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Cell fixation by light-triggered release of glutaraldehyde

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Cellfi-Flash: Cell Fixation by Light-Triggered Release of Glutaraldehyde

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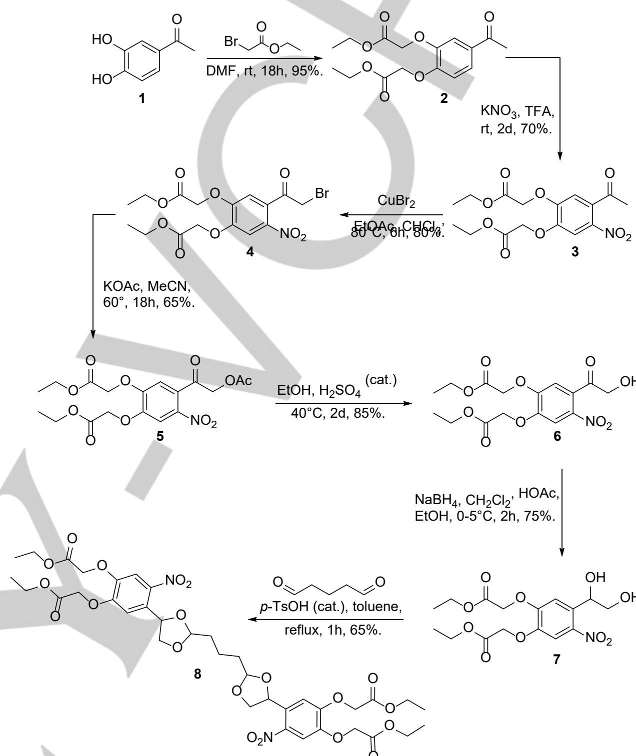
Abstract: Chemical fixation of living cells for microscopy is commonly achieved by cross-linking of intracellular proteins employing dialdehydes, prior to examination. We report a photo-cleavable protecting group for glutaraldehyde, resulting in a light-triggered and membrane permeable fixative, which is non-toxic prior to photo-cleavage. Lipophilic ester groups allow 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, microscopy-ready. In contrast to conventional glutaraldehyde fixation, tissue auto-fluorescence does not increase after fixation. Caged glutaraldehyde allows functional experiments on living cells in the light microscope, stopping events of interest in spatially confined volumes at defined times. Samples with individually stopped events can later be analysed in ultrastructural studies.

Since the first demonstration by Sabatini *et al.,* aldehydes are the most commonly used fixatives for light and electron microscopy due to their unrivalled ability to stabilize the fine structural details of cells and tissue 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 protocols, fixation with aldehydes is a diffusion-controlled process as the employed aldehydes have to enter the cell across their membrane, the process being 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 instantaneous physical fixation. These techniques, however, require special equipment and manipulating the sample at low temperatures (< -100°C).[6,7]



Scheme 1. Synthesis of caged glutaraldehyde **8**.

Yet, both fixation methods represent integral approaches, resulting 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 photo-cleavable protected glutaraldehyde into cells, which is activated by light for immediate fixation (cell fixation by flash-photolysis, Cellfi-flash).[8] Photo-cleavable protecting groups modulate molecular processes by light temporal and spatial resolution. Light as an external stimulus penetrates microscopy samples easily and thus allows access to otherwise hard to reach reaction centers.[9] The fixative is masked with a photo-cleavable functional group to block the crosslinking activity until irradiated.[10]

