Glycoconj J (2012) 29:445–452
DOI 10.1007/s10719-012-9425-y

Fluorescein-5-thiosemicarbazide as a probe for directly imaging of mucin-type *O*-linked glycoprotein within living cells

Ying Zhang · Yujiao Sun · Zhongfu Wang · Linjuan Huang

Received: 13 April 2012 / Revised: 13 June 2012 / Accepted: 25 June 2012 / Published online: 15 July 2012
© Springer Science+Business Media, LLC 2012

Abstract Mucin-type *O*-linked glycoproteins are known for regulating many aspects of cell activity but remains a challenge to detect under physiological conditions which is due to the diversity of *O*-glycosylation and the lack of universal method. Here a direct labeling strategy for *in situ* visualizing of mucin-type *O*-linked glycoproteins on living cells has been developed. The strategy utilizes the combination of metabolic engineering and chemical probing technologies. Treating cells with an unnatural sugar, 2-keto Ac_4GalNAc analogue (2-keto isostere of GalNAc) to generate keto groups upon cells, followed by chemoselective ligation of keto groups on cells with a fluorescent tag, fluorescein-5-thiosemicarbazide (FTSC), provides a promising platform to probing mucin-type *O*-glycosylation on living cells. The FTSC conjugates illustrated very similar fluorescent spectra as FITC, a fluorescent tag widely used in proteomics, indicating good compatibility with commonly used fluorescent equipments. The established method eliminated the need of an additional fluorescent amplification step. Cells after being treated with the method maintained a rather high level of viability of 84.3 %. Finally, the assay has been successfully applied to image the expression of mucin-type *O*-linked glycoproteins within CHO and HeLa cells.

Y. Zhang · Y. Sun · Z. Wang · L. Huang
Key Laboratory of Resource Biology and Biotechnology in Western China, Ministry of Education, College of Life Science, Northwest University,
Xi'an 710069, People's Republic of China

Z. Wang (✉) · L. Huang (✉)
College of Life Sciences, Northwest University,
229 Northern Taibai Road,
Xi'an 710069, People's Republic of China
e-mail: wangzhf@nwu.edu.cn
e-mail: huanglj@nwu.edu.cn

Keywords Fluorescein-5-thiosemicarbazide (FTSC) · Mucin-type *O*-glycosylation · Imaging · Living cells · 2-keto Ac_4GalNAc analogue

Abbreviations

FTSC	Fluorescein-5-thiosemicarbazide
DAPI	4′,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
keto sugar	2-keto Ac_4GalNAc analogue
FITC	Fluorescein isothiocyanate
ESI-MS	Electrospray ionization mass spectrometry
LC-MS	Liquid chromatography mass spectrometry
HPLC	High performance liquid chromatography

Introduction

Glycosylations, which are estimated to comprise 50 % of eukaryotic proteomes, play important roles in many biological processes [1, 2]. However, for most glycoproteins, the types of glycans attached to the polypeptide have not been defined. Usually, there are two major forms of glycosylation on proteins, *N*-linked (attached to Asn residue) and *O*-linked (attached to Ser/Thr residue) [3]. The *N*-linked glycans precursor are installed on protein by a single enzyme, oligosaccharyl transferase, which could transfer glycan to a well-known consensus sequence (Asn-X-Ser/Thr, X denotes any amino acid residue but proline) on protein [4]. This natural feature thus enables prediction of *N*-glycosylation by sequencing of protein or site-directed mutagenesis [5]. Whereas, *O*-linked glycosylations are initiated in the Golgi compartment by a family of polypeptide *N*-acetyl-α-galactosaminyl transferases (ppGalNAcTs, about 20 in human, 23 in mouse, 15 in *Drosophila*, and 9 in *Caenorhabditis*) [6–8], and no consensus recognition sequence for predicting the *O*-glycosylation makes prediction of *O*-glycosylation difficult

[9, 10]. But as attracted by the important biological role of mucin-type *O*-glycoproteins within cells [9], there is urge to develop prediction methods to bridge the gap between the large number of glycosylation on protein and systematically investigation with regard to glycosylation status [11].

Fortunately, the unnatural substrate tolerance of enzymes involved in carbohydrate biosynthesis pathways has been exploited to identifying mucin-type *O*-glycosylations through the incorporation of detectable reporters [1, 3, 12]. Sugar analogues bearing reactive functional groups (azide, keto or alkyne group) [13–15], which could be metabolically incorporated into the target glycoprotein, provides a promising platform to investigate mucin-type *O*-linked glycosylations at system or cell levels by various chemical ligations towards the reporter groups [13, 16–19]. However, the approaches mentioned above have suffered from some drawbacks, such as, for example, slow reaction kinetics of Staudinger-Bertozzi ligation [20], toxicity of the Cu(I) catalyzed azide-alkyne condensation (*i.e.*, click chemistry) [13] or strain-promoted [3+2] azide-alkyne cycloaddition against living cells [13, 19, 21]. Nevertheless, one chemoselective ligation between a ketone and amino or hydrazide residues, has facilitated the identification of mucin-type *O*-linked glycosylation [5, 22–24]. Feeding cells with substrate analogues such as acetylated 2-keto isostere of GalNAc (2-keto Ac_4GalNAc analogue), which could be metabolically incorporated into the target mucin-type *O*-glycoproteins [16, 25], followed by a chemoselective ligation with amino/hydrazide biotin and stained with FITC-avidin, allows detecting of target mucin-type *O*-linked glycosylation [16]. But, a major limitation of all this method is the need of an additional signal amplification step to profile and image of target glycosylation, resulting in time consumption and tedious procedures [3, 26].

