Cell Fixation Hot Paper

International Edition: DOI: 10.1002/anie.201612112
German Edition: DOI: 10.1002/ange.201612112

Cell Fixation by Light-Triggered Release of Glutaraldehyde

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Abstract: *Chemical fixation of living cells for microscopy is commonly achieved by crosslinking of intracellular proteins with dialdehydes prior to examination. We herein report a photocleavable protecting group for glutaraldehyde that results in a light-triggered and membrane-permeable fixative, which is nontoxic prior to photocleavage. Lipophilic ester groups allow for diffusion across the cell membrane and intracellular accumulation after enzymatic hydrolysis. Irradiation with UV light releases glutaraldehyde. The in situ generated fixative crosslinks intracellular proteins and preserves and stabilizes the cell so that it is ready for microscopy. In contrast to conventional glutaraldehyde fixation, tissue autofluorescence does not increase after fixation. Caged glutaraldehyde may in future enable functional experiments on living cells under a light microscope in which events of interest can be stopped in spatially confined volumes at defined time points. Samples with individually stopped events could then later be analyzed in ultrastructural studies.*

Since the first demonstration by Sabatini and co-workers, aldehydes have been the most commonly used fixatives for light and electron microscopy owing to their unrivalled ability to stabilize the fine structural details of cells and tissues prior to examination.[1,2] Among them, glutaraldehyde is the aldehyde of choice because of its high crosslinking reactivity, allowing superior structural preservation in combination with fast fixation.[3,4] However, in conventional procedures, fixation with aldehydes is a diffusion-controlled process as the employed aldehydes have to enter a cell across its membrane, a process that is not instantaneous. During that time lag, the chemical fixative can cause severe changes within the cell.[5] In contrast to conventional fixation with aldehydes, rapid freezing techniques (e.g., high-pressure freezing) allow for instantaneous physical fixation. These techniques, however, require special equipment and manipulation of the sample at low temperatures (below −100 °C).[6,7]

Yet, both fixation methods represent integral approaches that result in the fixation of the complete sample. Instantaneous fixation with the potential for high spatial resolution would be an important complement to the known fixation techniques. Our investigation started from the idea to introduce a photocleavable protected glutaraldehyde into cells that is activated by light for immediate fixation (cell fixation by flash photolysis, "Cellfi-flash").[8] Photocleavable protecting groups enable the modulation of molecular processes by light with both temporal and spatial resolution. Light as an external stimulus easily penetrates microscopy samples and thus has access to reaction centers that are otherwise difficult to reach.[9] The fixative is masked with a photocleavable functional group to block the crosslinking activity prior to irradiation.[10]

Various photocleavable protecting groups have been established and used to modulate the localization,[11] interaction,[12] and activity[13,14] of biomolecules. The ideal caged molecule should 1) be noncytotoxic, 2) provide high uncaging efficiencies, 3) have sufficient solubility in buffered solution, 4) be sufficiently membrane-permeable, and 5) feature a mechanism for accumulation and trapping of the compound after getting into the cell. For the design and synthesis of caged glutaraldehyde, we were inspired by work from the groups of Gravel and Ni and identified the structural motive of *ortho*-nitrophenylethylene glycol as a promising starting

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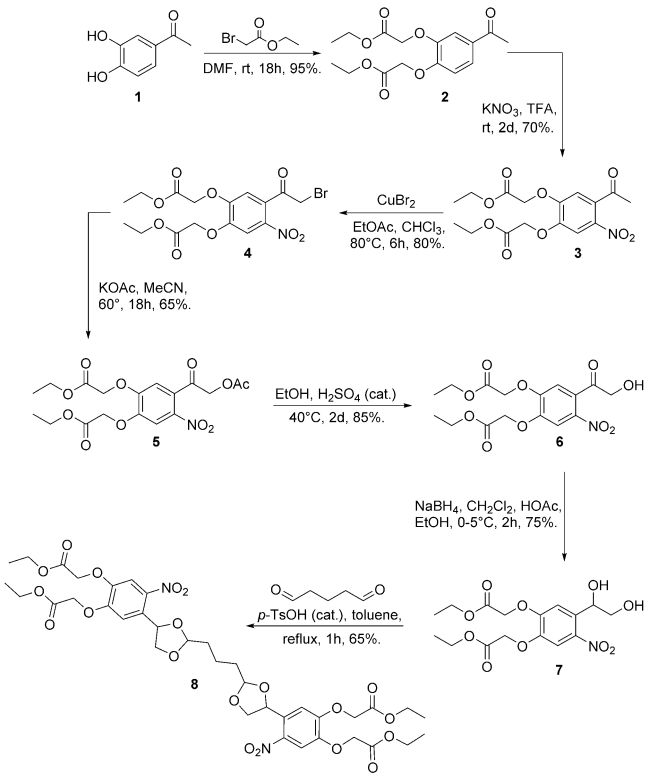
Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under: http://dx.doi.org/10.1002/anie.201612112.



