Published in final edited form as:

Photochem Photobiol Sci. 2012 March ; 11(3): 460–471. doi:10.1039/c2pp05342j.

Photoactivatable fluorophores and techniques for biological imaging applications

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Abstract

Photoactivatable fluorophores (PAFs) are powerful imaging probes for tracking molecular and cellular dynamics with high spatiotemporal resolution in biological systems. Recent developments in biological microscopy have raised new demands for engineering new PAFs with improved properties such as high two photon excitation efficiency, reversibility, cellular delivery and targeting. Here we review the history and some of the recent developments in this area, emphasizing our efforts in developing a new class of caged coumarins and related imaging methods for studying dynamic cell-cell communication through gap junction channels, and in extending the application of these caged coumarins to new areas including spatiotemporal control of microRNA activity *in vivo*.

1. Introduction

Photoactivatable fluorophores (PAFs) are also known as caged fluorophores or caged dyes. These are weakly or non-fluorescent molecules that can be photochemically converted to fluorescent dyes when excited at the appropriate wavelengths. Earlier interests in this area purely stemmed from a physicochemical perspective focusing on the mechanistic understanding of such transformations¹. Since the pioneering works of Ware, Krafft, Mitchison and coworkers^{2–5}, photoactivatable fluorescent dyes have generated a variety of important applications in biological research. The development of PAFs has been largely driven by the superb spatiotemporal resolution achievable through photoactivation and by the high sensitivity of fluorescence detection. Since the intensity, direction, dimension and even the pattern of a light beam can be easily manipulated, we can precisely control the onset and the site of photoactivation to create a sudden jump in fluorescence to label a cell, a cellular structure, or a biomolecule of interest. Subsequent optical imaging tracks the movement of the fluorescent label continuously to reveal the trajectory, speed, and timing of molecular or cellular dynamics in real time.

As a general introduction to this topic, we will first summarize classical caged fluorophores based on small synthetic dyes that have been successfully applied to biological imaging. We will then briefly describe recent developments in engineering photoactivatable fluorescent proteins, and discuss the unique advantages and limitations of these two classes of PAFs for biological imaging.

The focus of this review article is to describe recent efforts from our laboratory in developing a new class of caged coumarins for biological imaging applications. These caged coumarins exhibit a number of major advantages and improvements over the small caged

dyes previously available. We will summarize the design, photochemical property, and bioconjugation of these caged coumarins, and the development of several related imaging techniques, including LAMP, Infrared-LAMP, and Trojan-LAMP, for studying cell-cell communication through gap junction channels. In addition, we will describe our recent efforts of utilizing these caged coumarins to develop caged antisense reagents to control microRNA activity *in vivo* with high temporal and spatial resolution.

1a. PAFs based on small synthetic fluorophores

Caged dyes based on small synthetic fluorophores typically contain a photo-labile protecting group (caging group) based on 2-nitrobenzyl (NB) or its derivatives including 4,5-dimethoxy-2-nitrobenzyl (DMNB), 1-(2'-nitrophenyl)ethyl (NPE) and 1-(4',5'-dimethoxy-2'-nitrophenyl) ethyl (DMNPE) groups (Figure 1)^{6, 7}. Recent developments in small synthetic PAFs have expanded the caging chemistry beyond NB group⁷⁻¹¹, yet most of the caged probes are still built on commonly used fluorophores including fluorescein, rhodamine, coumarin and their derivatives. Caged fluoresceins are commercially available from Invitrogen.

Among the small synthetic PAFs, NB (or its derivatives) caged fluorescein, resorufin and rhodamine were the ones first developed^{4, 6}. They have been applied to biological imaging to study the assembly of tubulin and microtubule flux^{5, 12, 13}, actin microfilament dynamics^{14, 15}, hydrodynamic flows^{16, 17}, cell lineage during embryo development^{18, 19}, and intranuclear movements of poly(A) RNA²⁰⁻²². More recent applications of PAFs have extended beyond the realm of tracking molecular dynamics to new areas such as superresolution imaging⁹⁻¹¹.

1b. PAFs derived from fluorescent proteins

The first photoactivatable fluorescent protein was derived from green fluorescent protein (GFP) isolated from the jellyfish *Aequorea victoria*²³. The protein, PA-GFP, upon intense irradiation at 413 nm, enhances its fluorescence 100-fold (excitation wavelength 490 nm). Since then numerous protein-based PAFs have been developed²⁴⁻²⁶, covering spectral range from green to red. Another exciting development in this area is the generation of photoconvertible fluorescent proteins that can switch between two different colors²⁶. These novel probes make it possible to simultaneously follow the pre- and post-converted fluorescent proteins by multi-color imaging. Further along this line of development, improving the reversibility of photoswitching led to the creation of photochromic fluorescent proteins which can undergo reversible changes between two states²⁶⁻²⁸. These innovations provide a new family of imaging probes for characterizing intracellular protein dynamics²⁹, molecular transfers through intercellular bridges³⁰, cell tracking³¹, and superresolution imaging²⁸, etc.