Fluorescein-5-thiosemicarbazide, a fluorescent tag, proved friendly to living cells and illustrated idea fluorescent properties as fabulous fluorescein isothiocyanate (FITC) widely used in proteomics, could reacted with aldehydes or ketones under physiological conditions [12, 27–29]. In our prior work, we have successfully exploited FTSC as a fluorescent labelling tag for imaging of polysaccharides with reducing ends in living cells [30]. The strength of intrinsic reaction activity of FTSC towards 2-keto Ac_4GalNAc analogues inspires us to develop an innovative strategy for imaging of mucin-type *O*-linked glycosylation within living cells. The method combines the metabolic engineering strategy and a subsequent chemoselective ligation. A schematic procedure for imaging of sialylated glycoproteins on living cells is depicted in Fig. 1. Treating cells with 2-keto Ac_4GalNAc analogue to generate ketones upon cells, followed by fluorescent labelling of ketones with FTSC under physiological conditions [12, 27, 28], could facilitate imaging studies of mucin-type *O*-linked glycosylation within cells. The evaluation and development of the strategy has been described as below.

Material and methods

Materials

Fluorescein-5-thiosemicarbazide (FTSC), trypan blue, hydrazide-biotin and avidin-FITC from egg white and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), ammonium acetate and glacial acetic acid were obtained from Third Chemical Company of Tianjin (Tianjin, China). Acetonitrile and methanol were from Fisher Scientific Co (HPLC grade, USA). Milli-Q water (Millipore, Milford, MA, USA) was used as solvent. HeLa and CHO cells were kindly provided by Professor Jianli Liu at College of Life Science, Northwest University, PR China. RPMI-1640 and fetal bovine serum were the products of Gibco BRL (Gaithersburg, MD). All other chemicals were of analytical grade.

2-keto Ac_4GalNAc analogue was synthesized in our lab. Glycal was initially synthesized from natural sugar (Gal) through three simple reactions [31, 32]. Iodoacetoxylation of Glycal to afford the key intermediate, 2-iodo sugar, using NIS as the iodo donor. Substitution reaction of 2-iodo sugar with methallyltributyltin, followed by reaction with OsO_4-$NaIO_4$ to form final product, 2-keto Ac_4GalNAc analogue [33].

Apparatus

Image profiles were performed under a Fluoview FV1000 confocal microscope (Olympus, Tokyo, Japan) equipped with a FV10-MCPSU instrument and 405, 458, 488 and 568 nm lines of a krypton/argon laser, using Laser Sharp 2000 software (Bio-Rad Laboratories). Images were further processed by FV10-ASW software. Flow cytometry was measured on a FACSCalibur (BD Biosciences, Mountain View, CA) equipped with an argon laser (488 nm). For each measurement, about 10^4 cells were counted.

Cell culture

Cells were cultured in RPMI-1640 supplemented with 10 % FBS (fetal bovine serum), 100 U/mL penicillin, and 0.10 mg/mL streptomycin at 37 °C under 5 % CO_2. CHO or HeLa cells (about 1×10^3/per well) were seeded on glass-bottom cell culture dish special for confocal imaging use (35 mm, MatTek) and cultured in feeding medium in the presence or absence of 2-keto Ac_4GalNAc analogues (50 μM) for 2 days [25].

ESI-MS and LC-MS conditions

The LC-MS equipment consisted of a Surveyor LC pump, a Surveyor auto-sampler, a Photo Diode Array Spectrophotometer (PDA) detector plus, a Thermo Scientific LTQ XL

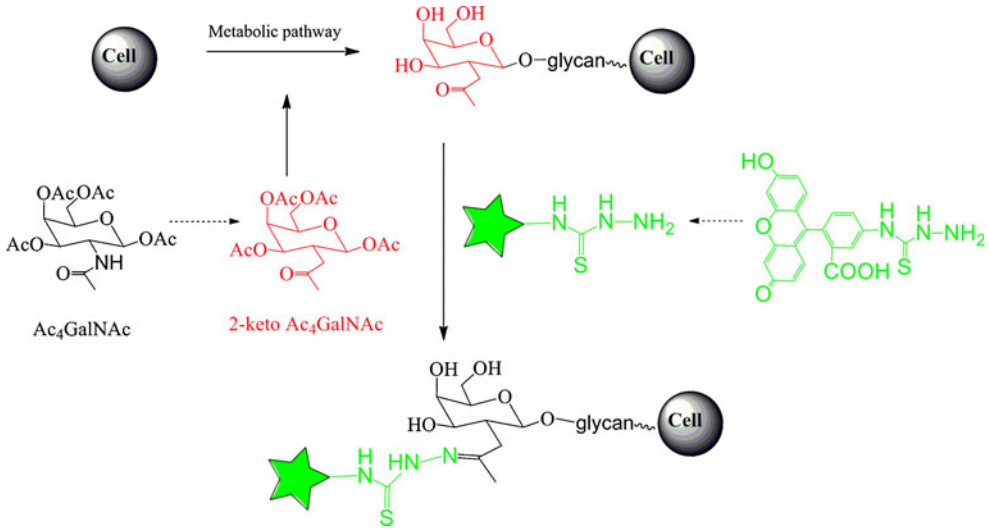

Fig. 1 Metabolic labelling-based FTSC ligation for fluorescence visualization of mucin-type *O*-linked glycoprotein within living cells

ion-trap mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA) equipped with an ESI ion source. Separation was obtained on a Waters RP-C18 column (5 μm, 150 mm×4.6 mm, Waters, Milford, MA, USA) with a PDA detector at a wavelength of 490 nm. Eluent A was 0.01 mol/L ammonium acetate buffer solution (pH=4.5) and eluent B was acetonitrile. A linear gradient (90–75 % eluent B in 30 min) at a flow rate of 1.0 mL/min was used for eluation. The column temperature was maintained at 25 °C. The injection volume of sample was 10 μL every time. Eluate from the column was introduced to ESI source at a post-column split ratio of 1:8 with a T-type valve when HPLC and ESI-MS were combined.

