

Imaging the Glycosylation State of Cell Surface Glycoproteins by Two-Photon Fluorescence Lifetime Imaging Microscopy**

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A cell surface protein's glycosylation state can profoundly influence its biological capabilities and can report on the physiological state of the underlying cell.^[1–7] Thus, visualization of particular protein glycoforms is an important though challenging goal. Most protein-directed imaging methods (e.g. green fluorescent protein (GFP) tags, fluorescent antibodies) are not sensitive to the glycosylation state of a protein. Our research group previously developed a method for imaging certain glycan structures on native glycoproteins by metabolic labeling with functionalized sugars. However, this glycan-targeted imaging method cannot reveal the identity of proteins to which the labeled glycans are attached.^[8–10] Imaging of a specific protein glycoform will require integration of the identifies of both the protein and the glycan.

Other groups have recently made strides towards this goal. Söderberg and co-workers used proximity ligation to detect a specific glycoform of the tumor marker MUC2.^[11] More recently, Haga et al. used azido sugar labeling of GFP-tagged proteins to image cell surface glycoproteins by Förster resonance energy transfer (FRET) fluorescence microscopy.^[12] Due to their reliance on GFP-tagged proteins, however, this method cannot image endogenous glycoproteins or proteins that are not amenable to fluorescent protein fusion. Even so, there are some limitations to a traditional FRET-based technique. The distance between the donor and acceptor fluorophores in a FRET experiment is related to

the efficiency of energy transfer and typically precludes the use of two large macromolecules, such as immunoglobulin G (IgG; > 10 nm).^[13] Another compounding factor for imaging of specific protein glycoforms is the discrepancy between protein copy number and glycan abundance. The difference in abundance between common types of glycans and a specific protein can be orders of magnitude on the cell surface.^[14] This large difference in relative number can complicate analyses in imaging applications. For example, in a typical FRET-based experiment, the donor fluorophore is excited, and emission from the acceptor fluorophore is monitored. In the case of high acceptor fluorophore concentration, acceptor bleed-through can occur causing a false positive FRET signal (see Figure 1 in the Supporting Information).

Herein, we present a new approach to image endogenous protein glycoforms using a combination of azido sugar labeling and two-photon fluorescence lifetime imaging microscopy (FLIM). We rely on a small (< 7 nm) targeting moiety, an antigen-binding fragment (Fab), to introduce the donor fluorophore and locate the protein component. We applied our previously developed glycan labeling strategy to introduce the acceptor fluorophore. In this scheme, cells were first incubated with an azido sugar, peracetylated *N*-azido-acetylmannosamine (Ac₄ManNAz), which is processed by the cellular machinery and incorporated into glycoproteins as azido sialic acid (SiaNAz). Subsequent bioorthogonal reaction with a cyclooctyne–fluorophore^[15,16] conjugate delivers the acceptor fluorophore within a minimal distance (Figure 1).

A common method for circumventing acceptor bleed-through is to focus on the donor fluorophore's emission in a FRET experiment. Energy transfer between the donor and acceptor fluorophore results in two major changes to the donor's physical properties. The first is reduction in emission from the donor. Imaging this photon reduction in a population of cells requires normalization by photobleaching of the acceptor to reveal the maximum amount of donor emission, a difficult and tedious task when the field of view contains numerous cells. The other change for the donor fluorophore upon energy transfer is a decrease in fluorescence lifetime.^[17] This time-dependent property is advantageous since no further experimentation or sample manipulation is necessary. We sought to utilize the decrease in fluorescence lifetime of the donor fluorophore associated with FRET to monitor the sialylation state of a given glycoprotein through two-photon FLIM.

