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Visualization of Protein-Specific Glycosylation inside Living Cells

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Dedicated to Professor Horst Kunz on the occasion of his 75th birthday

Abstract: *Protein glycosylation is a ubiquitous post-translational modification that is involved in the regulation of many aspects of protein function. In order to uncover the biological roles of this modification, imaging the glycosylation state of specific proteins within living cells would be of fundamental importance. To date, however, this has not been achieved. Herein, we demonstrate protein-specific detection of the glycosylation of the intracellular proteins OGT, Foxo1, p53, and Akt1 in living cells. Our generally applicable approach relies on Diels–Alder chemistry to fluorescently label intracellular carbohydrates through metabolic engineering. The target proteins are tagged with enhanced green fluorescent protein (EGFP). Förster resonance energy transfer (FRET) between the EGFP and the glycan-anchored fluorophore is detected with high contrast even in presence of a large excess of acceptor fluorophores by fluorescence lifetime imaging microscopy (FLIM).*

Protein glycosylation is an essential post-translational modification.[1] Modification of proteins with oligosaccharides, such as N-glycans (linked to asparagine) or mucin-type O-glycans (linked to serine and threonine), is accomplished in the endoplasmic reticulum (ER)–Golgi pathway. These glycoproteins are localized on the cell surface or secreted into the extracellular space. Besides this, many cytoplasmic, nuclear, and mitochondrial proteins are modified by a single *N*-acetylglucosamine (GlcNAc) residue attached to serine or threonine (*O*-GlcNAc modification, *O*-GlcNAcylation).[2] *O*-GlcNAcylation has been shown to regulate protein phosphorylation, degradation, localization, and protein–protein interactions, and to influence transcription.[3] Disorders of *O*-GlcNAcylation are involved in serious pathologies such as type 2 diabetes, cardiovascular disorders, and Alzheimer's disease.[4] Methods for imaging the glycosylation state of a specific protein inside living cells are essential for investigating the biological roles of *O*-GlcNAcylation. However, this important goal has not been achieved yet.

Previously, the glycosylation of specific proteins has been detected by biochemical methods and mass spectrometry after isolation, which disrupts the cellular context of the protein.[5] Conze et al. employed a proximity ligation assay to detect the glycosylation of MUC2.[6] Other groups have used metabolic glycoengineering (MGE) to fluorescently label carbohydrates in the whole glycome,[7] and attached a second fluorophore to a specific protein. Glycosylation of the protein is then monitored by using Förster resonance energy transfer (FRET) as a readout for proximity between the donor and acceptor fluorophore.[8] All of these approaches made use of azide–alkyne cycloadditions as bioorthogonal labeling reactions and were confined to the investigation of glycoproteins on the cell surface. However, the application of Cu^{I}-catalyzed[9] or strain-promoted[10] azide–alkyne cycloadditions to label glycoproteins inside living cells is limited.[11] Cu^{I} is cytotoxic and the cyclooctynes used in the strain-promoted version of the reaction have been shown to react with thiols,[12] which are present in the reducing environment of the cytosol in high concentrations.

Recently, inverse-electron-demand Diels–Alder (DAinv) reaction with either terminal alkenes[13] or methylcyclopropenes[14] as chemical reporter groups has been applied in MGE as an alternative bioorthogonal labeling strategy. Carbamate-linked methylcyclopropene derivatives turned out to be especially well suited for this purpose.[14c–e] The DAinv reaction[15] is highly selective and does not require catalysis by toxic heavy metals, and methylcyclopropenes have been shown to be stable in presence of thiols.[14a,16] These properties mean that DAinv appears to be a particularly suitable ligation method for intracellular applications. Among several carbohydrates that have been used to target protein *O*-GlcNAcylation,[14d,e,17] the recently developed peracetylated methylcyclopropene-tagged *N*-acetylglucosamine Ac_4GlcNCyoc undergoes a fast DAinv reaction with tetrazines.[14d,e]

Herein, we present the first approach for visualizing the glycosylation of specific intracellular proteins in living cells (Figure 1 A). We genetically fused enhanced green fluorescent protein (EGFP) to several proteins of interest and