Scheme 1. Synthesis of caged glutaraldehyde **8**. *p*-TsOH = *para*-toluenesulfonic acid, TFA = trifluoroacetic acid.

point.[15,16] The synthesis of caged glutaraldehyde **8** is shown in Scheme 1. The photocleavable protecting group **7** was prepared from 3,4-dihydroxyacetophenone (**1**) in six steps: O-alkylation with ethyl bromoacetate, nitration, α-bromination, acetylation of the α-bromoketone, α-hydroxylation, and subsequent reduction of hydroxyketone **6** afforded diol **7**. In the last step, acid-catalyzed addition of diol **7** to glutaraldehyde yielded caged glutaraldehyde **8** (caged-GA **8**) in 14% overall yield.

In aqueous phosphate buffer solution (0.1M, pH 7.4), caged-GA **8** displays two UV absorption peaks at $\lambda = 300$ nm and $\lambda = 342$ nm ($\varepsilon_{300\,nm} = 1.06 \times 10^3\,M^{-1}cm^{-1}$ and $\varepsilon_{342\,nm} = 1.03 \times 10^3\,M^{-1}cm^{-1}$, respectively; see the Supporting Information for the corresponding spectra). Photolysis of caged-GA **8** in aqueous phosphate buffer (0.1M, pH 7.4) upon irradiation at $\lambda = 300$ nm in a Rayonet RPR-200 photoreactor was fast and proceeded with a quantum efficiency of $\phi = 0.30$, which was determined by chemical actinometry.[17] The course of the photolysis reaction was followed by ultra-performance liquid chromatography mass spectrometry (UPLC-MS; Figure 1). Along with the decrease in the amount of caged-GA **8**, an increase in the amount of cleavage product **10** and the monoprotected glutaraldehyde **9** was observed (see the Supporting Information for the corresponding spectra). Acetoxymethyl (AM) esters are commonly used when membrane permeability and intracellular accumulation are desired.[18] Based on studies by Thodi and co-workers,[19] we investigated whether our ethyl esters are also cleaved by esterases. Assuming that caged-GA **8** is lipophilic enough to permeate the cell membrane, intracellular cleavage of the ethyl esters would generate carboxylates, which are anionic at physiological pH and not membrane-permeable anymore. Thus hydrolyzed caged-GA **8** would be trapped within the cell, ensuring continuous intracellular accumulation. To probe whether ethyl esters are cleaved by esterases, we used porcine liver esterase and lipase B extracted from *Candida antarctica* as two representative esterases at various enzyme/substrate concentrations, and the reaction mixtures were subsequently subjected to UPLC-MS analysis. Complete consumption of the tetracarboxylic ester was observed within 1 h for all enzyme/substrate ratios. For porcine liver esterase, mainly tri- and tetracarboxylic acids were detected, while lipase B of *Candida antarctica* exclusively hydrolyzed until the dicarboxylic acid stage (Figure 2; see the Supporting Information for the corresponding spectra). The cytotoxicity of caged-GA **8** was tested first by live/dead staining. The presence of only green fluorescent cells demonstrated that cells survive treatment with the compound for several hours. As the fluorescence intensity of the treated cells after incubation with caged-GA **8** was lower than that of nonincubated samples, we assume that caged-GA **8** bearing two nitro groups, which could be responsible for the observed fluorescence quenching, is membrane-permeable without apparent cytotoxic side effects (see the Supporting Information).[20]