Overexpression of the integrin $\alpha_v\beta_3$ subtype is observed in a variety of cancers and is often correlated with invasiveness due to its pro-angiogenic function.^[18] Integrin $\alpha_v\beta_3$ possesses

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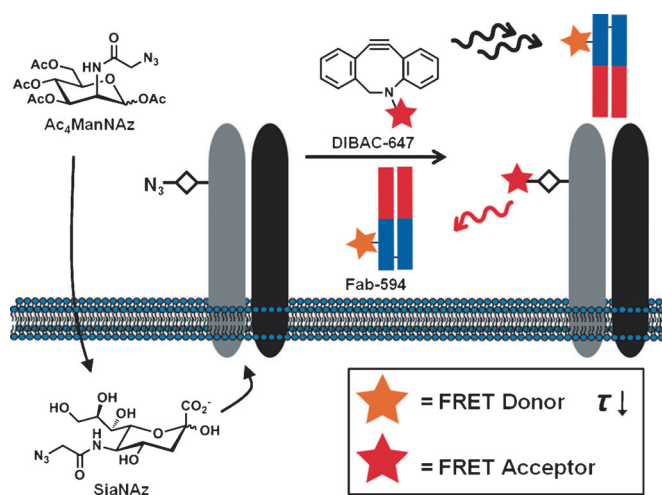


Figure 1. A technique for imaging the glycosylation state of glycoproteins through the use of metabolic labeling and two-photon fluorescence lifetime imaging microscopy (FLIM). Cells or tissues are fed the azido sugar, Ac_4ManNAz , which is metabolized to SiaNAz , and SiaNAz is then incorporated in sialylated glycoproteins. The azide functionality is reacted with an appropriate bioorthogonal probe bearing an acceptor fluorophore to report on the localization of sialic acid residues. In a subsequent step, the protein scaffold of the glycoprotein is targeted with a donor fluorophore-labeled Fab fragment. The presence of both the FRET donor and acceptor fluorophores on a specific glycoprotein is monitored by changes in the fluorescence lifetime (τ) of the donor after two-photon excitation on a per-pixel basis.

four reported and nine potential N-glycosylation sites on the α subunit and two reported and four potential sites on the β subunit. Recently, several reports have suggested that the glycosylation state of integrin $\alpha_v\beta_3$ can dramatically alter its migratory and angiogenic functions. In two separate reports, sialylation of integrin $\alpha_v\beta_3$ was found to be required for cell proliferation, migration, and regeneration in wound-healing assays.^[19,20] Panjwani and co-workers have shown that $\alpha_v\beta_3$'s glycosylation state also affects VEGF- and bFGF-mediated angiogenesis through an interaction with galectin-3.^[21] Motivated by the importance of $\alpha_v\beta_3$'s glycosylation state, we chose to pursue this integrin as a proof-of-concept glycoprotein for imaging its sialylation status.

We first investigated whether endogenous integrin $\alpha_v\beta_3$ is sialylated in the U87MG glioblastoma cell line, which is known to express the heterodimer complex at high levels.^[22] $\alpha_v\beta_3$ was immunoprecipitated from U87MG lysate, and the presence of sialic acid on both subunits was assessed by lectin blotting. $\alpha_2,3$ - and $\alpha_2,6$ -linked sialosides can be distinguished by blotting with *Maackia amurensis* agglutinin (MAA) and *Sambucus nigra* lectins (SNA), respectively. After immunoprecipitation, we observed MAA binding to both integrin subunits, suggesting they are sialylated with $\alpha_2,3$ -linked residues (Figure 2 A).