ESI-MS data were obtained using a Thermo Scientific LTQXL ion-trap mass spectrometer in positive-ion mode, with following conditions: sheath gas flow rate, 40.0 arb; auxillary gas flow rate, 4.5 arb; spray voltage, 5 kV. The heated capillary and voltage were maintained at 350 °C and 48 V, respectively. The full-scan mass spectra from *m/z* 150–1500 were recorded with a scan speed of 3 scans/s.

Conjugation of FTSC with 2-keto Ac_4GalNAc analogue

FTSC was dissolved in PBS buffer (pH 7.0, 100 μM) as standard solution. Standard solution of 2-keto Ac_4GalNAc analogue was prepared in DMSO (100 μM). To a 2 mL glass tube containing 1 μL of 2-keto Ac_4GalNAc solution, 5 μL of FTSC standard solution buffer was added. The tube was shaken briefly, sealed, and heated at a water bath at 37 °C for 3 h. The conjugations of FTSC with 2-keto Ac_4GalNAc were characterized by ESI-MS. The effects of different reaction time on the conjugation reaction were also investigated. Four tubes containing reaction buffer were shaken briefly, sealed, and heated at a water bath at 37 °C for 0.25, 0.5, 1, 2, 3 and 4 h, respectively. The amounts of 2-keto Ac_4GalNAc-FTSC derivatives were detected and measured by LC-MS and on-line ESI-MS.

FTSC and DAPI staining

The appearances of glycosylation-associated ketones were detected by a FTSC ligation with minor modifications [27–29]. Briefly, the 2-keto Ac_4GalNAc-treated cells were fixed and subjected to chemoselective ligation with FTSC dissolved in PBS buffer (37 °C, pH 7.0, 100 μM, 1 h). After reaction was completed, the FTSC buffer was totally removed. Then the cells were washed thoroughly with cold PBS buffer three times to wash away the unattached FTSC and free 2-keto Ac_4GalNAc analogue. After that, cell nuclei were stained with DAPI according to the instructions of product. Then the treated cells were merged in PBS buffer and imaged under a Fluoview FV1000 confocal microscope.

Fluorescence spectral studies

The fluorescent emission spectra of the sample were recorded on a FL-4600 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Samples were dissolved in PBS (phosphate buffered solution, pH=7.5) at concentration of 0.01×10^{-6} mol/L. Working solution of each sample was prepared by diluting corresponding standard solution with PBS buffer solution.

Specificity of the established method

CHO, a cell line which could deliver 2-keto Ac_4GalNAc analogue to mucin-type *O*-linked glycoconjugates on cell surfaces [25], was chosen as model cells. Four groups of cells were cultured on glass plates specially for confocal laser (a density of about 1×10^3 per well). One group of cells was cultured in presence of 2-keto Ac_4GalNAc analogues. Other two groups of cells were subjected to FTSC ligation (100 μM, pH 7.0, 37 °C, for 60 min) after incubation in absence or presence of 2-keto Ac_4GalNAc analogue. While

cells treated with medium only were used as control group. Nuclei of cells were stained with DAPI according producer's instruction book. Subsequently, the prepared cells were imaged under a Fluoview FV1000 confocal microscope. After imaging, the treated cells were subjected to flow cytometric quantification on a FACSCalibur.

Evaluation of cell viability

The viability of CHO cells treated with incubation of 2-keto Ac_4GalNAc analogue (50 μM, 2 day) and/or 100 μM FTSC (in PBS, pH 7.0 at 37 °C) for different time course was determined by counting live cells in a haemocytometer under a microscope by trypan blue exclusion (n=3) [34]. Briefly, the CHO cells were seeded in 6-well plate at a density of 1×10^3 per well (n=3). The cells were cultured in absence or presence of 2-keto Ac_4GalNAc analogue (50 μM, 2 days) and/or 100 μM FTSC (in PBS, pH 7.0 at 37 °C) for different time course (0.5, 1 and 2 h). When the reactions were completed, the reaction buffer solutions were removed from the dash and the culture medium was added to the cells. The cells were digested and then trypan blue was added into medium per well and gently stirred for a while (3–5 min). Then the viability of treated cells was determined by counting live cells in a haemocytometer under a microscope by trypan blue exclusion (n=3).

Application of the established method to image of mucin-type *O*-linked glycosylation within living cells

CHO and HeLa cells were seeded on glass plates, which are special for confocal laser (a density of about 1×10^3 per well) and cultured in presence of 2-keto Ac_4GalNAc analogue (50 μM, 2 days). After that, cells were washed with PBS to remove the excess unnatural keto sugar substrate. Then cells were fixed, and labelled with FTSC (100 μM FTSC in PBS, pH 7.0 at 37 °C) for 1 h. Nuclei were stained with DAPI according to the instruction of product. Then the cells were merged in PBS buffer solution and subjected to confocal microscopy analysis.

Results

This strategy involves the incubation cells with unusual substrate to generate a chemical reporter group (keto), followed by a fluorescent staining step, as shown schematically in Fig. 1. Chemical reporter, 2-keto Ac_4GalNAc analogue were introduced into mucin-type *O*-glycosylation residues using the cell's own biosynthetic machinery, and then chemoselectively conjugated with a commercially available fluorescent probe, FTSC, under physiological condition [12, 29]. FTSC, which has bright green fluorescence and desirable compatibility with commonly used fluorescence equipments [30], exhibited none obvious cytotoxicity against living cells [30]. Since the aldehydes and/or ketones are usually absent from cells [35], thus the strategy provides a promising platform to imaging of mucin-type *O*-glycosylation within cells.

ESI-MS analysis of FTSC ligation with 2-keto Ac_4GalNAc analogue

The ligation of 2-keto Ac_4GalNAc with FTSC was carried out *via* a condensation reaction, in which the thiosemicarbazide group of FTSC was covalently conjugated to ketone group of 2-keto Ac_4GalNAc analogue to afford stable thiosemicarbazones [12, 27], as depicted in Fig. 2a.