Because photoactivatable fluorescent proteins can be conveniently fused to other proteins of interest using the standard technique of molecular cloning, these genetically encoded PAFs quickly gain popularity and become widely used in biological imaging to study protein movements or interactions in living cells. Further, by fusing photoactivatable fluorescent proteins with peptide sequences that confer binding or targeting specificity to cellular proteins or organelles, we can confine the expression of these probes to selected cellular compartments to further facilitate studying protein dynamics or cellular structures at unprecedented spatiotemporal resolution.

1c. A comparison of small synthetic PAFs and protein based PAFs

While these engineered protein-based PAFs offer exciting new strategies for imaging protein dynamics in living cells, they do not replace low molecular weight fluorescent dyes prepared

from synthetic chemistry. First, the small molecular size of synthetic probes is expected to cause less perturbation on protein functions or activities. Second, the photolytic mechanism of NB and related protecting groups has been thoroughly studied and is well known^{32–35}. This facilitates rational design and engineering of new PAFs by synthetic chemistry. Third, new protein labeling strategies that combine chemistry and genetics have emerged which makes it possible to tag proteins with small synthetic dyes in live cells with excellent specificity and fast kinetics³⁶. Fourth, biomolecules other than proteins, oligonucleotides or RNA for example^{20, 21}, can be more conveniently labeled with synthetic dyes than photoactivatable fluorescent proteins. Finally, certain biological studies require using PAFs that are much smaller than photoactivatable fluorescent proteins (~ 26 KDa). Cell-cell coupling through gap junction channels, for example, only allow molecules with molecular weight less than ~ 1200 Da to pass through. In this case, only small PAFs can be used as a fluorescent tracer to study cell-cell dye diffusion across gap junction channels. We expect that both classes of PAFs, protein-based and small synthetic molecules, will continue to generate complementary applications in biological imaging.

2. A new class of caged coumarins for biological imaging applications

2a. Desired properties of synthetic PAFs

Prior to the development of this new class of caged coumarins, small synthetic PAFs previously available, including caged fluorescein, resorufin, and rhodamine, have a number of drawbacks which limit their applications in biological imaging. To overcome these limitations, it requires engineering new PAFs with a number of improvements. These include:

1. Higher photo-stability of parent fluorophores. Both fluorescein and resorufin are fairly prone to photo-bleaching⁶. This introduces uncertainties in the quantification and makes it difficult to image labels repeatedly over a period of time. In addition, highly reactive oxygen species that are toxic to cells are generated during photobleaching.
2. Improved hydrophilicity and biocompatibility. Caged rhodamine, fluorescein and resorufin are hydrophobic aromatic compounds that are poorly soluble in water. This causes at least two problems for bio-labeling. First, the low solubility decreases the reaction rate and labeling efficiency in aqueous buffers. To increase their solubility, solutions containing a high percentage of organic solvents such as DMSO or DMF need to be used. However, an excess of organic solvents may disrupt protein conformation or reduce protein activity. The second problem is that proteins labeled with these hydrophobic caged fluorophores tend to aggregate in water⁶.
3. Enhanced uncaging efficiency by UV light illumination. At wavelength 350 nm or above, the photolytic efficiency of previously reported caged fluorophores is quite low^{4, 6}. Since commonly used caged rhodamine or fluorescein contain two 2-nitrobenzyl caging groups per molecule, this further reduces the efficiency of photolysis because both caging groups need to be photolyzed in order to generate one fluorescent molecule. New caged fluorescent molecules with high uncaging efficiency are desirable because we can minimize the side effects of UV illumination on live specimens.
4. Higher sensitivity to two photon excitation. A recent development in the photouncaging field was the introduction of the two-photon uncaging technique³⁷. Two photon excitation involves exciting a chromophore by two infrared photons with a combined energy equivalent to a single photon of about half the wavelength³⁸. Because the event requires nearly simultaneous absorption of two

photons, it needs very high fluxes of light. Such a high photon flux can only be achieved at the focus of an objective of high numerical aperture when using a pulsed laser as the excitation source. This localized excitation offers an intrinsic optical sectioning which was first exploited for three dimensional imaging using two photon laser scanning microscopy (2PLSM)³⁹. By comparison with confocal laser scanning microscopy, 2PLSM avoids exciting samples out of the focal plane, thus decreases photo-bleaching or photo-toxicity. Other advantages of 2PLSM include imaging at greater depth due to the increased penetration of infrared light, and improved sensitivity of detection due to the omission of a pinhole in front of the detector³⁸. The concept and the technique of two photon excitation has subsequently been applied to photo-uncaging^{37, 40, 41}. Again, because two photon excitation has a quadratic dependence on light intensity, and because light intensity falls off rapidly away from the focus, two photon excitation only photolyzes caged molecules in a highly restricted focal volume, $\sim 1 \mu\text{m}^3$. This high three dimensional selectivity of photo-activation has important applications for studying local signaling events in cells, for example, activation of glutamate receptors in dendritic spines⁴², Ca^{2+} signaling near Ca^{2+} channels in cardiac myocytes⁴⁰, and restricted Ca^{2+} signals in the nucleus⁴³ or in the cytosol⁴⁴, etc. Moreover, by scanning the laser beam across a small area in the plane of focus, two photon uncaging can be applied to activate a cell or a sub-population of cells with excellent three dimensional selectivity in dissected tissues or even in live animals.