Our FRET scheme is predicated on SiaNAz incorporation into the N-glycans of integrin $\alpha_v\beta_3$ (Figure 1). To confirm this, we supplemented cells with either Ac_4ManNAz or Ac_4ManNAc for 3 days. We then incubated cells with a biotin-conjugated phosphine, a bioorthogonal probe for

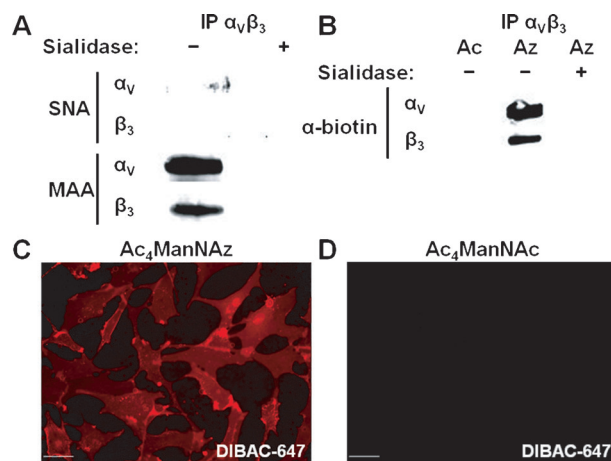


Figure 2. Incorporation of SiaNAz into integrin $\alpha_v\beta_3$ and U87MG cell surface glycoconjugates. A) Lectin blot of α and β subunits of immunoprecipitated $\alpha_v\beta_3$ with $\alpha_2,6$ (SNA) and $\alpha_2,3$ (MAA) sialic acid binding lectins in the presence or absence of sialidase. B) Reactivity of $\alpha_v\beta_3$ subunits with phosphine-biotin. Cells were fed either Ac_4ManNAz (Az) or Ac_4ManNAc (Ac) for 3 days and incubated with phosphine-biotin, a probe for the presence of azides, in the presence or absence of sialidase. C,D) Fluorescence microscopy of U87MG cells treated with either Ac_4ManNAz (C) or Ac_4ManNAc (D) and then DIBAC-647 (C,D). Scale bars: 50 μm .

the azido functionality.^[9] Cell lysates were subjected to immunoprecipitation with an anti-integrin $\alpha_v\beta_3$ antibody, and SiaNAz was detected by western blotting. Azide-dependent labeling was observed for both the alpha and beta chains of integrin $\alpha_v\beta_3$ (Figure 2 B), suggesting that SiaNAz is present on both subunits of the heterodimer. We also confirmed that the azide-specific signal was indeed due to SiaNAz residues by digesting the lysates with sialidase, whereupon we observed a reduced signal on both integrin subunits (Figure 2 B).

We next turned our attention to the selection of the proper FRET pair for the dual labeling strategy. Neuronal cell lines, such as U87MG, are known to exhibit strong flavin- and riboflavin-associated autofluorescence centered at 540–550 nm.^[23] To avoid cellular autofluorescence, we focused on donor fluorophores with emission above 600 nm. We ultimately chose the Alexa Fluor 594 (594) and Alexa Fluor 647 (647) fluorophore combination since Alexa Fluor 594's emission maximum is 617 nm, and the Förster radius for the pair is 8.5 nm (see the Supporting Information).

Preparation of the donor Fab fragment conjugate commenced by treating the monoclonal antibody against integrin $\alpha_v\beta_3$ (LM609 clone) with a peptidase that cleaves the full-length IgG between the Fab and Fc segments. The Fab fragment was separated from the Fc segment and unreacted IgG by incubation with protein A resin, and in a subsequent step the lysine residues were reacted with Alexa Fluor 594 N-hydroxysuccinimidyl ester to furnish the Fab-594 conjugate (see the Supporting Information). The acceptor cyclooctyne was also synthesized from commercially available reagents, dibenzazacyclooctyne (DIBAC) amine and Alexa Fluor 647 N-hydroxysuccinimidyl ester (see the Supporting Information). The ease of construction of both the donor, Fab-594,