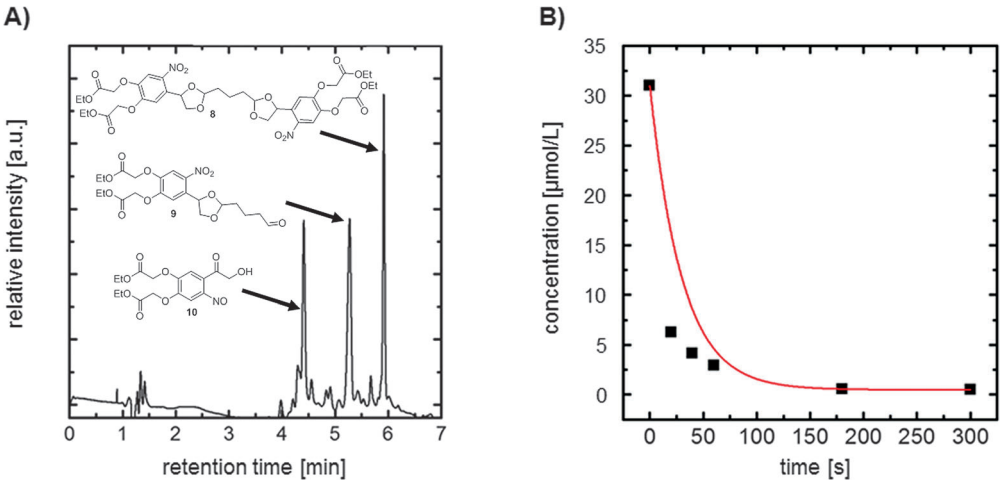


Figure 1. A) UPLC-MS elugram for the photolysis of caged glutaraldehyde **8** after irradiation at $\lambda = 300$ nm for 20 s. B) Decrease in the concentration of caged-GA **8** during photolysis. The corresponding HPLC traces can be found in the Supporting Information.

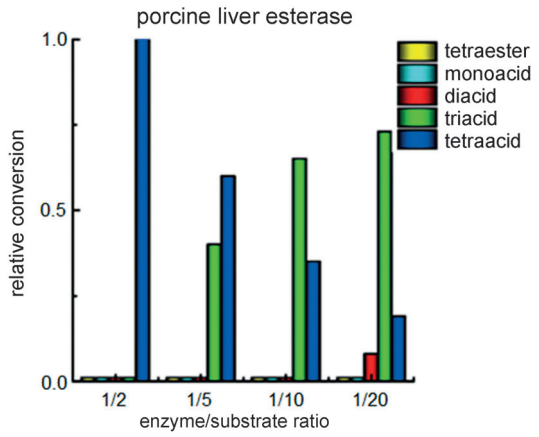


Figure 2. Enzymatic ester hydrolysis of caged-GA **8** at different enzyme/substrate ratios by porcine liver esterase. Complete consumption of the tetraester was observed. The data for enzymatic ester hydrolysis by lipase B extracted from *Candida antarctica* and the corresponding HPLC traces can be found in the Supporting Information.

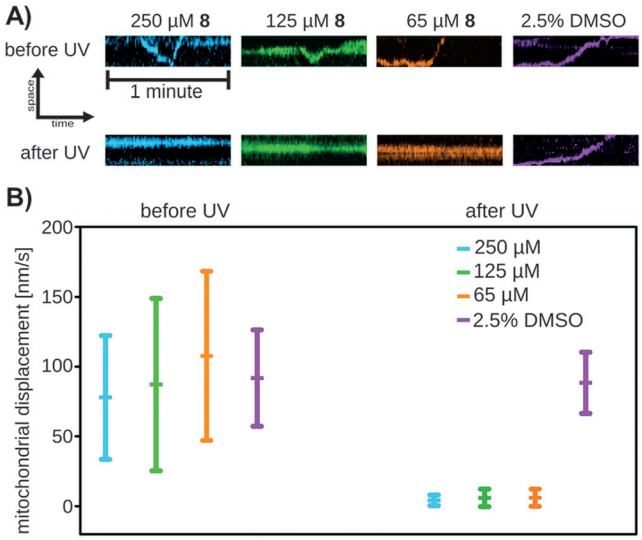


Figure 3. A) Kymographs of the mitochondrial motion in Hela cells and B) mitochondrial velocity in HeLa cells exposed to 250 µM (blue), 125 µM (green), or 65 µM (orange) of caged-GA **8** or 2.5% DMSO solution (violet) before (left) and after (right) irradiation at $\lambda = 300$ nm. The velocities are given as the average of six mitochondrial traces with the standard deviations shown as error bars.