The application potential of activating caged dyes by two photon excitation is far from being fully realized. A major obstacle has been the lack of caged probes with high two photon uncaging cross section (δ_u). Although two photon excitation limits photodamage to the focal plane, the possibility of bleaching and damage within this region remains, especially when the scanning laser power at the specimen exceeds 20 mW^{45, 46}. Ideally, caged probes should possess sufficiently high δ_u value (> 0.1 Goepfert-Mayer, $1 \text{ GM} = 10^{-50} \text{ cm}^4 \cdot \text{s/photon}$) to allow efficient two photon photolysis at laser powers that are non-destructive to live cells. In addition, after photolysis, the uncaged dyes should have high two photon excitation action across section ($\sigma_{2\text{PE}}$, product of the two photon absorption cross section and fluorescence quantum yield) so that the parent fluorophores can be imaged efficiently by 2PLSM. Last but not least, the optimal excitation wavelength for two photon imaging should be “orthogonal” to the two photon uncaging wavelength, so when observing fluorescence of the uncaged dye by two photon imaging, the two photon excitation light does not cause additional photolysis of caged fluorophores. PAFs reported previously such as caged fluorescein or caged rhodamine have unmeasurably low δ_u .

5. Facile chemistry to facilitate probe derivatization for cellular delivery or bioconjugation. The technique of microinjection has been applied to deliver synthetic PAFs inside cells. While this method has been proven to be effective for many biological studies, it is nevertheless invasive and can harm cells. Further, microinjection can only be applied to a limited number of cells at a time. Small PAFs that can cross cell membranes by passive diffusion would allow labeling a population of cells rapidly and non-invasively. Finally, to label biomolecules with PAFs, it would be desirable to incorporate facile derivatization chemistry into these compounds to introduce reactive groups or tagging moieties for bioconjugation^{36, 47}.
6. To follow molecular dynamics (either intracellular or intercellular diffusion) by the technique of photo-uncaging, photo-released fluorophores should have minimum binding to cellular proteins, nucleic acids or other cytosolic factors. This is an

important requirement to ensure that photo-released fluorophores can diffuse freely in the cytosol so that rates of dye diffusion can be conveniently measured and quantified.

2b. A new class of caged coumarins suitable for biological imaging

Towards the goal of engineering new PAFs with the improvements outlined above, we recently developed a new class of caged coumarins with a number of major advantages for cellular imaging (Figure 2 and Table 1)⁴⁸:

1. Bright and stable fluorescence of parent fluorophores. These NPE-caged dyes employed 7-hydroxy-coumarin 3-carboxamide (HCC) or 7-hydroxy-6-chloro-coumarin 3-carboxamide (HCCC) as fluorophore. Both HCC and HCCC fluoresce strongly in aqueous solutions and both are fairly resistant to photo-bleaching. HCCC, for example, has a extinction coefficient (ϵ) of 44,000 M⁻¹cm⁻¹ at 408 nm and emits near 450 nm with a fluorescence quantum yield (Q_f) of 93%. A comparison of HCCC with the widely used enhanced cyan fluorescent protein (ECFP) and its improved version Cerulean⁴⁹ shows the favorable optical properties of these coumarin fluorophores for biological imaging.
Fluorophore	$\lambda_{\text{abs}}(\text{nm})$	$\epsilon(\text{M}^{-1}\text{cm}^{-1})$	$\lambda_{\text{em}}(\text{nm})$	Q_f	Brightness ($Q_f\epsilon$)
HCCC	408	44,000	450	0.93	40,920
ECFP	433	29,000	476	0.37	10,730
Cerulean	433	43,000	475	0.62	26,660
2. High contrast enhancement after uncaging. Masking 7-hydroxy of HCCC or HCC with the NPE group quenches its fluorescence to a negligible level ($Q_f < 0.3\%$), so overall the fluorescence intensity of NPE-caged coumarins enhanced over 3000-fold (combined increases in Q_f and $\epsilon^{408\text{nm}}$) upon photolysis. This brings forth the advantage of very low background signal prior to uncaging and a robust fluorescence enhancement post uncaging.
 3. Extraordinarily high sensitivity to UV photolysis. The most remarkable feature of these caged coumarins is their high uncaging efficiency. Table 1 lists structures of some example caged coumarins, their extinction coefficients, uncaging quantum yields (Q_u), and uncaging cross sections (product of $\epsilon \cdot Q_u$) at 365 nm. The uncaging cross sections of these NPE-HCCC at 365 nm exceeds 6,000, about 100 times higher than caged fluorescein⁴.
 4. Remarkably high two photon uncaging efficiency. Both NPE-HCCC and NPE-HCC can be photolyzed efficiently by infrared light through the process of two photon excitation (Table 1). Their δ_u values exceed 0.35 GM^{48, 50}, sufficiently high for live cell uncaging applications (section 2d)
 5. Both coumarin and NPE caging group can be easily modified to facilitate cellular delivery (sections 2c, 2d) or bioconjugation (sections 2e, 2f).