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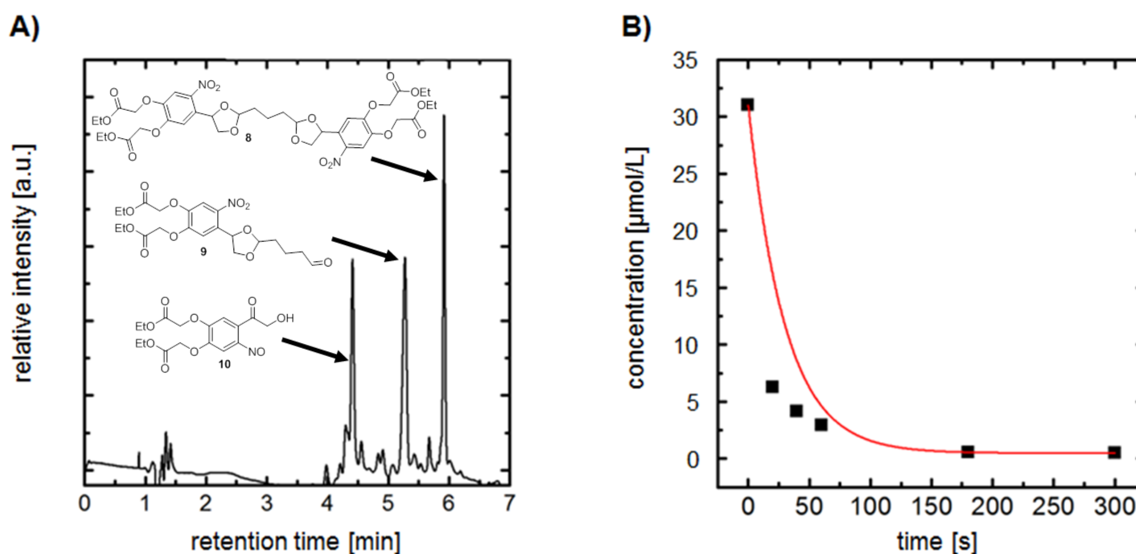


Figure 1. A) UPLC-MS elugram during photolysis of caged glutaraldehyde **8** after 20 seconds irradiation at λ = 300 nm. **B)** Time resolved decrease of caged glutaraldehyde **8** (caged-GA **8**) during photolysis. Corresponding HPLC spectra can be found in the SI.

Various photo-cleavable protecting groups are established and allow modulation of localization,[11] interaction[12] and the activity[13,14] of biomolecules. The ideal caged molecule should (1) be non-cytotoxic, (2) provide high uncaging efficiencies, (3) have sufficient solubility in buffered solution, (4) sufficient membrane permeability and (5) a mechanism for accumulation and trapping of the compound after entering the cell. For the design and synthesis of caged glutaraldehyde we were inspired by work from Gravel *et al.* and Ni *et al.* and identified the structural motive of *o*-nitrophenylethylene glycol as promising starting point.[15,16] The synthesis of caged glutaraldehyde **8** is displayed in Scheme 1. The photo-cleavable 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 the hydroxyketone **6** afforded the diol **7**. In the last step acid catalyzed addition of the diol **7** to glutaraldehyde yielded caged glutaraldehyde **8** (caged-GA **8**) in 14% overall yield. In aqueous phosphate buffer solution (0.1 M, pH = 7.4) caged-GA **8** displays two UV absorption peaks at λ = 300 nm (ε_{300nm} = 1.06 x 10^3 $M^{-1}cm^{-1}$) and λ = 342 nm ($\varepsilon_{342\ nm}$ = 1.03 x 10^3 $M^{-1}cm^{-1}$, see SI for corresponding spectra). Photolysis of caged-GA **8** in aqueous phosphate buffer (0.1 M, pH = 7.4) irradiated at λ = 300 nm in a Rayonet RPR-200 photo reactor displayed fast photo-cleavage with a quantum efficiency of ϕ = 0.30, determined by chemical actinometry.[17] The course of the photolysis reaction was followed by UPLC-MS, displayed in Figure 1. In combination with a decrease of caged-GA **8**, an increase of the cleaved product **10** and the mono-protected glutaraldehyde **9** was observed (see SI for corresponding spectra). Acetoxymethyl (AM) esters are commonly used when membrane permeability and intracellular accumulation is desired.[18] Based on studies by Thodi *et al.*[19], we investigated if our ethyl esters are also esterase-cleaved. Assuming that caged-GA **8** is lipophilic enough to permeate the cell membrane, intracellular cleavage of our ethyl esters would generate carboxylates, anionic at physiological pH, not membrane permeable any more. Thus, hydrolyzed caged-GA **8** would be trapped within the cell, ensuring continuous intracellular accumulation. To probe if ethyl esters are cleaved by esterases, we tested porcine liver esterase and lipase B extracted from *Candida antarctica* as two representative esterases with varying enzyme to substrate concentrations, accompanied by subsequent UPLC/MS analysis. Complete consumption of tetra-carboxylic ester was observed within 1 h for all enzyme/substrate ratios. For porcine liver esterase mainly tri- and tetra-carboxylic acid was detected, while lipase B of *Candida antarctica* exclusively hydrolyzes until the di-carboxylic stage (Figure 2; for corresponding spectra see SI).