To investigate whether the ketone group at 2-keto Ac_4GalNAc analogue could conjugate with FTSC *in vivo*, 2-keto Ac_4GalNAc analogue was incubated with FTSC under physiological conditions, and the products were characterized by ESI-MS in positive ion mode. As shown in Fig. 2b, signal peak at *m/z* 792.22, 814.12 and 836.17, respectively, were assigned to the 2-keto Ac_4GalNAc-FTSC thiosemicarbazones in $[M+H]^+$, $[M+Na]^+$ and $[M+2Na]^+$ ion forms (M denotes molecular weight of 2-keto Ac_4GalNAc-FTSC thiosemicarbazones), exactly consistent with its structure in Fig. 2a. Lower signal peak at *m/z* 422.11 observed in the profile, is due to the presence of low level of the free FTSC. No other byproduct including native 2-keto Ac_4GalNAc analogue was observed in the map. Data demonstrated that the fluorescent labelling of 2-keto Ac_4GalNAc analogue with FTSC could be achieved under physiological conditions, presenting its potential in living cells.

Optimization of condensation reaction of FTSC towards 2-keto Ac_4GalNAc analogues

In our previous study, the condensation reaction of FTSC with aldehydes could be completed with 20 min. However, the condensation reaction of FTSC towards 2-keto Ac_4GalNAc analogue might be harder and slower than that of aldehydes because of steric hindrance. Although the condensation of FTSC and 2-keto Ac_4GalNAc analogue by condensation reaction shows great potential, as detected by ESI-MS, it may require further optimization with concern of reaction time to become compatible with *in vivo* imaging. The condensations of FTSC with 2-keto Ac_4GalNAc analogue under different time periods (0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 h) were analyzed by HPLC.

As shown in Fig. 2c, the peak areas of the FTSC labeled 2-keto Ac_4GalNAc analogue increased by the prolongation of reaction time up to 1 h, but did not change much thereafter. Taking into account the reaction yield and reaction time consumption, a time course of 1 h proved suitable for

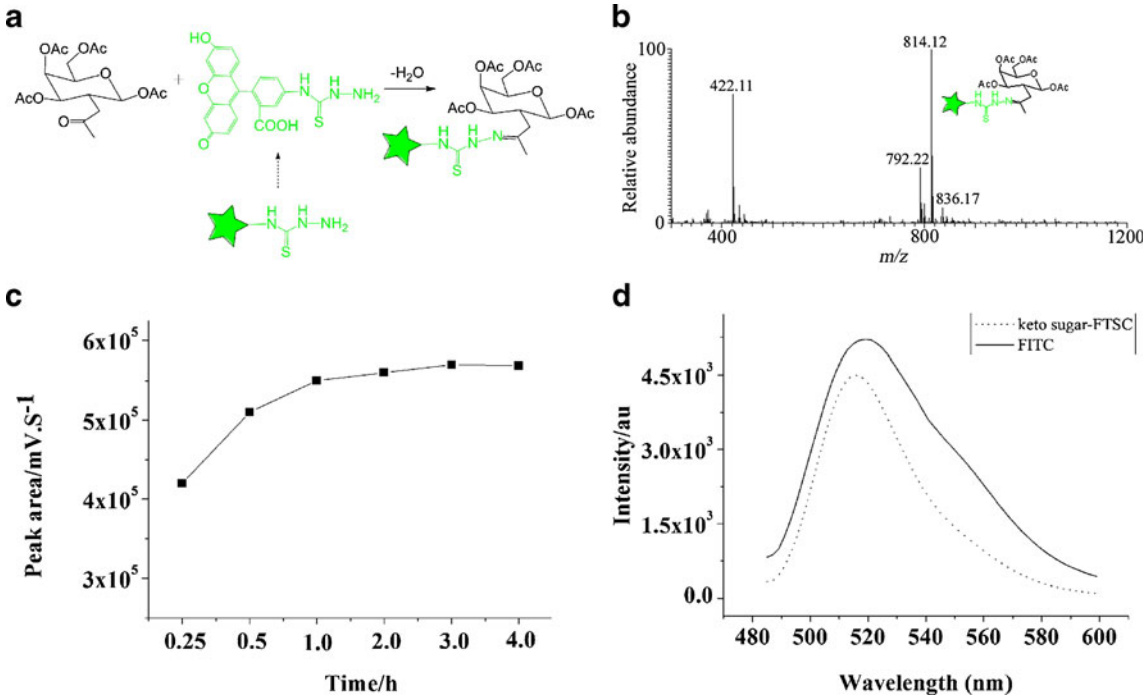

Fig. 2 **a** Condensation reaction of FTSC towards 2-keto Ac_4GalNAc analogue; **b** 2-keto Ac_4GalNAc analogue-FTSC zones analyzed by ESI-MS; **c** Effects of reaction time on reaction yields of FTSC with 2-keto Ac_4GalNAc under physiological condition; **d** Fluorescent emission profile of 2-keto Ac_4GalNAc analogue-FTSC zones and FITC

FTSC ligation towards ketone group of 2-keto Ac_4GalNAc analogue under physiological condition.

Fluorescence spectral studies

The emission fluorescence spectra of 2-keto Ac_4GalNAc analogue-FTSC zones had been recorded as clearly shown in Fig. 2d. At a excitation wavelength of 495 nm, the fluorescence emission maxima of free FITC tag and 2-keto Ac_4GalNAc analogue-FTSC derivatives were 523 and 518 nm, respectively. Fluorescent profile indicated that conjugation of the FTSC to 2-keto Ac_4GalNAc analogue did not change its spectral properties. Data shown that fluorescence properties of FTSC labeled 2-keto Ac_4GalNAc analogue was very similar with the widely used fluorescent tag FITC, demonstrating that the FTSC tag has its desirable compatibility with commonly used fluorescence equipments and potential application to imaging of 2-keto Ac_4GalNAc analogues.

