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Size-matched alkyne-conjugated cyanine fluorophores to identify differences in protein glycosylation

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Abstract

Currently, there are few methods to detect differences in protein post-translational modifications (PTMs) in a specific manner from complex mixtures. Thus, we developed an approach that combines the sensitivity and specificity of Click Chemistry with the resolution capabilities of 2D DIGE. In “Click-DIGE”, post-translationally modified proteins are metabolically labeled with azido-substrate analogs, then size- and charge-matched alkyne-Cy3 or alkyne-Cy5 dyes are covalently attached to the azide of the PTM by Click Chemistry. The fluorescently-tagged protein samples are then multiplexed for 2DE analysis. Whereas standard DIGE labels all proteins, Click-DIGE focuses the analysis of protein differences to a targeted subset of post-translationally modified proteins within a complex sample (i.e. specific labeling and analysis of azido-glycoproteins within a cell lysate). Our data indicate that 1) Click-DIGE specifically labels azido-proteins, 2) the resulting Cy-protein conjugates are spectrally distinct, and 3) the conjugates are size- and charge-matched at the level of 2DE. We demonstrate the utility of this approach by detecting multiple differentially expressed glycoproteins between a mutant cell line defective in UDP-Galactose transport and the parental cell line. We anticipate that the diversity of azido-substrates already available will enable Click-DIGE to be compatible with analysis of a wide range of PTMs.

Keywords

DIGE; Click Chemistry; glycosylation; post-translational modification; azido-GalNAc

Analysis of differences in protein expression between biological samples (e.g. cancerous tissue vs. healthy tissue) can be achieved by two-dimensional difference gel electrophoresis (DIGE) [1–3]. In DIGE, different complex protein samples are labeled with size- and charge-matched, spectrally distinct fluorescent dyes. Samples from one group are labeled with one dye (Cy3) while samples from the other group are labeled with a second dye (Cy5). Then a labeled sample from one group is mixed with a labeled sample from the second group, and the mixture is applied to a single two-dimensional gel, which separates proteins according to their mass and isoelectric point. Additional gels are run to analyze additional replicates from each sample group. Successive fluorescence imaging at the excitation

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Conflict of Interest Statement

The authors declare no conflicts of interest.

wavelengths of each of the two dyes enables identification of the proteins expressed in each sample. Thus, a difference in the fluorescence intensities at a specific coordinate within a 2D gel indicates that the protein at that coordinate is differently expressed between the two samples. To standardize comparison in multi-gel experiments, two-color DIGE workflows commonly include a labeled internal standard, which is prepared by pooling aliquots of all the samples and labeling the resulting mix with one of the fluorophores. The remainder of each sample is then labeled with a second fluorophore. The labeled internal standard is then added to each labeled individual sample. Each gel that is then run thus contains one sample and the internal standard. This facilitates the precise alignment of the protein patterns among multiple gels.

A fundamental aspect of any DIGE experiment is that size- and charge-matched Cy3 and Cy5 fluorescent dyes are used to ideally label every single protein present within each sample. To accomplish this, at least one lysine or cysteine residue of every protein is labeled via NHS-ester reactive or maleimide reactive Cy dyes, respectively [3] [4]. Here we describe the use of size- and charge-matched Cy dyes bearing an alkyne reactive group (Fig. 1). These dyes covalently label biological molecules carrying an azido group by forming an azide-alkyne linkage catalyzed by azide-alkyne cycloaddition (“Click Chemistry”).

Unlike NHS-ester or maleimide Cy dyes, which label all proteins within a sample, alkyne-functionalized Cy dyes specifically label only azido-proteins. Thus the key advantage of Click-DIGE over the existing DIGE approach is the ability to exquisitely focus the analysis of protein differences to a targeted subset of proteins within a complex sample (i.e. to analyze only azido-labeled glycoproteins within a total cell lysate) (Fig. 1A). These alkyne Cy dyes could be used for both the classic, two-color DIGE workflow, in which samples from one group are labeled with one dye while samples from the second group are labeled with the second dye, or in standardized, two-color workflows in which one dye labels the internal standard and the other dye labels the samples.

It is important to note that with the existing DIGE approach, a difference in a protein spot's Cy dye intensity between samples indicates a **protein expression** difference between samples. With Click-DIGE, a difference in a protein spot's Cy dye intensity between samples indicates a potential **protein post-translational modification** (i.e. glycosylation) difference between samples. In Click-DIGE, a difference in fluorescence intensity of a protein spot between samples may indicate 1) the protein is more extensively modified in one sample, or 2) the protein is modified equally in both samples, but one sample contains more copies of the modified protein. For PTMs in which proteins can be modified at multiple sites (i.e. O-GlcNAc or mucin-type glycosylation), a spot with different fluorescence between samples could also indicate a difference in the protein's PTM site occupancy between samples. Running both a classic DIGE and a Click-DIGE experiment would aid in distinguishing the source of the difference because the two techniques provide complementary information on protein expression and protein post-translational modifications, respectively.