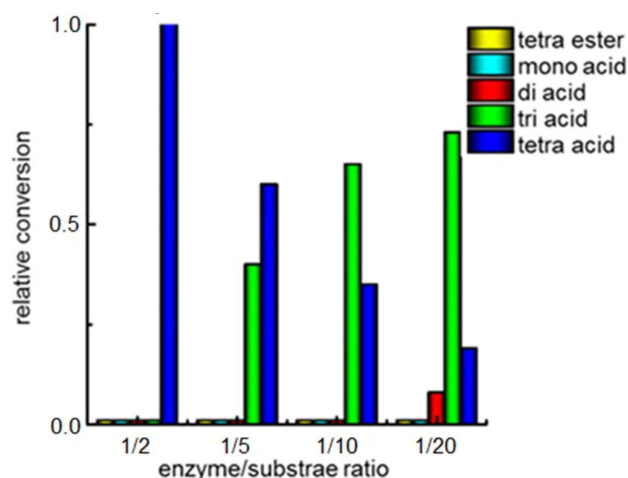


Figure 2. Enzymatic ester hydrolysis at different enzyme/substrate ratios of caged-GA **8** by porcine liver esterase. Complete consumption of the tetra ester was observed. Enzymatic ester hydrolysis by lipase B extracted from *Candida antarctica* and corresponding HPLC spectra can be found in the SI.

The cytotoxicity of caged-GA **8** was tested first by live dead staining. The presence of only green fluorescent cells demonstrates that cells survive treatment with the compound for several hours. Since the fluorescence intensity of the treated cells after incubation with caged-GA **8** is lowered compared to non-incubated samples, we assume that caged-GA **8**, bearing two nitro-groups responsible for the observed fluorescence quenching, is membrane permeable without apparent cytotoxic side effects (see SI).[20]

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 an overnight incubation with caged-GA **8** mitochondrial movement was recorded before and after uncaging. Uncaging was performed by irradiation with UV light at λ = 300 nm for 2 min in a homemade reaction chamber bearing eight lamps of a Rayonet RPR-200 photo reactor. At all concentrations (250 μM, 125 μM, 65 μM) uncaging stopped mitochondrial movement (see Figure 3 and attached video file), while mitochondrial motility of untreated control cells was unaffected by exposure to UV light (Figure 3). The finding that mitochondrial morphology and motility is unaffected by 12 h exposure to caged-GA **8** is an additional indication of the uncleaved compound's low cytotoxicity, since mitochondria tend to fractionate under stress.

We compared the fixation kinetics of caged-GA **8** (concentrations as indicated above) and commonly employed aldehydes, i.e. glutaraldehyde (**GA**) and para-formaldehyde (**PFA**), at different concentrations by evaluating the decrease of mitochondrial motility over time. Mitochondria were instantaneously (in less than 1 min) immobilized by *para*-formaldehyde. Rapid fixation using glutaraldehyde was observed only at a minimum concentration of 200 μM. In the lowest concentration of glutaraldehyde – equivalent to the lowest concentration of caged-GA **8**