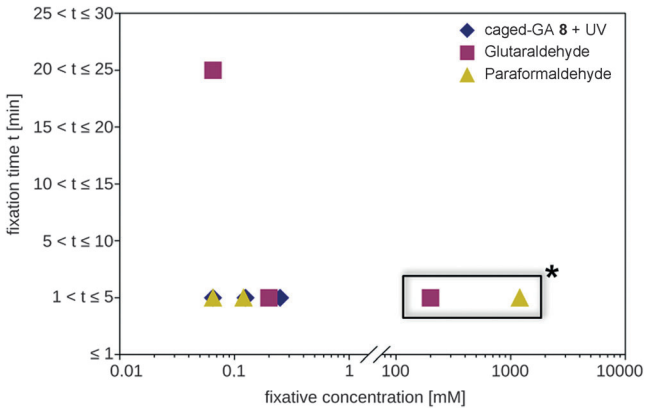


Figure 4. Decrease in the mitochondrial motility of HeLa cells exposed to either caged-GA **8** (250 µM, 125 µM, 65 µM) and UV treatment, GA (200 mM, 200 µM, 65 µM), or PFA (1.2 M, 120 µM, 65 µM). For each time span, the motion of six mitochondrial traces was analyzed per fixative concentration. * Concentrations of GA and PFA routinely used in cell biology.

To assess the applicability of caged-GA **8** as a cellular fixative, we tracked the movement of fluorescently stained mitochondria in living cells by fluorescence microscopy. After overnight incubation with caged-GA **8**, excess (not cell-attached or not cell-internalized) caged-GA **8** was washed out (see the Supporting Information). After washing, mitochondrial movement was recorded before and after uncaging. Uncaging was performed by irradiation with UV light at $\lambda =$ 300 nm for 2 min in a homemade reaction chamber bearing eight lamps of a Rayonet RPR-200 photoreactor. At all concentrations (250 µM, 125 µM, 65 µM), uncaging stopped the mitochondrial movement (see Figure 3 and Movie S1) while the mitochondrial motility of untreated control cells was unaffected by exposure to UV light (Figure 3). The finding that both the mitochondrial morphology and motility are unaffected by 12 h exposure to caged-GA **8** is an additional indication of the low cytotoxicity of the uncleaved compound as mitochondria tend to fractionate under stress.

We compared the fixation kinetics of caged-GA **8** (concentrations as indicated above) and commonly employed aldehydes, namely glutaraldehyde (GA) and paraformaldehyde (PFA), at different concentrations by monitoring the decrease in mitochondrial motility with time. Mitochondria were instantaneously (in less than 1 min) immobilized by PFA. Rapid fixation using glutaraldehyde was observed only at a minimum concentration of 200 µM. At the lowest GA concentration, equivalent to the lowest concentration of caged-GA **8**, mitochondrial motility stopped only after 20 min. Caged-GA **8** instantly stalled mitochondrial movement upon its uncaging (see Movie S1) at all concentrations tested. Based on these experiments, caged-GA **8** is on par with PFA and superior to glutaraldehyde at the lowest concentrations tested (Figure 4). 200 mM GA or 1.2 M PFA, concentrations routinely used in cell biology, increase cellular autofluorescence as indicated by the lower contrast after fixation with PFA or GA (Figure 5D,F). The increase in autofluorescence for $\lambda = 640$ nm (red) excitation is about 40–60% with PFA, and amounts to a factor of 20 for GA (Figure 5G). In contrast, caged-GA **8** did not elevate cellular autofluorescence after uncaging (Figure 5G).

Light microscopy revealed that cells exposed to caged-GA **8** are covered with micellar structures (see Figure 5 and the Supporting Information). We analyzed their size by negative-stain electron microscopy, which revealed that the size of the micellar structures ranged from about 500 nm to almost 6 µm (see the Supporting Information).

In conclusion, we have synthesized a caged glutaraldehyde derivative that is efficiently uncaged upon exposure to UV light and rapidly fixes mammalian cells. In contrast to