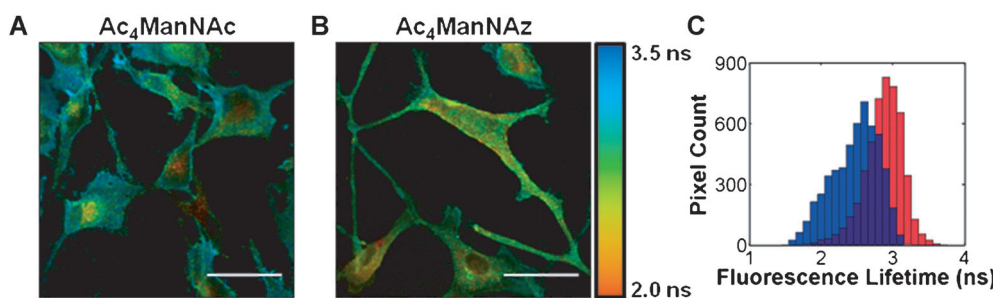


Figure 3. FLIM imaging of sialylated integrin $\alpha_v\beta_3$. A,B) Two-photon FLIM images of U87MG cells treated with either Ac_4ManNAc (A) or Ac_4ManNAz (B), followed by DIBAC-647 and Fab-594. FLIM images are the heat maps of τ values determined per pixel (see accompanying legend, right). C) Histogram of cell surface τ values for cells treated with either Ac_4ManNAc (red bars) or Ac_4ManNAz (blue bars) and then incubated with DIBAC-647 and Fab-594. Scale bars: 50 μm .

and the acceptor, DIBAC-647, targeting moieties underscores the versatility of this method and its applicability to other glycoprotein targets.

After the treatment of U87MG cells with Fab-594, we observed specific cellular labeling of membrane-associated $\alpha_v\beta_3$ that could be inhibited by the presence of full-length anti- $\alpha_v\beta_3$ IgG (see Figure 2 in the Supporting Information). We also sought to determine if DIBAC-647 was a faithful bioorthogonal probe for azides. Towards this end, we fed cells either Ac_4ManNAz or Ac_4ManNAc for 3 days and then incubated them with DIBAC-647. Cell-surface labeling occurred only in cells administered Ac_4ManNAz (Figure 2 C,D).

After confirming the labeling of $\alpha_v\beta_3$'s protein backbone with the FRET donor Fab-594 and SiaNAz residues with the FRET acceptor DIBAC-647, we focused on monitoring FRET using two-photon FLIM. The two-photon FLIM FRET technique entails exciting the donor fluorophore using near-IR/IR femtosecond pulses and monitoring the time-dependent fluorescence decay in the nanosecond regime.^[17] In the case when an acceptor fluorophore is in close proximity to the donor fluorophore, the excited state of the donor is depleted through energy transfer, which ultimately results in the reduced fluorescence lifetime (τ) of the donor molecule. We observed that Fab-594 had an *in vitro* characteristic τ value of 3.09 ns, which was determined by fitting the fluorescence decay of Fab-594 to a single exponential (see Figure 3 in the Supporting Information). In FLIM, the fluorescence emission for each pixel of an image is monitored in a time-dependent manner. The emission data are then fitted to extract the τ values, and these are displayed as a heat-map image.^[17]

FLIM images were obtained by incubating U87MG cells with either Ac_4ManNAz or Ac_4ManNAc for 3 days and then labeling cells with DIBAC-647 and Fab-594, sequentially. Cells that were treated with Ac_4ManNAc had an average τ value of 2.91 ns (Figure 3 A). The cell-surface regions had a relatively constant τ value, as represented by the deep blue color in the image in Figure 3 A. On the other hand, in cells treated with Ac_4ManNAz , the average τ value decreased to 2.60 ns (Figure 3 B), which is consistent with cell-surface FRET^[24] between Fab-594 and DIBAC-647; the cell-surface τ values appeared far more heterogeneous than in Figure 3 A,

as evident by green and yellow clusters. Collectively, these data indicate that cell surface integrin $\alpha_v\beta_3$ is sialylated on U87MG cells. We further analyzed the τ values by constructing a histogram of the cell surface τ values for both Ac_4ManNAc and Ac_4ManNAz cell populations (Figure 3 C). The decrease in the τ values from cells labeled with only the Fab-594 donor versus cells with both the Fab-594 donor and the DIBAC-647 acceptor was also accompanied by a switch from a unimodal (red) to a multimodal distribution (blue). These changes may reflect variations in the amount of sialylation on individual integrin $\alpha_v\beta_3$ molecules. As well, cells lacking Fab-594 but labeled with the DIBAC-647 acceptor displayed minimal signal (Figure 4 A).