To better understand the outstanding uncaging efficiency of NPE-caged coumarin, we investigated its mechanism of photolysis. HPLC and mass spectrometry analysis of the photolyzed products confirmed that uncaging of NPE-HCCC generated the parent coumarin and 2-nitrosoacetophenone (Figure 2), suggesting that the photolysis followed the known photolytic pathway of 2-nitrobenzyl group through an *aci*-nitro intermediate³²⁻³⁵. Since NPE group itself absorbs weakly above 360 nm ($\epsilon^{365\text{nm}} < 400 \text{ M}^{-1}\text{cm}^{-1}$; trace c of Figure

3), the high uncaging cross section of NPE-HCCC or NPE-HCC must result from the strong absorption of caged coumarin chromophore near 360 nm (trace *a* of Figure 3). This suggests that either the photonic energy absorbed by coumarin moiety was funneled to NPE cage or NPE-coumarin together acts as a unit to absorb light. In either case, coumarin and NPE group need to be coupled in order for the absorbed energy to photolyze the NPE group efficiently. The proposed coupling was supported by comparing the absorption spectrum of NPE-HCCC (trace *a* of Figure 3) with that of 6-chloro-7-methoxy-coumarin 3-carboxamide (trace *b* of Figure 3) or with the absorption spectrum of mixed 6-chloro-7-methoxy-coumarin 3-carboxamide and 1-(2-nitrophenyl)ethanol (trace *d* of Figure 3). Among them, only NPE-HCCC showed a strong absorption at wavelengths above 400 nm, suggesting coupling between NPE and HCCC. We do not think that the coupling occurs through the Förster's type of resonance energy transfer because the NPE group absorbs at shorter wavelengths than coumarin absorbs or emits. Instead, it is more likely that the NPE group and coumarin form an unconventional conjugated system which functions as a unit during photolysis. We termed the process "substrate-assisted photolysis" in which the coumarin moiety acts as an antenna to enhance light harvesting efficiency of the coumarin cage⁴⁸. This mechanism of photolysis is distinct from that of 8-bromo-7-hydroxycoumarin (Bhc) or 8-bromo-7-hydroxyquinoline (BHQ), in which the photolytic reaction has been proposed to proceed from the singlet excited state⁵¹. Another important distinction between these caged coumarins is that, in NPE-coumarin, coumarin is the caged substrate; whereas in Bhc or BHQ, coumarin or quinoline serves as the caging group.

2c. Apply a cell membrane permeable caged coumarin to develop LAMP assay for studying cell-cell coupling through gap junction channels

To exploit the superb photochemical and fluorescent properties of NPE-caged coumarins for cellular imaging, we developed a cell membrane permeable derivative of NPE-HCCC, NPE-HCCC2/AM (Figure 4). When added to cells at micromolar concentration, this neutral and lipophilic compound can cross cell membranes by passive diffusion. Once inside cells, intracellular esterases cleave the acetoxymethyl (AM) ester to generate NPE-HCCC2, a charged and hydrophilic molecule that is trapped in the cytosol. This irreversible trapping can increase intracellular loading of NPE-HCCC2 to concentrations much higher than what is supplied in the extracellular solution. Among numerous mammalian cells tested, including human primary fibroblasts, rodent primary pancreatic acinar cells or beta cells, cultured Hela cells, NIH3T3 fibroblasts, COS cells, CHO cells, etc, NPE-HCCC2/AM could be loaded into all these cells to high concentrations. After loading, we perform repetitive photo uncaging to generate stepwise increase in cellular coumarin fluorescence, whose intensity remained stable^{48, 52}, confirming that HCCC2 is chemically and metabolically stable inside cells. Moreover, diffusion of HCCC2 in the cytosol is rapid. When HCCC2 was locally photo-released at one end of a cell, it diffused across the entire cell and equilibrated in the cytosol in just several seconds⁵², suggesting that HCCC2 has minimum binding to cytosolic factors.

To apply NPE-HCCC2/AM to biological studies, we subsequently developed an imaging assay to follow cell-cell coupling through gap junction (GJ) channels. Intercellular communication through GJ channels plays important roles in maintaining cell homeostasis and synchronizing physiological functions of cells⁵³⁻⁵⁵. In vertebrates, gap junctions are formed from connexin channels that typically allow the passage of small molecules with molecular weight less than ~ 1200 Da. Each connexin channel is comprised of two hemichannels (connexon) contributed from each cells of a coupled cell pair (Figure 5A). The molecular unit of connexin channels is the connexin protein. Six circumferentially arranged connexin proteins form a hemichannel which connects with another hemi-channel from the

opposing cell in the extracellular space (Figure 5A). So far more than 20 connexin genes have been identified in human⁵⁶.