Specificity of the established method

To illustrate the specificity of the method, CHO cells, a cell line with high activity of incorporation of unusual substrate, 2-keto Ac_4GalNAc analogue, into its *O*-glycosylation glycoprotein using biosynthesis pathway, was chosen. Cells were subjected to the designed strategy, incubation with 2-keto Ac_4GalNAc analogue, followed by FTSC ligation. While cells treated with medium, 2-keto Ac_4GalNAc analogue incubation and FTSC ligation only, were used as controls. Then the fluorescent signals on treated cells were recorded under two-photo laser scanning microscope. As shown in Fig. 3a, by co-localization imaging with DAPI (blue, nuclei specific dye), CHO cells treated with medium and 2-keto Ac_4GalNAc analogue incubation only, displayed no signals of fluorescence, while cells treated with FTSC ligation only exhibited rather low level (background level) of fluorescent signal. As expected, cells treated with 2-keto Ac_4GalNAc analogue and FTSC incubation, illustrated bright green fluorescence signals (bottom right in Fig. 3a) within cells, presenting the potential of FTSC ligation towards 2-keto Ac_4GalNAc analogues within living cells. By co-localization with DAPI (blue, nucleus staining), cells exhibited bright green fluorescence signals not only on cell surface, but also inside of cells, in agreement with a previous report [25, 36, 37]. Lack of the green fluorescence signals on medium, 2-keto Ac_4GalNAc analogue or FTSC ligation treated cells revealed that the established method has specificity to image of mucin-type *O*-glycosylation glycoprotein within living cells.

After imaging by confocal microscopy, the treated CHO cells were subjected to flow cytometric quantifications (n=3, see Fig. 3b). Medium, 2-keto Ac_4GalNAc analogue or FTSC ligation-treated CHO cells shown only background levels of fluorescence (FI=4.9, 4.6 and 18.7), whereas 2-keto Ac_4GalNAc analogue and FTSC staining-treated CHO cells exhibited remarkable high level of fluorescent

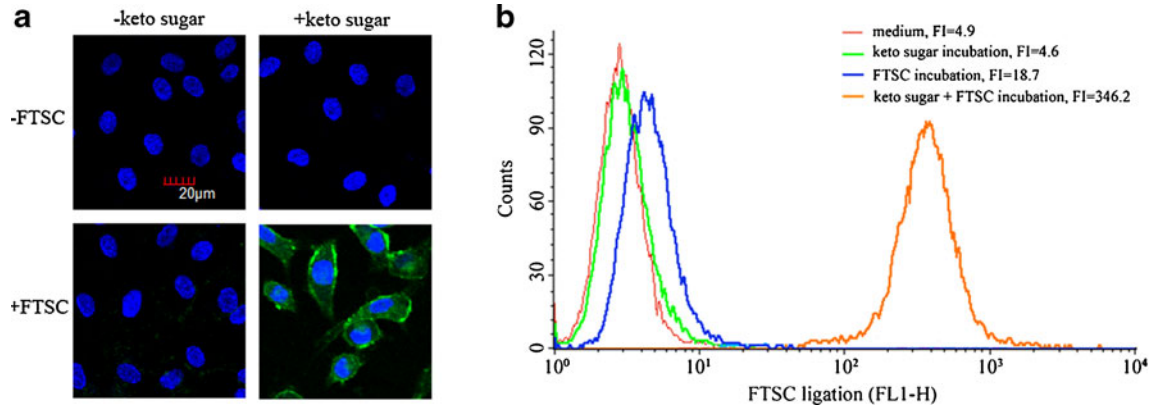

Fig. 3 Imaging of untreated and 2-keto Ac_4GalNAc analogue-incubated (50 μM, 2 days) CHO cells subjected to ligation with FTSC (100 μM, 37 °C, 1 h) or not by two-photo laser scanning (**a**) and (**b**) Flow cytometric quantification of fluorescent intensity of untreated and 2-keto GalNAc analogue-incubated (50 μM, 2 days) CHO cells subjected to ligation with FTSC (100 μM, 37 °C, 1 h) or not. The cells treated with medium, 2-keto Ac_4GalNAc analogue incubation and FTSC ligation only, were used as controls

intensity (FI) increases (FI=346.2, when compared to controls). Data got by flow cytometric quantification further confirmed the high specificity of the strategy towards mucin-type *O*-linked glycoproteins *in vivo*, consistent with results obtained by confocal laser microscopy, further confirming the specificity of the method.

Cell viability analysis

Viability of CHO cells treated with 2-keto Ac_4GalNAc analogue incubation and/or FTSC ligation was determined by trypan blue exclusion test. As can be clearly seen from Fig. 4, compared to control group, the treated cells could maintain rather high level of viability rates during 2-keto Ac_4GalNAc analogue incubation or FTSC incubation only. The viability levels of CHO cells treated with 2-keto Ac_4GalNAc analogue incubation and FTSC ligation displayed a little bit of decrease and reduced with a time-dependence manner. However, upon incubation of 2-keto Ac_4GalNAc analogue treated-cells with FTSC ligation at a time course of 1 h, the cells maintained a rather high level of 84.2 %, demonstrating a time course of 1 h was suitable for FTSC ligation towards keto of 2-keto Ac_4GalNAc analogues within living cells. Data showed that the established assay, which exhibited slight impact on cell viability, would be appropriate for imaging of mucin-type *O*-glycoproteins within living cells.