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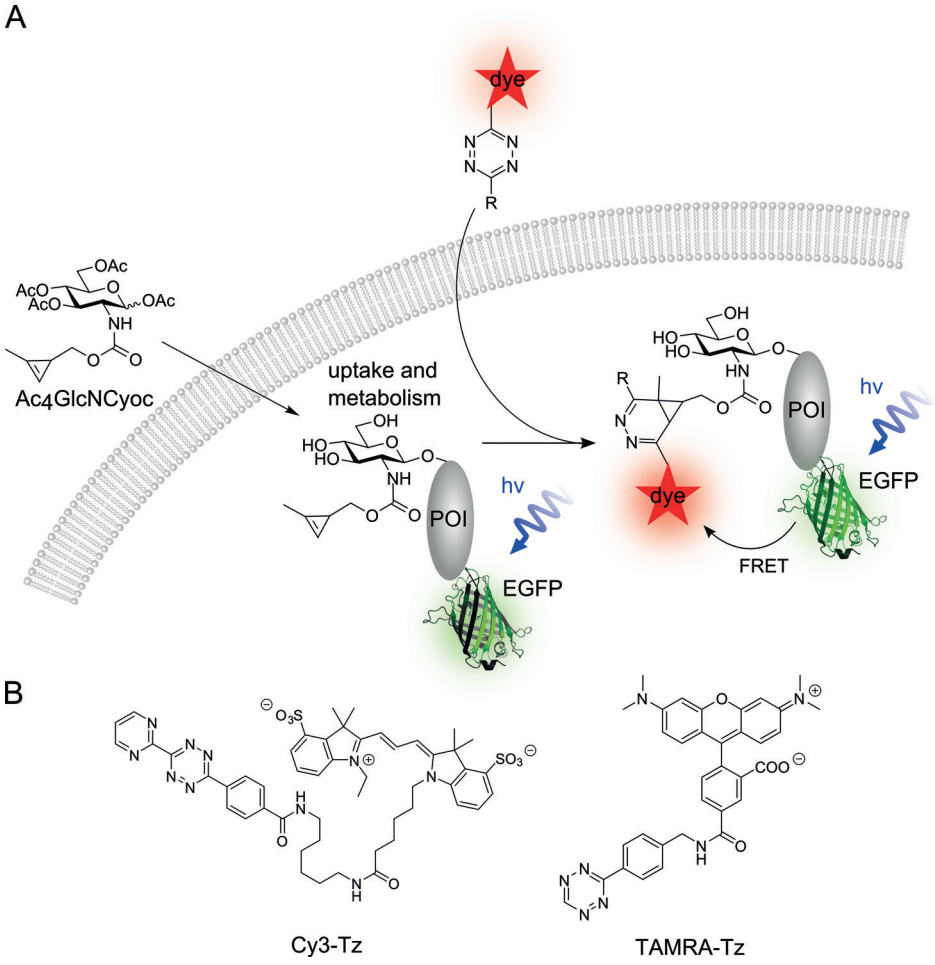

Figure 1. Experimental strategy. A) Peracetylated *N*-acetylglucosamine with a methylcyclopropene tag (Ac_4GlcNCyoc) is taken up by cells, deacetylated by non-specific esterases, and incorporated into the cellular glycome, including the protein of interest (POI), which is fused to EGFP. Subsequently, all proteins that have successfully incorporated GlcNCyoc are labeled with dye–tetrazine conjugates in a specific DAinv reaction. The fluorescence lifetime of the donor (EGFP) can be used to assess the glycosylation of a protein of interest by measuring FRET between the donor EGFP and the acceptor dye–tetrazine conjugate. B) Chemical structures of Cy3–tetrazine (Cy3–Tz) and TAMRA–tetrazine (TAMRA–Tz).

performed MGE by using Ac_4GlcNCyoc. An acceptor fluorophore was attached glycome-wide to the metabolically incorporated sugar by DAinv reaction. Only glycosylation of the protein of interest leads to FRET between the EGFP donor and the acceptor dye. We show that FRET can be detected with high contrast even in presence of a large excess of acceptor fluorophore by fluorescence lifetime imaging microscopy (FLIM). Using this strategy, we succeeded in visualizing the glycosylation of the intracellular proteins OGT, Foxo1, p53, and Akt1.