Since abnormal cell coupling has been implicated in a number of diseases including cardiac arrhythmia, deafness, neuronal demyelination, and cataracts⁵⁷, there have been extensive efforts devoted to identifying cellular mechanisms that regulate the molecular permeability of connexin channels. The investigation of the gating of connexin channels by cellular biochemical changes has been hampered in part by the lack of a non-invasive and quantitative technique to assay cell coupling. Assays previously available for studying cell-cell coupling through gap junction channels include both electrical and optical methods⁵⁵. Limitations of these methods include invasiveness and cell damage, low temporal resolution, incompatibility with multi-color imaging, and requirement for special technical expertise, etc^{52, 55}.

To tackle the challenge, we developed a non-invasive imaging assay, termed LAMP (local activation of a molecular fluorescent probe), for studying dynamic GJ coupling in intact cell populations. The assay consists of four steps (Figure 5B): (1) Loading cells with cell permeable NPE-HCCC2/AM; (2) Locally uncaging of trapped NPE-HCCC2 in a cell (or sub-population of cells) to generate highly fluorescent HCCC2 to mark donor cells; (3) Capture the dynamics of HCCC2 (molecular weight 450, well below the molecular weight cut-off of connexin channels) transfer through gap junction channels by digital fluorescence microscopy; and (4) Calculate rates of dye diffusion using Fick's equation which describes the kinetics of dye passage in a system of two compartments separated by a membrane⁵².

An example of LAMP assay is shown in Figure 6 in which we measured the rate of HCCC2 diffusion between a pair of coupled human fibroblasts. Because NPE-HCCC2 can be loaded inside cells to high concentrations without causing any observable side effect, we can perform multiple uncagings to *repeatedly measure the rates of dye transfer in the same coupled cell pairs*. This is a very important feature of LAMP assay because rates of cell-cell dye transfer among *different* pairs of cells vary over a wide range. In order to detect changes in molecular permeability of gap junction channels and to determine whether a cellular change can alter the gating property of connexin proteins, it is essential to assay dye transfer rates *in the same coupled cell pairs* before and after a biochemical stimulation in order to address if and how such an event affects cell coupling. Exploiting this important and unique property of LAMP assay, we demonstrated that Ca^{2+} influx through store operated calcium channels potentially inhibited GJ coupling⁵², likely mediated through a high subplasmalemmal Ca^{2+} activity and colocalization of store operated Ca^{2+} channel and connexin channel⁵⁸.

In short, NPE-caged coumarins including NPE-HCCC2/AM enables us to develop a non-invasive imaging assay, LAMP, that provides quantitative and dynamic information on cell coupling. Since HCCC2 emits blue fluorescence, it spectrally complements a variety of fluorescent sensors emitting from green to red. The combinatory use of these probes allow multi-color imaging to follow cellular biochemistry and cell coupling simultaneously^{52, 58}.

The success of LAMP development in cultured cells prompted us to extend this method to study GJ coupling in tissues where physiological cell-cell contacts are maintained. Given that NPE-caged coumarins can be efficiently photolyzed by two photon excitation near 740 nm (Table 1)⁴⁸, we explored the possibility of integrating two photon imaging with two photon uncaging in same experiments to monitor cell-cell dye transfer in 3D.

Characterization of the wavelength dependence of two photon photolysis revealed that both NPE-HCCC and NPE-HCC were most sensitive to two photon excitation around 740 nm, and their δ_u values dropped to unmeasurable above 790 nm (Figure 7, top black traces). The two photon excitation cross sections (σ_{2PE}) of their parent fluorophores, HCCC and HCC,

reached maxima of over 25 GM at 790 nm (Figure 7, bottom blue lines). Thus, after locally uncaging NPE-caged coumarin at 770 nm or below, we can perform two-photon laser scanning microscopy of HCCC2 at excitation wavelengths 790 nm or above without causing additional photolysis of NPE-HCCC2. We termed this combined two photon uncaging and imaging technique Infrared-LAMP for studying dye movements and cell coupling in 3D in tissues⁵⁰.

In addition to NPE-caged coumarin, new synthetic caged fluorophores with high two photon uncaging efficiency have recently been developed. PENB-DDAO, for example, is a photoactivatable acridinone derivative⁵⁹. The photolyzed product acridinone emits in red so it spectrally complements coumarin or other dyes emitting at shorter wavelengths. Future improvements in its hydrophilicity or solubility would be desirable for biological imaging.

2d. Type 1 conjugates of NPE-caged coumarin derivatized through coumarin moiety

The very high uncaging cross section of NPE-caged coumarins appears to be unique for HCC or HCCC fluorophore. To extend this development to prepare caged fluorophores emitting different colors, we designed a new caged green dye, CCC-1 (caged coumarin-calcein, Figure 8 and Table 1) based on the principle of fluorescence resonance energy transfer (FRET)⁶⁰. CCC-1 contains NPE-HCC and calcein connected by a cyclohexyl linker. Among green emitting xanthenes dyes, we chose calcein as the energy acceptor not only because of the extensive spectral overlap between coumarin emission and calcein excitation but also because of its good water solubility and long cytoplasm retention, a property that has been exploited for labeling and long-term tracking of biological samples⁶¹.