We further sought to evaluate whether the observed FRET arose from SiaNAz residues on integrin $\alpha_v\beta_3$ itself, or

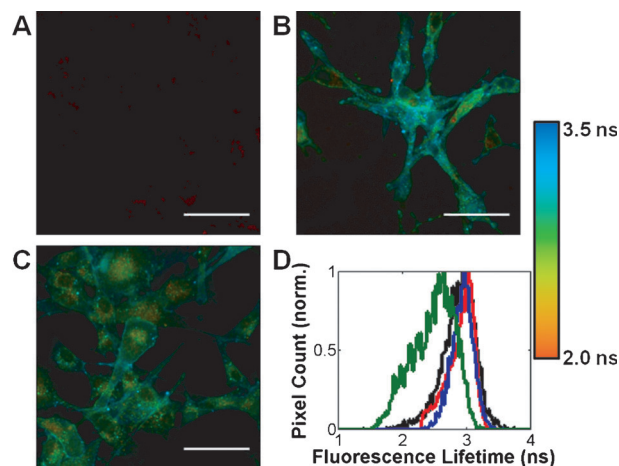


Figure 4. FLIM FRET is dependent on the proximity of the acceptor dye to Fab-594 and the target protein's glycosylation state. FLIM images of A) Ac_4ManNAz -fed U87MG cells treated with DIBAC-647 alone, B) U87MG cells modified with DPPE-647 and then incubated with Fab-594, and C) Ac_4ManNAz -fed U87MG cells first treated with sialidase and then incubated with DIBAC-647 and Fab-594. FLIM images are the heat maps of τ values determined per pixel (see accompanying legend, right). D) Histogram of cell surface τ values for cells treated with either Ac_4ManNAc (black line), DPPE-647 (red line), Ac_4ManNAz and sialidase (blue line), or Ac_4ManNAz (green line) and then incubated with DIBAC-647 (black, blue, and green) and Fab-594 (black, red, blue, and green). Scale bars: 50 μm .

alternatively, from nearby sialic acid-modified glycoproteins or glycolipids. We reasoned that by adding an acceptor fluorophore to cell surfaces nonspecifically, while guaranteeing that the acceptor moiety was not bound to our integrin-of-interest, we could differentiate between sialylated integrin-dependent FRET and background FRET. Cells were first incubated with dipalmitoyl phosphatidylethanolamine (DPPE)-647, which incorporates the acceptor into cell membranes uniformly, and then followed with Fab-594 labeling. No decrease in τ values was observed in this case as cell surfaces appeared to maintain a similar hue to that of cells that only possess the Fab-594 fragment (Figure 4B,D). Thus, we reasoned that sialic acid residues on nearby proteins or lipids do not influence the FRET signal originating from $\alpha_v\beta_3$ complexes. We also tested whether the observed FRET was a sialylation state-dependent phenomenon. We treated Ac_4ManNAz -fed U87MG cells with a sialidase to cleave SiaNAz residues from cell surface glycoconjugates. The cells were further treated with DIBAC-647 and incubated with Fab-594. Sialidase treatment abolished the decrease in τ (Figure 4C,D), indicating that SiaNAz residues on integrin $\alpha_v\beta_3$ were critical for the observed FRET.