The enzyme *O*-GlcNAc transferase (OGT)[18] served as a model protein to establish our method. OGT catalyzes the β-*O*-glycosidic attachment of UDP-GlcNAc at serine or threonine residues of proteins resulting in *O*-GlcNAcylation (UDP = uridine diphosphate). It is located in the cytoplasm and the nucleus and has been shown to *O*-GlcNAcylate itself.[19] Other types of glycosylation have not been reported for OGT.

After having optimized the incorporation of Ac_4GlcNCyoc and determined suitable labeling conditions (Figures S1, S2), we ensured modification of OGT by Ac_4GlcNCyoc. To this end, HEK293T cells were transfected with an EGFP–OGT expression vector. Control experiments were performed with cells transfected with an expression vector for EGFP. Cells were then treated with 100 μM Ac_4GlcNCyoc or Ac_4GlcNAc for 20 hours. Ac_4GlcNAc was used as control since it cannot react with the dye–tetrazine (Tz) conjugate. After cell lysis, immunoprecipitation was performed with an anti-GFP antibody to extract EGFP–OGT or EGFP from the cell lysates. The immunoprecipitates were labeled with Cy3–Tz, western blot analysis was performed, and glycosylated proteins were detected by measuring the fluorescence of Cy3 (Figure 2A). Immunoblotting against GFP confirmed equal amounts of EGFP–OGT and EGFP for Ac_4GlcNCyoc- or Ac_4GlcNAc-treated probes. For EGFP–OGT-transfected cells, Cy3 fluorescence at about 135 kDa was only present in the Ac_4GlcNCyoc-treated samples. For EGFP-transfected cells, no fluorescence signal for Cy3 was detected at 30 kDa. This demonstrates that EGFP is not glycosylated and the Cy3 fluorescence for EGFP–OGT is solely due to the modification of OGT with Ac_4GlcNCyoc. We thus used EGFP as a negative control in further experiments. Overall, these results show that incorporation of Ac_4GlcNCyoc can be utilized to monitor the glycosylation of a specific intracellular protein by DAinv chemistry.

For western blot analysis, we applied Cy3–Tz (Figure S2C). To detect the glycosylation of OGT with FRET microscopy inside living cells, we chose the cell-permeable TAMRA–Tz dye (Figure 1B), which does not influence cell viability (Figure S3). Treatment with 50 μM Ac_4GlcNCyoc or Ac_4GlcNAc for 20 hours only slightly reduced cell viability to 81–85%, while 100 μM led to 53% and 74% viable cells, respectively (Figure S4).

Since traditional FRET is based on sensitized emission of the acceptor, it requires elaborate controls to correct for cross-excitation and spectral bleed-through. For intracellular applications, spectral readout of FRET is hampered by background signal resulting from an excess of acceptor dye.