We determined Q_u of CCC-1 to be 30% at 365 nm and its uncaging cross section to be 6000 $M^{-1}cm^{-1}$, over a hundred times higher than that of a caged fluorescein⁴. The photo-released CC-1 displayed a predominate green emission with a FRET efficiency of about 95%. Further, CC-1 has a molecular weight of 943, which still falls within the molecular cutoff of gap junction channels. This would allow combining CCC-1 with NPE-caged coumarins (or their neutral AM esters) in the same experiments to study the size and/or charge selectivity of different connexin channels by two-color LAMP assay.

Based on this development, we prepared two bioconjugates of NPE-caged coumarin by derivatizing the coumarin moiety. Exploiting the reactivity of iminodiacetates towards amines⁶², we reacted dextran amines with NPE-HCC2 or CCC-1 to form, respectively, NPE-HCC2-Dextran⁶³ or CCC-1-Dextran⁶⁰ in one step (Figure 9A). Upon photolysis, both compounds¹⁹ released dextran-dye conjugates which can be useful as cell lineage tracers, or as a fluorescent marker to confirm a successful two photon uncaging in 3D⁶³. We termed these bioconjugates type 1 to distinguish from type 2 bioconjugates in which biomolecules were linked to caged fluorophores via the caging group (Figure 9B and section 2e). A unique feature of caged FRET dyes such as CCC-1 is that we can localize these caged probes by directly exciting the acceptor near 490 nm prior to photolysis. This should help us mapping the distribution of the label in 3D prior to photolysis in order to precisely define the area of photolysis.

2e. Type 2 conjugates of NPE-caged coumarin and Trojan-LAMP assay for tracking gap junction coupling *in vivo*

To study the physiological regulation and function of cell-cell gap junction communication, a non-invasive imaging technique for tracking the spatiotemporal dynamics of cell coupling in living organisms would be invaluable. As we illustrated in previous sections, while small PAFs such as NPE-caged coumarins are powerful probes for imaging gap junction coupling in cultured cells and in dissected tissues, these probes are nevertheless limited in two aspects

if they are to be applied to living organisms: poor cellular loading and short cellular retention time. When AM esters are applied to live animals either by soaking or feeding, they generally penetrate very poorly through the skin barrier or are only taken up by epithelial cells lining the intestine. Moreover, once inside cells, small molecules tend to slowly leak out of cytosol or become compartmentalized in cellular organelles, making it difficult to image them over a long time.

To overcome these obstacles, we developed a new type of bioconjugates of caged dyes, namely type 2 bioconjugates of PAFs, for imaging cell coupling in small model organisms⁶⁴. Dextran-CANPE-HCC is the first member of this new class of caged dyes (Figure 9B). Unlike type 1 bioconjugates in which a macromolecular carrier is linked to a PAF through the fluorophore (Figure 9A), type 2 bioconjugates have the macromolecular carrier connected to the caging group. The design brings forth the combined advantages of lengthening cellular retention time of caged fluorophore without losing the ability to photo-release a small dye, HCC, that can quickly diffuse through GJ channels.

To synthesize Dextran-CANPE-HCC, we modified NPE group by introducing a carboxylate at the 4-position of the phenyl ring to generate 1-(4-carboxy-2-nitrophenyl)ethyl group. The activated ester of this compound was then conjugated with dextran amine to form Dextran-CANPE-HCC, in which CANPE stands for the modified caging group, 1-(4-carbamoyl-2-nitrophenyl)ethyl. Dextran-CANPE-HCC retains the high photolytic efficiency of caged coumarin, with a UV uncaging cross-section of $5,000 \text{ M}^{-1}\text{cm}^{-1}$ at 365 nm and a two photon uncaging cross section of 0.5 GM at 740 nm⁶⁴. As an initial test, we applied the compound to a model organism, *Caenorhabditis elegans* (*C. elegans*), that offers several advantages for *in vivo* imaging of junctional coupling, including small size and limited number of cells (~ 1 mm in length with ~ 1,000 cells in adult worms), transparency, and invariant cell lineage and developmental program. After injection into the gonad (the reproductive organ of *C. elegans*), Dextran-CANPE-HCC was transmitted to the progenies of the injected worm. Even at millimolar concentration, the compound was well tolerated by the labeled embryos and was well retained inside cells throughout embryonic development and even after worms hatched. There was no observable toxic effect on animal behavior or development. Using Dextran-CANPE-HCC and localized uncaging (Trojan-LAMP), we characterized the pattern of junctional cell coupling in early developing *C. elegans* embryos and identified a dramatic remodeling of cell coupling among early blastomeres. By the time an embryo developed to the late 4-cell stage, the germ-line blastomere already became uncoupled to somatic cells⁶⁴, suggesting a unique cytoplasmic environment important for maintaining germ line potential. Further, since Dextran-CANPE-HCC is well retained in cells and can be injected at high concentration, we were able to uncage Dextran-CANPE-HCC (either by UV laser or by two photon excitation) in the terminal bulb of the pharynx in hatched larvae (Figure 10). To our knowledge, this represented the first attempt of imaging dynamic gap junctional coupling at cellular resolution in an intact organ of a living animal. The study revealed rapid dye transfer and thus strong coupling between pharyngeal muscle cells.