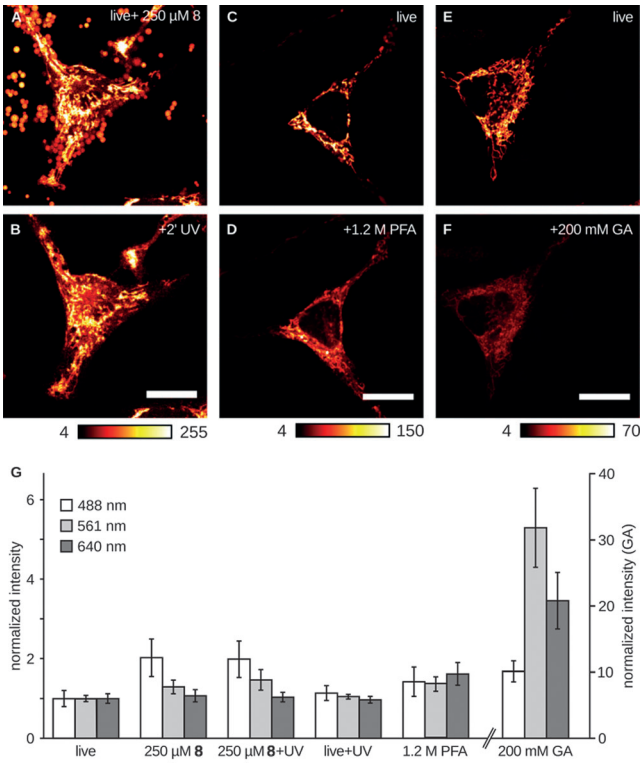


Figure 5. Cellular autofluorescence in HeLa cells stained with MitoTracker Deep Red before (A, C, E) and after fixation using B) 250 mM caged-GA **8** and 2′ UV treatment, D) 1.2 M (4%) PFA, and F) 200 mM (2%) GA. G) Autofluorescence in unstained HeLa cells upon excitation in the blue/green (488 nm, light bars), green/orange (561 nm, gray bars), and red (640 nm, dark bars) spectral region. The intensities per cell were normalized against the autofluorescence of live untreated cells. At least 20 cells were quantified per condition; error bars represent one standard deviation. The elevated autofluorescence in the blue/green spectral channel for **8** is due to photolabile caging groups. Please note that (A)–(F) show the mixed signal of autofluorescence and MitoTracker (640 nm) while (G) indicates the contribution of the autofluorescence of unstained cells. Based on these data, we concluded that uncaging **8** does not generate autofluorescence.

conventional aldehyde fixation, glutaraldehyde released from caged-GA **8** does not give rise to strong tissue-related autofluorescence as the concentrations necessary for effective fixation are much lower than those conventionally applied with most other fixatives. Our compound can be applied to living cells without cytotoxic side effects prior to light activation. This might indicate that diffusion of the caged-GA **8** compound is less hindered than that of conventionally applied glutaraldehyde, especially at long incubation times. Caged-GA **8** opens an avenue to functional experiments on living cells under a light microscope as events of interest can be stopped in spatially confined volumes. Such samples with many individually stopped events are useful for ensuing ultrastructural studies.[21] The "Cellfi-flash" approach complements other recently developed processes that improve the spatial resolution in 3D light microscopy of cells.[22,23] These approaches could be combined to allow event-triggered fixation with very high temporal and spatial resolution. To refine our compound further, we will focus on protecting groups that can be cleaved off by light of longer wavelengths or two-photon absorption to avoid the use of potentially harmful UV light. In addition, we will further investigate the uptake mechanism of the caged glutaraldehyde derivative **8** across the cell membrane by selectively inhibiting the cellular endocytic machinery.

Acknowledgements

This work was financially supported within the leading-edge cluster "Forum Organic Electronics" as part of the High-Tech Strategy for Germany of the Federal Ministry of Education and Research (FKZ 13N11701) and the collaborative consortium "MorphiQuant-3D" (FKZ 13GW0044). K.M.S. thanks the Chemical Industry Fund of the German Chemical Industry Association (VCI) for a scholarship.

Conflict of interest

The authors declare no conflict of interest.

Keywords: cell fixation · glutaraldehyde · microscopy · mitochondrial motion · photocleavage

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Manuscript received: December 13, 2016
Revised: February 23, 2017
Final Article published: ■■ ■■, ■■■■

Communications

Cell Fixation

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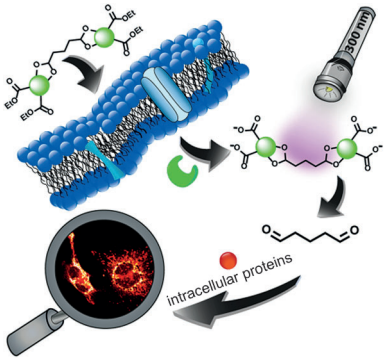
Cell Fixation by Light-Triggered Release of Glutaraldehyde



Cellfi-Flash: A new fixative for light microscopy of living cells was obtained by functionalizing glutaraldehyde with a photocleavable protecting group. Ester substituents ensure that the masked compound can enter the cell and accumulate therein after esterase-mediated hydrolysis. Incubated cells are instantly fixed and ready for microscopy upon irradiation whereas conventional aldehyde fixation is diffusion-controlled.