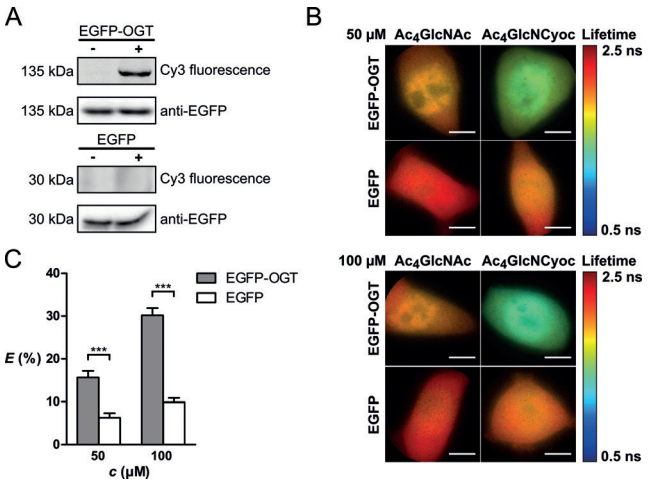

Figure 2. Glycosylation of OGT visualized in living cells. A) Incorporation of $Ac_4GlcNCyoc$ was biochemically confirmed. HEK293T cells were transfected with expression vectors for EGFP–OGT or EGFP, treated with 100 µM $Ac_4GlcNAc$ (−) or $Ac_4GlcNCyoc$ (+) for 20 h, and lysed. EGFP–OGT and EGFP were immunoprecipitated from whole-cell lysates by using an anti-GFP antibody and then labeled with Cy3–Tz. Cy3 fluorescence was detected by western blot. Equal amounts of precipitated EGFP fusion proteins were verified by using an anti-GFP antibody. B) Pseudocolored and intensity-weighted fluorescence modulation lifetime images of EGFP–OGT- and EGFP-transfected cells are displayed. HEK293T cells were treated with either 50 µM or 100 µM $Ac_4GlcNAc$ or $Ac_4GlcNCyoc$ and labeled with TAMRA–Tz. Scale bars: 10 µm. C) Fluorescence modulation lifetimes (τ) from three experiments with five cells each using sugar concentrations (c) of 50 µM and 100 µM were averaged and apparent FRET efficiencies (E) were calculated as: $(1-\tau_{Ac4GlcNCyoc}/\tau_{Ac4GlcNAc})\,100\,\%$. Mean values ± standard error of the mean (SEM) are depicted. Statistical significance was assessed with a two-way ANOVA and Bonferroni posttest. The degree of significance is *** $p < 0.001$.

We used fluorescence lifetime imaging (FLIM) as an alternative approach. The donor fluorescence lifetime is a measure of energy transfer because it decreases in the presence of an additional deactivation pathway such as FRET. We chose the frequency-domain wide-field approach because it is especially well suited for measurements in living cells. Owing to the fast parallel acquisition of data for all pixels, many cells can be imaged in a short time, thereby allowing statistical comparison.

HEK293T cells were transfected with EGFP–OGT or EGFP, treated with $Ac_4GlcNAc$ or $Ac_4GlcNCyoc$ for 20 hours, and incubated with TAMRA–Tz. Fluorescence lifetime images are presented in pseudocolor and are intensity weighted, with the color of a pixel corresponding to its fluorescence lifetime and the brightness to its intensity (Figure 2B). Remarkably, the fluorescence lifetime of EGFP–OGT strongly decreased upon treatment with $Ac_4GlcNCyoc$, compared to treatment with $Ac_4GlcNAc$. The effect is clearly visible at 50 µM sugar concentration, but is even more pronounced with 100 µM (Figures 2B and Figure S5).

The fluorescence lifetimes of EGFP–OGT slightly differ from those of EGFP even in the absence of $Ac_4GlcNCyoc$ (Figures 2B and Figure S5). This can be explained by two effects: The fluorescence lifetime can be sensitive to local parameters (viscosity, temperature, ion concentration, pH value, and oxygen concentration), which can be different for EGFP fusion proteins.[20] Additionally, unspecific labeling of proteins with TAMRA–Tz can lead to background FRET, which is present in both $Ac_4GlcNCyoc$- and $Ac_4GlcNAc$-treated cells. For the analysis of FRET efficiencies, we therefore compared the fluorescence lifetimes of $Ac_4GlcNCyoc$- and $Ac_4GlcNAc$-treated cells for each protein (Figure 2C). The FRET efficiency for EGFP–OGT was 16% with 50 µM and 30% with 100 µM sugar. Since EGFP is not labeled with the acceptor fluorophore, we used it as negative control to estimate the extent of intermolecular FRET to neighboring proteins that have incorporated the modified sugar and are specifically labeled with TAMRA–Tz. The FRET efficiencies for EGFP were below 10% for both sugar concentrations, thus indicating that intermolecular FRET is significantly weaker than protein-specific intramolecular FRET. However, FRET between an EGFP-tagged protein and its glycosylated interaction partner cannot be excluded under conditions of close proximity between the two fluorescent labels.

To substantiate our data, we additionally determined the FRET efficiencies by acceptor-photobleaching experiments on fixed cells (Figure S6). The apparent FRET efficiencies measured in EGFP–OGT- and EGFP-transfected cells were in good agreement with our FLIM results and show glycosylation of OGT within living cells.

To assess the generality of the established approach, we selected five additional intracellular proteins (the forkhead transcription factor Foxo1, the tumor suppressor p53, the serine/threonine kinase 1 Akt1, the cytoplasmic actin-binding protein vinculin, and the calcium/calmodulin-dependent protein kinase CAMK4), which exhibit a broad variety of cellular functions. For all of them, the only reported glycosylation is *O*-GlcNAcylation (Table S1).