2f. Light controlled silencing of microRNA with oligonucleotide conjugates of caged coumarin

To fully exploit the superb photochemical property of NPE-caged coumarin, we considered extending their applications beyond tracking molecular movements. Since biomolecules other than proteins, including oligonucleotides, can be conjugated with small synthetic caged dyes more easily than with photoactivatable fluorescent proteins, we first explored applying these NPE-caged coumarins to prepare oligonucleotide conjugates as caged antisense reagents against microRNAs⁶⁵.

MicroRNAs (miRNAs) are single strand RNA molecules ~ 22 nucleotides long that play important roles in many biological processes through regulating gene expression^{66–68}. In animal cells, miRNAs act by suppressing the translation and/or the stability of messenger RNAs (mRNAs) through a process involving partial complementary base-pairing with sequences at the 3'-untranslated region of mRNAs⁶⁹. To study functions of miRNAs, antisense reagents against miRNAs, so called antagomirs or antimirs, have been developed as a reverse genetics tool⁷⁰. Applying antimirs to inhibit miRNAs is limited in its spatial and temporal resolution, so it remains challenging to inactivate a specific microRNA in a selected cell or time. To gain precise spatial and temporal control of manipulating miRNA activity, we recently developed photo-activatable antisense oligonucleotides against miRNAs (caged antimirs, or cantimirs) based on NPE-caged coumarin.

Exploiting the principle of photo-triggered hybridization^{71–74}, we constructed cantimirs from two strands of 2'-O-methyl oligoribonucleotides. One strand is an antisense oligoribonucleotide with sequence complementary to a specific miRNA⁷⁵, and the other is a blocking strand of shorter length complementary to the 3'-terminus of the antisense strand (Figure 11). To link them together, we synthesized a bifunctional caged coumarin linker containing an amine reactive NHS ester and a thiol reactive maleimide. When covalently connected by the caged coumarin linker, the short blocking strand binds tightly with the complementary antisense strand to block its antisense activity. Photolysis splits the caged linker, weakens the interaction between blocking and antisense strands, and promotes the antisense oligonucleotide hybridizing with its target miRNA (Figure 11). Insert figure 11 here

These cantimirs manifested very high uncaging efficiency like their parent NPE-caged coumarins (Table 1). *In vivo* testing of these cantimirs in *C. elegans* demonstrated low antisense activity in their caged form and very potent inhibition of microRNA post uncaging. Further, by controlling the timing of inhibition at different developmental stages, we demonstrated that a transient activity of *Isy-6*, a microRNA of *C. elegans* controlling the asymmetric neuronal differentiation program⁷⁶, just prior to the comma stage of embryonic development is sufficient to specify the identity of a pair of gustatory neurons⁶⁵.

3. Conclusions and outlook

NPE-caged coumarins are a new class of small PAFs manifesting a number of desired properties for imaging and caging applications in live cells. Based on our initial discovery that NPE-HCC and NPE-HCCC possess superbly high uncaging cross sections, we have developed a series of cell membrane permeable derivatives or novel bioconjugates of NPE-caged coumarins. These compounds were successfully applied to develop several related imaging techniques, including LAMP, Infrared-LAMP and Trojan-LAMP, for tracking dynamic cell-cell coupling through gap junction channels in cultured cells, in dissected tissues, and in live animals. Further exploration of these caged coumarins has extended their applications to new areas such as spatiotemporal control of miRNA activity.

Genetically encoded PAFs are new and exciting PAFs with great potentials for biological studies, yet they do not necessarily replace small synthetic caged dyes. Recent developments in protein labeling technology have generated a number of methods that combines chemistry and genetics to achieve selective labeling of engineered proteins in a cellular environment³⁶, offering new approaches to tag proteins with small synthetic PAFs^{77, 78}. NPE-caged coumarins described here, and new caged fluorophores capable of reversible switching without releasing any photolytic byproduct^{79, 80} or those based on dyes suitable for single molecule imaging⁸¹ will continue to generate new and exciting applications in biological research.

Acknowledgments

We acknowledge grant supports from the National Institute of Health (R01GM077593) and the Cancer Prevention and Research Institute of Texas (RP100456).

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Caged fluorescein

Caged resorufin

Figure 1.
Examples of classical caged fluorophores based on NB or related caging groups.

Figure 2.

Structures, photochemical and fluorescent properties of NPE-caged coumarins, NPE-HCCC and NPE-HCC. Parameters shown here are for NPE-HCCC. Parameters for NPE-HCC are shown in Table 1. Q_f , fluorescence quantum yield; Q_u , uncaging quantum yield; $\epsilon^{365\text{ nm}}$, extinction coefficient at 365 nm; $\delta_u^{740\text{ nm}}$, two photon uncaging cross section at 740 nm.