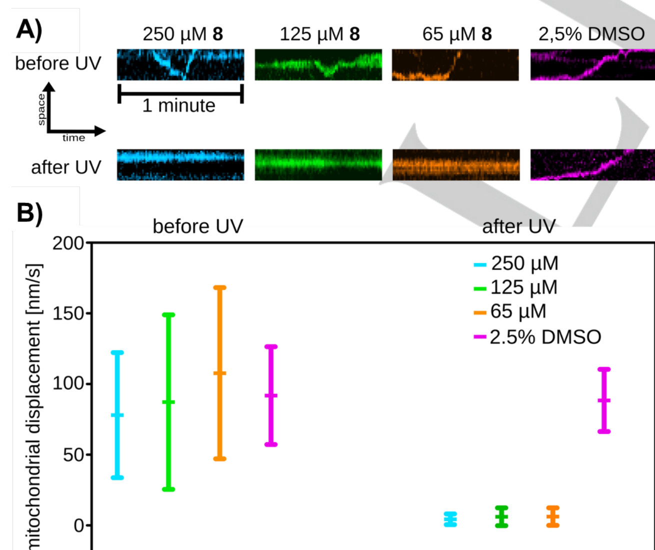


Figure 3. A) Kymographs of mitochondrial motion in Hela cells and **B)** mitochondrial velocity in HeLa cells exposed to 250 μM (blue), 125 μM (green), 65 μM (orange) of caged-GA **8** or 2.5% DMSO (violet), before (left) and after irradiation (right) at λ = 300 nm. Velocities are displayed as the average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

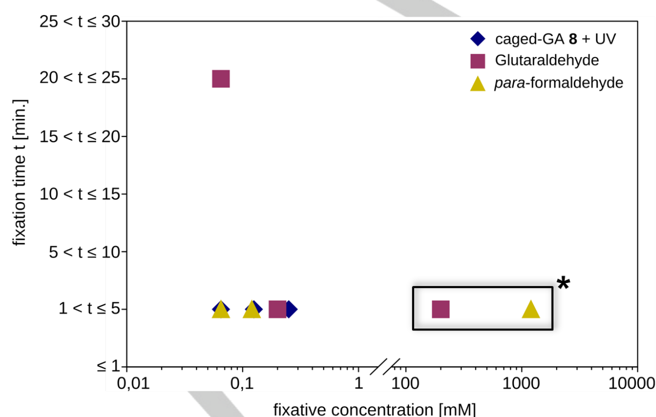


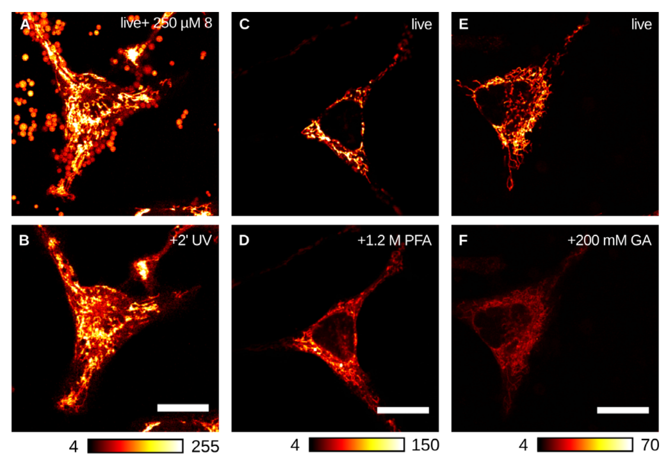
Figure 4. Decrease of mitochondrial motility in HeLa cells exposed to either caged-GA **8** and UV treatment (250 μM, 125 μM, 65 μM), glutaraldehyde (**GA**, 200 mM, 200 μM, 65 μM) or *para*-formaldehyde (**PFA**, 1.2 M, 120 μM, 65 μM). For each time span, the motion of 6 mitochondrial traces was analyzed per fixative concentration. *Concentration of **GA** and PFA routinely used in cell biology.