We performed immunoprecipitation of the corresponding EGFP fusion proteins to assess incorporation of $Ac_4GlcNCyoc$. Immunoprecipitation with a GFP-directed monoclonal antibody demonstrated that Foxo1–EGFP, p53–EGFP, Akt1–EGFP, and CAMK4–EGFP were modified with $Ac_4GlcNCyoc$, whereas EGFP–Vinculin was not (Figures 3A and Figure S7A). Upon performing FLIM–FRET microscopy with living cells, notable changes in the fluorescence lifetimes were detected for Foxo1–EGFP, p53–EGFP, and Akt1–EGFP upon treatment with 50 µM $Ac_4GlcNCyoc$ (Figures 3B and Figure S8A). The fluorescence lifetimes decreased even more prominently with 100 µM $Ac_4GlcNCyoc$ (Figures S8B, S9A).

The localization of Foxo1–EGFP was mainly cytoplasmic, p53–EGFP was exclusively found in the nucleus, and Akt1–EGFP was present in both the cytoplasm and nucleus (Figure 3B). Whereas the fluorescence lifetimes of Foxo1–EGFP and p53–EGFP were spatially invariant throughout the cell, the fluorescence lifetime of Akt1–EGFP decreased more strongly in the nucleus compared to in the cytoplasm (Figure 3B). Interestingly, it is reported that nuclear localization of Akt1 may be regulated by *O*-GlcNAcylation.[21] With a quantitative analysis of our live-cell FLIM–FRET data, we were able to confirm this biochemical observation (Fig-

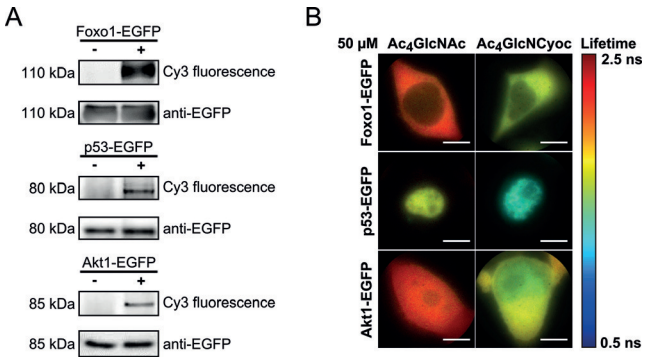

Figure 3. Glycosylation of Foxo1, p53, and Akt1 visualized in living cells. A) Incorporation of $Ac_4GlcNCyoc$ into Foxo1–EGFP, p53–EGFP, and Akt1–EGFP was biochemically confirmed. HEK293T cells were transfected with expression vectors for EGFP fusion proteins, treated with 100 μM $Ac_4GlcNAc$ (−) or $Ac_4GlcNCyoc$ (+) for 20 h, and lysed. EGFP fusion proteins were immunoprecipitated from whole cell lysates by using an anti-GFP antibody and then labeled with Cy3–Tz. Cy3 fluorescence was detected. Equal amounts of precipitated EGFP fusion proteins were verified by using an anti-GFP antibody. B) Representative fluorescence modulation lifetime images of Foxo1–EGFP-, p53–EGFP-, and Akt1–EGFP-transfected cells are displayed. HEK293T cells were treated with 50 μM of either $Ac_4GlcNAc$ or $Ac_4GlcNCyoc$ and labeled with TAMRA–Tz. Scale bars: 10 μm.