Figure 3. Absorption spectra of equimolar NPE-HCCC (*a*), 6-chloro-7-methoxy-coumarin 3-carboxamide (*b*), 1-(2-nitrophenyl)ethanol (*c*), and a 1:1 mixture of 6-chloro-7-methoxy-coumarin 3-carboxamide and 1-(2-nitrophenyl)ethanol (*d*). The spectra were taken in pH 7.3 Mops buffer (20 mM). Adapted from ref. 48 with permission.

Figure 4. Structure and mode of action of a caged and cell membrane permeable coumarin, NPE-HCCC2/AM. Adapted from ref. 48 with permission.

Figure 5.

(A) Connexin, connexon and gap junction channel between a coupled cell pair. (B) Schematic of the LAMP technique for studying gap junction coupling. Black dots represent NPE-HCCC2. In step (1), cells were loaded with NPE-HCCC2/AM; Step (2), NPE-HCCC2 was photolyzed with a restricted light beam in a selected cell to generate HCCC2; Step (3), track HCCC2 diffusion to neighboring coupled cells by fluorescence microscopy. Steps (2) and (3) can be repeated multiple times, either by uncaging the same or a different donor cell, in order to track changes in cell coupling strength.

Figure 6. LAMP, a non-invasive and quantitative imaging assay of gap junctional communication. **(A)** Time courses of fluorescence intensities of HCCC2 in a pair of coupled human fibroblasts. Three episodes of UV flashes were applied to a cell (cell *a*, donor) at times indicated by the arrows at the top. Prior to the third uncaging, a GJ coupling blocker, α -Glycyrrhetic acid (α -GA, 10 μ M), was added. It stopped dye transfer. **(B1–B5)** Selected fluorescence images of coupled cells taken at times indicated by dashed arrows in **(A)**. To aid visualization of both donor and recipient cells, they were briefly illuminated by UV light at the beginning (**B1**). **(C1, C2)** Quantification of HCCC2 transfer rates using data from **(A)** and Fick's equation. The slopes of the fitted lines (linear least-square fits) gave rates of dye transfer after first (k_1) and second (k_2) uncagings (r^2 values of linear fittings in parentheses).

Figure 7.

Two-photon uncaging spectra (top) of NPE-HCCC (triangle, X = Cl) and NPE-HCC (circle, X = H) and two photon excitation spectra of HCCC (triangle, X = Cl) and HCC (circle, X = H). Adapted from ref. 50 with permission.

Figure 8. Structures of a caged FRET dye, caged coumarin-calcein (CCC-1), and its photolyzed product CC-1.

A Type 1 bioconjugate **B** Type 2 bioconjugate **Figure 9.**

Two distinct types of bioconjugates of small synthetic PAFs. (A) In type 1 dextran conjugates of caged coumarin, fluorophore and dextran remain linked after photolysis. (B) In type 2 dextran conjugates of caged coumarin, fluorophore is separated from the dextran carrier upon uncaging.

Figure 10.

Trojan-LAMP assay of cell junctional coupling *in vivo*. (A–D) Rapid dye transfer between pharyngeal muscle cells in a living *C. elegans* larvae. A pulse of UV laser was delivered to a spot within the terminal bulb (indicated by the arrow in DIC image, A, scale bar = 10 μ m) at time 0. Three additional pulses of UV laser were then delivered at 4, 11, and 16 sec.

Coumarin fluorescence images at different time points were shown (B–D, image D was taken after moving the stage of the microscope to fit the entire pharynx into the viewing area of a CCD camera). 1, terminal bulb; 2, isthmus; 3, metacarpus; 4, procorpus. Adapted from ref. 64 with permission. (E) Schematic of the anatomy of the pharynx of *C. elegans*. Eight groups of pharyngeal muscle cells, pm1 to pm8, are coupled by gap junction channels.

Figure 11. Structure, preparation, and mode of action of caged antimirs (cantimirs) for the spatial and temporal control of miRNA activity. Adapted from ref. 65 with permission.

Table 1
Structures and photochemical properties of caged coumarins and their applications

Structure	Name	Q_u	$\epsilon^{365\text{ nm}}(\text{M}^{-1}\text{cm}^{-1})$	$\epsilon^{365\text{ nm}}Q_u(\text{M}^{-1}\text{cm}^{-1})$	$\delta_n^{740\text{ nm}}(\text{GM})$	Feature	Application	Ref
NPE-HCC1/Me	0.53	18,400	9752	0.68			48
NPE-HCCC2/AM	0.33	20,000	6600	0.36	Cell permeable	LAMP, infrared-LAMP	48, 50, 52, 58
CCC-1	0.3	20,000	6000		FRET	LAMP	60
Dextran-CANPE-HCC	0.27	18,400	5000	0.5	Dextran conjugate (Type 2)	Trojan-LAMP	64
bifunctional caged linker	0.37	20,000	7350		thiol and amine reactive	Cantimir	65