in achieving one's research goals and dedication to long-term scientific vision, even when not yet popular.

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AUTHOR INFORMATION

Corresponding Author

Graham C. R. Ellis-Davies − *Department of Neuroscience, Icahn School of Medicine at Mount Sinai, New York, New York 10029, United States;* orcid.org/0000-0003-4179-5455; Email: graham.davies@mssm.edu

Author

Kenneth A. Jacobson − *Laboratory of Bioorganic Chemistry, National Institute of Diabetes & Digestive & Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, United States;* orcid.org/0000-0001-8104-1493

Complete contact information is available at: https://pubs.acs.org/10.1021/jacsau.4c00779

Notes

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