A unique attribute of this platform is that it can monitor the glycosylation state of endogeneous glycoproteins as well as image deep within a specimen, owing to the two-photon mode of excitation. We have previously shown that human prostate adenocarcinoma tissue slices can metabolically incorporate azido sugars into their glycoproteins.^[25] Prostate cancers are known to undergo an upregulation in integrin $\alpha_v\beta_3$,^[26] which provided us an opportunity to apply our imaging technique to human tissue slices. Grade 3–4 precision-cut adenocarcinoma tissue slices derived from an 8 mm core of a radical prostatectomy were cultured for 3 days in either Ac_4ManNAz or Ac_4ManNAc . We first determined that $\alpha_v\beta_3$ immunoprecipitated from tissue lysate, like $\alpha_v\beta_3$ derived from U87MG lysate, was recognized by MAA (see Figure 5A in the Supporting Information) and incorporated SiaNAz residues into its glycans (see Figure 5B in the Supporting Information). Using fluorescence microscopy, Fab-594 and DIBAC-647 were found to label prostate tissue slices in a $\alpha_v\beta_3$ - and azide-dependent manner (see Figures 6 and 7 in the Supporting Information), respectively. We next applied our FLIM FRET strategy to the tissue slices by first staining with DIBAC-647 and then with Fab-594. Ac_4ManNAc -treated tissue slices displayed the characteristic Fab-594 τ value (Figure 5A,D), as evident by the blue color. The Ac_4ManNAz -treated tissue slices showed a decrease in τ values (Figure 5B,D) that could be abrogated by treatment with sialidase (Figure 5C,D), again consistent with FRET and the presence of sialylated integrin $\alpha_v\beta_3$ embedded within the tissue slices. These data show that we can probe protein glycosylation with increasing molecular precision.

In conclusion, we have developed a FLIM FRET-based method for visualizing the glycosylation state of specific glycoproteins. The method overcomes intrinsic limits of classic FRET imaging, which can be undermined by bleed-through. As well, we can now also image discrete protein glycoforms in whole tissue slices. The method therefore adds to a growing toolkit for characterizing cell surface glycomes

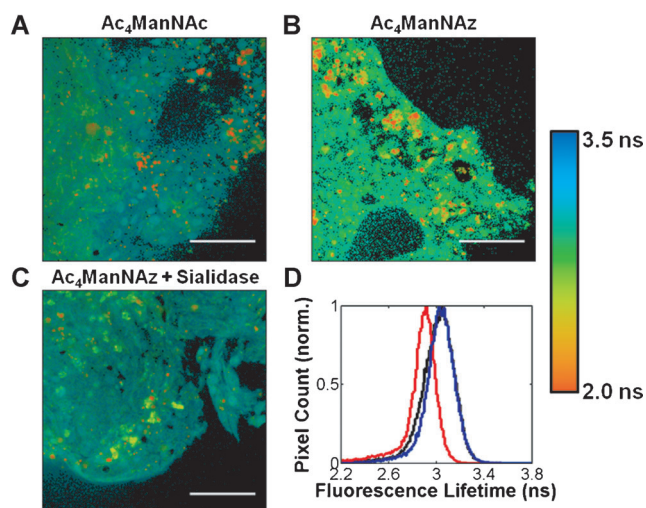


Figure 5. FLIM images and lifetime histogram of grade 3–4 human prostate adenocarcinoma tissue slices. Tissue slices were incubated with either Ac_4ManNAc (A), Ac_4ManNAz (B), or Ac_4ManNAz and sialidase (C) and then treated with DIBAC-647 and Fab-594. FLIM images are the heat maps of τ values determined per pixel, (see accompanying legend, right). D) Histogram of τ values for tissues treated with either Ac_4ManNAc (black line), Ac_4ManNAz and sialidase (blue line), or Ac_4ManNAz (red line) and then incubated with DIBAC-647 and Fab-594. Scale bars: 50 μm .

with molecular precision. We plan on utilizing this technique in the future for imaging the cognate cell surface ligands for secreted lectins as well as for diagnostic purposes in monitoring the glycosylation status of specific glycoproteins.

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