– mitochondrial motility stopped only after 20 min. Caged-GA **8** instantly stalled mitochondrial movement upon its uncaging (see attached video file) at all concentrations tested. Based on these experiments, caged-GA **8** is on par with *para*-formaldehyde and superior to glutaraldehyde at the lowest concentrations tested (Figure 4). 200 mM glutaraldehyde or 1.2 M *para*-formaldehyde – concentrations routinely used in cell biology – strongly increase cellular auto-fluorescence, indicated by the lowered contrast after fixation with *para*-formaldehyde or glutaraldehyde (Figure 5, **D** and **F**). In contrast to that, caged-GA **8** did not elevate cellular auto-fluorescence after uncaging, obvious in the bar graph (Figure 5 **D**).

Light microscopy reveals, that cells exposed to caged-GA **8** are covered with micellar structures (see Figure 5 and SI). The uptake mechanism of these structures into the cells was investigated. We analyzed their size by negative stain electron microscopy revealing a size range of the micellar structures of roughly 500 nm up to almost 6 μm (see SI).

In conclusion, we synthesized caged glutaraldehyde that is efficiently uncaged by exposure to UV light and rapidly fixes mammalian cells. Contrary to conventional aldehyde fixation, glutaraldehyde released from caged-GA **8** does not give rise to strong tissue related auto-fluorescence, as concentrations necessary for effective fixation are much lower than those conventionally applied. 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 – especially when applied at long incubation times – is less hindered than conventionally applied GA. Caged-GA **8** opens an avenue to functional experiments on living cells in the light microscope, stopping events of interest in spatially confined volumes. Such samples with many individually stopped events are useful for later ultrastructural studies.[21] Cellfi-flash complements other

recent novel chemistry to improve achievable spatial resolution in 3D light microscopy of cells.[22, 23] In future these approaches could be combined to allow event-triggered fixation at highest



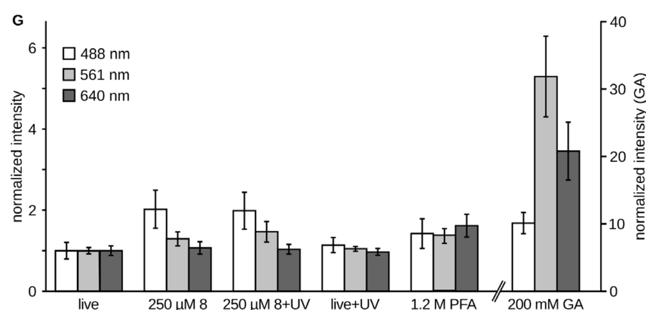


Figure 5. Cellular auto-fluorescence in HeLa cells stained with MitoTracker DeepRed before (**A**,**C**,**E**) and after fixation (**B**,**D**,**F**) using (**A**,**B**) 250 µM caged GA **8** and 2' UV treatment, (**C**,**D**) 1.2 M (4%) para-formaldehyde (**PFA**) and (**E**,**F**) 200 mM (2 %) glutaraldehyde (**GA**). Uncaging **8** does not generate auto-fluorescence. (**G**) Auto-fluorescence 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. Intensities per cell were normalized against auto-fluorescence encountered in live, untreated cells. At least 20 cells per condition were quantified; error bars represent one standard deviation. Elevated auto-fluorescence in the blue/green spectral channel for **8** is due to photo-labile caging groups.

temporal and spatial resolution. To refine our compound further, we will focus on protecting groups that can be cleaved off by light of longer wavelength or two-photon absorption, thus eliminating the use of potentially harmful UV light. Additionally we will further investigate on the uptake mechanism of our caged-GA **8** across the cell membrane by selectively inhibiting the cellular endocytic machinery.

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Keywords: cell fixation • caged glutaraldehyde • light-triggered release • mitochondrial motion • microscopy

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Cellfi-flash: A streamlined synthesis for a new type of fixative for light microscopy of living cells is presented, based on a light-cleavable protecting group for glutaraldehyde. Ester substitution ensures the cellular uptake and intracellular accumulation after hydrolysis elaborated by comparable *in vitro* enzyme studies. *In vivo*, incubated cells are instantly fixed and microscopy ready after irradiation compared to conventional diffusion-controlled aldehyde fixation.

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