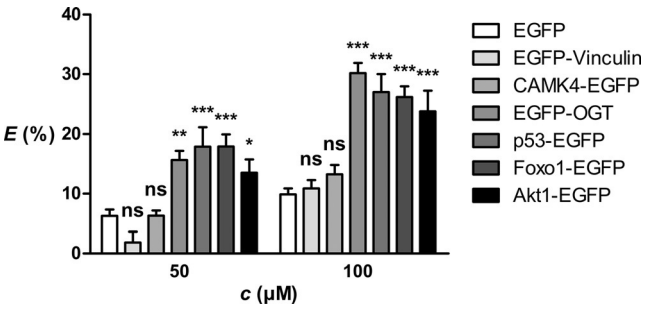

Figure 4. Comparison of the FRET efficiencies of all of the studied proteins. Fluorescence modulation lifetimes from 15 cells from 3 independent experiments, using sugar concentrations (*c*) of 50 μM or 100 μM, were averaged and apparent FRET efficiencies (*E*) were calculated. Mean values ± SEM are depicted. Statistical significance was assessed with a two-way ANOVA and a Bonferroni posttest. The degree of significance is ns = not significant, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

ure S9B). The spatially resolved visualization of Akt1 glycosylation demonstrates the power of this microscopy approach.

Both a sufficiently high glycosylation level and close proximity between the EGFP tag and the glycosylation site are prerequisites for the observation of significant intramolecular FRET. Examples for which these conditions were not met are CAMK4–EGFP and EGFP–Vinculin. For CAMK4–EGFP, modification with $Ac_4GlcNCyoc$ was detected by western blot (Figure S7A), but its fluorescence lifetime was not significantly affected (Figures S7B, S8). Presumably, the fluorescent label(s) at the glycosylation site(s) and the EGFP tag were not close enough. EGFP–Vinculin modification with $Ac_4GlcNCyoc$ could not be detected by western blot (Figure S7A). Consequently, just a slight change in its fluorescence lifetime was observed (Figures S7B, S8). These two examples additionally indicate that unspecific intermolecular FRET does not interfere with our FLIM–FRET measurements.

The FRET efficiencies for all studied EGFP fusion proteins are summarized in Figure 4. For EGFP–OGT, Foxo1–EGFP, p53–EGFP, and Akt1–EGFP, the FRET efficiencies are well above the background FRET obtained from EGFP at both concentrations.

An inherent issue of MGE experiments is the question of the exact fate of the modified sugars. For instance, it has previously been shown that monosaccharides can be enzymatically epimerized and incorporated into glycans other than the targeted glycans.[17b–d] Therefore, we limited our choice of proteins investigated within this study to examples where the only reported glycosylation is *O*-GlcNAcylation (Table S1). To ensure that the incorporation of $Ac_4GlcNCyoc$ is dependent on OGT activity, we overexpressed EGFP–OGT, which led to increased incorporation of the modified sugar (Figure S10A). Moreover, treatment of the cell lysates with $Ac_4$5SGlcNAc,[22] a known OGT inhibitor, resulted in reduced incorporation of $Ac_4GlcNCyoc$ (Figure S10B). Pratt and co-workers have shown that pentynyl groups can be transferred from 1-deoxy-*N*-pentynyl glucosamine to lysine side chains with the formation of stable amide bonds.[23] A comparable transfer for a carbamate structure such as GlcNCyoc would result in a base-stable carbamate linkage to lysine side chains. Mild base-induced β-elimination is an established method to remove O-linked glycans bound to serine and threonine residues while leaving N-linked glycans (and carbamates) intact.[24] We thus investigated whether the protein modification resulting from metabolic incorporation of $Ac_4GlcNCyoc$ is sensitive to base treatment. Lysates of $Ac_4GlcNCyoc$-treated cells were incubated with biotin-Tz and the pH was subsequently adjusted to 12. This resulted in the disappearance of both the *O*-GlcNAc and biotin signals on western blots (for details and control experiments, see Figure S11). These data indicate that $Ac_4GlcNCyoc$ is enzymatically incorporated into *O*-GlcNAcylated proteins.

In conclusion, we have established an approach for visualizing the glycosylation of specific proteins inside living cells. The ability to resolve the localization-dependent glycosylation status of a protein was shown for Akt1. Our strategy has the potential to enable analysis of the influence of external stimuli on the localization and glycosylation of proteins. The observation of time-dependent events in real time is possible with wide-field FLIM, however, it has to be considered that labeled *O*-GlcNCyoc might not be processed in the same way as native *O*-GlcNAc residues. We demonstrate that our strategy is generally applicable to a range of proteins providing that an EGFP tag can be fused in close proximity to the glycosylation site, and we believe that it provides an innovative tool for a deeper understanding of how glycosylation regulates the function of intracellular proteins.

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