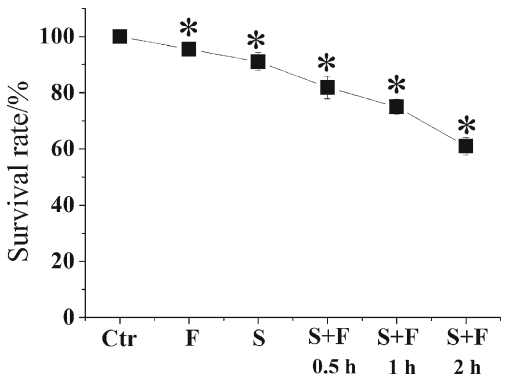

Fig. 4 Viability analysis of CHO cells treated with 2-keto GalNAc analogue incubation and/or FTSC ligation for different time periods by trypan blue exclusion test. F, FTSC ligation (pH 7.0, 100 μM, 37 °C); S, 2-keto GalNAc analogue incubation (50 μM, 2 days); S+F, 2-keto GalNAc analogue-treated CHO cells subjected to ligation with FTSC under different reaction time courses (0.5, 1 and 2 h). The viability values are expressed as percent of control, comprising CHO cells maintained in feeding medium, pH 7.4, for 2 days, 37 °C. * denotes $p<0.05$

Application of method to image of mucin-type *O*-linked glycoprotein within living cells

To illustrate the potential of the method for *in vivo* imaging the expression of mucin-type *O*-linked glycoproteins, two cell lines, CHO and HeLa cells, were treated with assay and further imaged under confocal laser microscopy. To examine the sub-cellular location of mucin-type *O*-linked glycoproteins, a dye specific for cell nucleus staining and locating (DAPI), was employed in co-localization study. As shown in Fig. 5, bright green fluorescent signals (middle panel) were found in both the membrane, nucleus and cytoplasm of two cell lines by co-localization with DAPI (left panel) under two-photo laser scanning. Overlapping of the fluorescent images (right panel) arising from FTSC ligation and DAPI displayed zonal co-localizations of the target mucin-type *O*-linked glycoproteins on cell surface, cytoplasm and nucleus of two cells. However, the fluorescent intensity of mucin-type *O*-linked glycoprotein residues on CHO cells showed a little higher level over that of HeLa cells, consistent with reference [25]. Images of fluorescence profiles indicated that the method has potential application to image of mucin-type *O*-linked glycoproteins within cells. The ability of method to selectively image and locate bioactive molecules, mucin-type *O*-linked glycoproteins within cells will help elucidate the functions and mechanisms of *O*-glycoprotein, as required for glycomics.

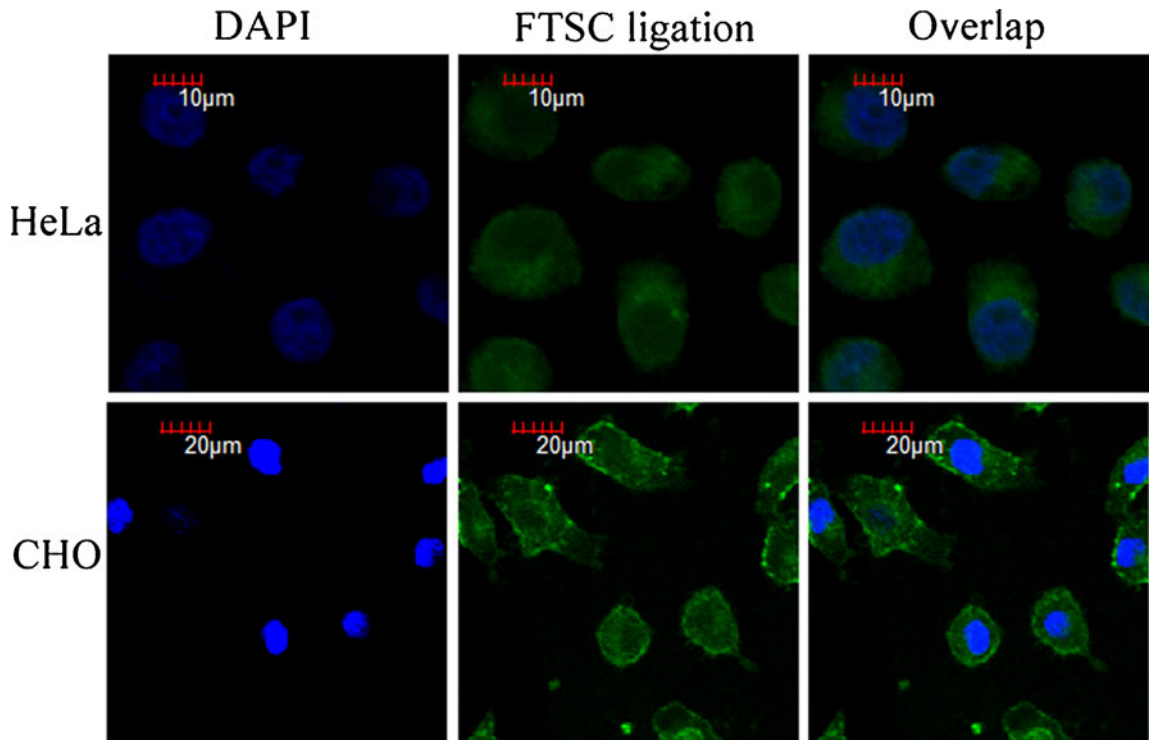

Fig. 5 Confocal microscopy images of the CHO and HeLa cells subjected to the established assay. Nucleus was stained with DAPI (blue, nuclei specific dye). Left panel, cellular localization of cell nuclei; Middle panel, cellular localization of mucin-type *O*-linked glycoproteins; Right panel, overlap of the two images (left and middle panels)

Discussions

Our results have demonstrated the development of an innovative strategy, which combined the oligosaccharide metabolic engineering and a chemoselevtive ligation, for selectively imaging of mucin-type *O*-linked glycoprotein within living cells. Treating cells with 2-keto sugar (2-keto Ac_4GalNAc analogue) incubation, followed by staining with a commercially available fluorescent tag (FTSC), permits selectively imaging of mucin-type *O*-linked glycoproeins within living cells. FTSC, which contains a thiosemicarbazide group, could react with ketones to form stable thiosemicarbazones under physiological conditions. The keto sugar-FTSC conjugates illustrated very similar fluorescent spectra as FITC, a fluorescent tag widely used in proteomics, indicating its potential compatibility with commonly used fluorescent equipments. Cells after treated with the method maintained a rather high level of viability (84.3 %).

Our studies also included a comparison between our assay and previous labeling method [16]. Results indicated that imaging profile of established-method treated CHO cells was very similar to that treated by previous method, indicating that our assay has potential to imaging of mucin-type *O*-linked glycosylation on living cells. Compared to previous method, the established assay eliminated the need of an additional fluorescent amplification step and employed a cheaper fluorescent tag (FTSC), which is commercially available. Results acquired from comparison studies illustrated that the established method makes the imaging of mucin-type *O*-linked glycoprotein within cells simpler, cheaper and faster than the previous report.

The method has been successfully applied to image the expression of mucin-type *O*-linked glycoproteins on CHO and HeLa cells. By *in vivo* imaging, expressions of mucin-type *O*-linked glycoproteins were found on cell surface, cytoplasm and nucleus. And the abundances of mucin-type *O*-linked glycoproteins on CHO cells was a little higher than that of HeLa cells, consistent with previous studies [25, 36, 37]. However, the mechanism of difference in expression abundance of mucin-type *O*-linked glycosylation between cells remains unknown.

The established assay for imaging of mucin-type *O*-linked glycoprotein within living cells possesses some advantages including: (i) Our method allows directly imaging of mucin-type *O*-linked glycoprotein, avoiding an additional signal amplification step; (ii) The FTSC conjugates, exhibited similar fluorescence spectra as widely used fluorescent tag FITC, presenting good compatibility with commonly used fluorescent equipments in proteomics study; (iii) FTSC ligation shows high chemoselective ligation reactivity towards ketones to form stable thiosemicarbazones under physiological conditions; (iv) High reproducibility and simplicity of the assay make it suitable for imaging of mucin-type *O*-linked glycoproteins on any type of cells.

Acknowledgments This work was supported by National Natural Science Foundation of China (NSFC) (30870548, 31071506, 31170773), Foundation of Key Laboratory of Resource Biology and Biotechnology in Western China, Ministry of Education in Northwest University (KH09019) and Program for New Century Excellent Talents in Universities (NCET-08-0893).

References

1. Rabuka, D., Hubbard, S.C., Laughlin, S.T., Argade, S.P., Bertozzi, C.R.: A chemical reporter strategy to probe glycoprotein fucosylation. J. Am. Chem. Soc. **128**, 12078–12079 (2006)
2. Timmer, M.S.M., Stocker, B.L., Seeberger, P.H.: Probing glycomics. Curr. Opin. Chem. Biol. **11**, 59–65 (2007)
3. Prescher, J.A., Bertozzi, C.R.: Chemical technologies for probing glycans. Cell **126**, 851–854 (2006)
4. Dempski Jr., R.E., Imperiali, B.: Oligosaccharyl transferase: gatekeeper to the secretory pathway. Curr. Opin. Chem. Biol. **6**, 844–850 (2002)
5. Dube, D.H., Prescher, J.A., Quang, C.N., Bertozzi, C.R.: Probing mucin-type O-linked glycosylation in living animals. Proc. Natl. Acad. Sci. **103**, 4819–4824 (2006)
6. Ten Hagen, K.G., Fritz, T.A., Tabak, L.A.: All in the family: the UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferases. Glycobiology **13**, 1R–16R (2003)
7. Peng, C., Togayachi, A., Kwon, Y.-D., Xie, C., Wu, G., Zou, X., Sato, T., Ito, H., Tachibana, K., Kubota, T., Noce, T., Narimatsu, H., Zhang, Y.: Identification of a novel human UDP-GalNAc transferase with unique catalytic activity and expression profile. Biochem. Biophys. Res. Commun. **402**, 680–686 (2010)
8. Bennett, E.P., Mandel, U., Clausen, H., Gerken, T.A., Fritz, T.A., Tabak, L.A.: Control of mucin-type O-glycosylation: a classification of the polypeptide GalNAc-transferase gene family. Glycobiology **22**, 736–756 (2012)
9. Hang, H.C., Bertozzi, C.R.: The chemistry and biology of mucin-type O-linked glycosylation. Bioorgan. Med. Chem. **13**, 5021–5034 (2005)
10. Jung, E., Veuthey, A.-L., Gasteiger, E., Bairoch, A.: Annotation of glycoproteins in the SWISS-PROT database. Proteomics **1**, 262–268 (2001)
11. Vocadlo, D.J., Hang, H.C., Kim, E.-J., Hanover, J.A., Bertozzi, C.R.: A chemical approach for identifying O-GlcNAc-modified proteins in cells. Proc. Natl. Acad. Sci. **100**, 9116–9121 (2003)
12. Prescher, J.A., Bertozzi, C.R.: Chemistry in living systems. Nat. Chem. Biol. **1**, 13–21 (2005)
13. Hsu, T.-L., Hanson, S.R., Kishikawa, K., Wang, S.-K., Sawa, M., Wong, C.-H.: Alkynyl sugar analogs for the labeling and visualization of glycoconjugates in cells. Proc. Natl. Acad. Sci. **104**, 2614–2619 (2007)
14. Dehnert, K.W., Beahm, B.J., Huynh, T.T., Baskin, J.M., Laughlin, S.T., Wang, W., Wu, P., Amacher, S.L., Bertozzi, C.R.: Metabolic labeling of fucosylated glycans in developing zebrafish. ACS Chem. Biol. **6**, 547–552 (2011)
15. Chang, P.V., Dube, D.H., Sletten, E.M., Bertozzi, C.R.: A strategy for the selective imaging of glycans using caged metabolic precursors. J. Am. Chem. Soc. **132**, 9516–9518 (2010)
16. Mahal, L.K., Yarema, K.J., Bertozzi, C.R.: Engineering chemical reactivity on cell surfaces through oligosaccharide biosynthesis. Science **276**, 1125–1128 (1997)
17. Kayser, H., Zeitler, R., Kannicht, C., Grunow, D., Nuck, R., Reutter, W.: Biosynthesis of a nonphysiological sialic acid in different rat organs, using N-propanoyl-D-hexosamines as precursors. J. Biol. Chem. **267**, 16934–16938 (1992)
18. Keppler, O.T., Horstkorte, R., Pawlita, M., Schmidt, C., Reutter, W.: Biochemical engineering of the N-acyl side chain of sialic acid: biological implications. Glycobiology **11**, 11R–18R (2001)
19. Saxon, E., Bertozzi, C.R.: Cell surface engineering by a modified sStaudinger reaction. Science **287**, 2007–2010 (2000)
20. Hangauer, M.J., Bertozzi, C.R.: A FRET-based fluorogenic phosphine for live-cell imaging with the staudinger ligation. Angew. Chem. Int. Edit. **47**, 2394–2397 (2008)
21. Agard, N.J., Baskin, J.M., Prescher, J.A., Lo, A., Bertozzi, C.R.: A comparative study of bioorthogonal reactions with azides. ACS Chem. Biol. **1**, 644–648 (2006)
22. Chang, P.V., Prescher, J.A., Hangauer, M.J., Bertozzi, C.R.: Imaging cell surface glycans with bioorthogonal chemical reporters. J. Am. Chem. Soc. **129**, 8400–8401 (2007)
23. Hang, H.C., Yu, C., Kato, D.L., Bertozzi, C.R.: A metabolic labeling approach toward proteomic analysis of mucin-type O-linked glycosylation. Proc. Natl. Acad. Sci. **100**, 14846–14851 (2003)
24. Khidekel, N., Ficarro, S.B., Clark, P.M., Bryan, M.C., Swaney, D.L., Rexach, J.E., Sun, Y.E., Coon, J.J., Peters, E.C., Hsieh-Wilson, L.C.: Probing the dynamics of O-GlcNAc glycosylation in the brain using quantitative proteomics. Nat. Chem. Biol. **3**, 339–348 (2007)
25. Hang, H.C., Bertozzi, C.R.: Ketone isosteres of 2-N-acetamidosugars as substrates for metabolic cell surface engineering. J. Am. Chem. Soc. **123**, 1242–1243 (2001)
26. Tai, H.-C., Khidekel, N., Ficarro, S.B., Peters, E.C., Hsieh-Wilson, L.C.: Parallel identification of O-GlcNAc-modified proteins from cell lysates. J. Am. Chem. Soc. **126**, 10500–10501 (2004)
27. Zhang, Z., Smith, B.A.C., Wang, L., Brock, A., Cho, C., Schultz, P.G.: A new strategy for the site-specific modification of proteins *in vivo*. Biochemistry **42**, 6735–6746 (2003)
28. Sah, P.P.T., Daniels, T.C.: Thiosemicarbazide as a reagent for the identification of aldehydes, ketones, and quinones. Recueil des Travaux Chimiques des Pays-Bas. **69**, 1545–1556 (1950)
29. Tamilarasu, N., Zhang, J., Hwang, S., Rana, T.M.: A new strategy for site-specific protein modification: analysis of a tat peptide−TAR RNA interaction. Bioconjug. Chem. **12**, 135–138 (2001)
30. Zhang, Y., Wang, Z., Zhang, X., Zhou, W., Huang, L.: One-pot fluorescent labeling of saccharides with fluorescein-5-thiosemicarbazide for imaging polysaccharides transported in living cells. Carbohydr. Res. **346**, 2156–2164 (2011)
31. Lafont, D., Boullanger, P.: Syntheses of l-glucosamine donors for 1,2-trans-glycosylation reactions. Tetrahedron Asymmetry **17**, 3368–3379 (2006)
32. Zhao, J., Wei, S., Ma, X., Shao, H.: A simple and convenient method for the synthesis of pyranoid glycals. Carbohydr. Res. **345**, 168–171 (2010)
33. Cai, L., Guan, W., Chen, W., Wang, P.G.: Chemoenzymatic synthesis of uridine 5′-diphospho-2-acetonyl-2-deoxy-α-D-glucose as C_2-carbon isostere of UDP-GlcNAc. J. Org. Chem. **75**, 3492–3494 (2010)
34. Zeng, Y., Ramya, T.N.C., Dirksen, A., Dawson, P.E., Paulson, J.C.: High efficiency labeling of glycoproteins on living cells. Nat. Methods **6**, 207–209 (2009)
35. De Bank, P.A., Kellam, B., Kendall, D.A., Shakesheff, K.M.: Surface engineering of living myoblasts via selective periodate oxidation. Biotechnol. Bioeng. **81**, 800–808 (2003)
36. Boyce, M., Carrico, I.S., Ganguli, A.S., Yu, S.-H., Hangauer, M.J., Hubbard, S.C., Kohler, J.J., Bertozzi, C.R.: Metabolic cross-talk allows labeling of O-linked β-N-acetylglucosamine-modified proteins via the N-acetylgalactosamine salvage pathway. Proc. Natl. Acad. Sci. **108**, 3141–3146 (2011)
37. Clark, P.M., Dweck, J.F., Mason, D.E., Hart, C.R., Buck, S.B., Peters, E.C., Agnew, B.J., Hsieh-Wilson, L.C.: Direct in-gel fluorescence detection and cellular imaging of O-GlcNAc-modified proteins. J. Am. Chem. Soc. **130**, 11576–11577 (2008)