Cy3 and Cy5 alkyne reagents have been described and used in a similar experimental approach[5, 6]. However both previously described Cy3 and Cy5 reagent sets differ in mass

by 26 Da, which is a mass difference large enough to cause dye-dependent differences in labeled protein size and thus result in a high false positive rate for differences between samples. This is particularly problematic for smaller proteins and for proteins with multiple labeling sites, such as glycoproteins, many of which are heavily modified with multiple monosaccharides. The Cy3 and Cy5 alkyne reagents used here (custom-synthesized by Lumiprobe (Hallandale Beach, FL)), differ in mass by only 2 Da (Fig. 1B), which is identical to the mass difference between Cy3 and Cy5 maleimide or NHS-ester reactive dyes used in the existing and well-established DIGE workflows. As a proof-of-principle of the utility of these alkyne-Cy dyes for analyzing proteins with multiple labeling sites, we applied these alkyne-Cy dyes to analyze differences in glycosylation between biological samples that were metabolically labeled with azido-sugars.

For alkyne-Cy3 and alkyne-Cy5 dyes to be useful, they must meet several criteria. First, they must react only with azido-labeled proteins and not with proteins lacking the azido-label. Second, proteins labeled with alkyne-Cy3 and proteins labeled with alkyne-Cy5 must have non-overlapping fluorescence excitation and emission profiles. Third, the two alkyne-fluorophores must generate size- and charge-matched protein conjugates.

We determined that alkyne-Cy3 and alkyne-Cy5 specifically tag azido-labeled proteins and are spectrally distinct by incubating a Chinese hamster ovary cell line (Pro5, obtained from the ATCC and maintained as recommended) with media containing 50 μ M of either the natural sugar N-acetylgalactosamine (GalNAc) or its azido-analog (azido-GalNAc, Life Technologies) for 48 hours. We and others have previously determined by mass spectrometry that azido-GalNAc is incorporated into O-GlcNAcylated proteins [7, 8]. Lysates were prepared and reacted with either alkyne-Cy3 or alkyne-Cy5 via Click Chemistry as described [7]. Mock reactions received water in place of the CuSO₄ catalyst solution. Samples were separated by SDS-PAGE, and gels were sequentially imaged with a Typhoon laser imager for Cy5 (633nm excitation; 670nm bandpass emission) and Cy3 (532nm excitation; 580nm bandpass emission). Proteins were electroblotted onto PVDF and post-stained with Sypro Ruby total protein stain to confirm protein loading within the lanes. Sypro Ruby staining was detected with a Bio-Rad VersaDoc 4000 imager (excitation 300nm, 520nm longpass emission).

The Cy3 and Cy5 fluorescence found in the azido-GalNAc samples was substantially greater than that found in either the Click Chemistry reacted GalNAc sample or the mock-reacted azido-GalNAc sample, indicating that alkyne-Cy3 and alkyne-Cy5 specifically label azido-proteins (Fig. 1C). Furthermore, the presence of robust Cy5 fluorescence only in Cy5-labeled sample lanes (and vice versa for Cy3) indicates that the Cy5 and Cy3 probe-protein conjugates have non-overlapping fluorescence profiles and can be differentially detected within a single gel.

The cyanine-conjugated alkynes used in this study are theoretically expected to give no background. The applicability of Click Chemistry stems from the selective reactivity of azides and alkynes only with each other, which makes them ideal bio-orthogonal reactive groups. Since its discovery in 2002 [9], copper-catalyzed azide-alkyne cycloaddition (Click Chemistry) has been widely used to profile enzymatic activity [10, 11] and to label nucleic

acids [12, 13], proteins [14, 15] and glycoconjugates [16]. Furthermore, because the functional group (i.e. alkyne) is the same in both dyes (alkyne-Cy3 and alkyne-Cy5), any non-specific, background labeling that may occur would be expected to occur equally. In our hands, we do observe a trace amount non-specific alkyne-Cy labeling in our samples whenever the alkyne-Cy dye is added, and no variation is apparent between the faint background of alkyne-Cy3 vs. alkyne-Cy5 (Fig. 1C, lanes 5 and 6 vs. 9 and 10). Importantly, because the purpose of the Click-DIGE approach is to identify differences in PTMs (i.e. alkyne-Cy dye labeling) between samples, we are looking for a change above background labeling in one of the samples. Thus these trace levels of background labeling should not be an issue for analysis.

To determine whether the two alkyne-fluorophores generate size- and charge-matched protein conjugates, we tested for co-migration in a 2D gel of Cy3-alkyne-labeled proteins with Cy5-alkyne-labeled proteins from the same lysate. CHO cells (Lec8) were incubated with azido-GalNAc as above. One aliquot of cell lysate was reacted via Click Chemistry with Cy3-alkyne, while another aliquot was reacted with Cy5-alkyne. Samples were resuspended in 8M urea, 2M thiourea, 1% CHAPS, 9 mg/ml DTT, 0.57% 3–10 ampholytes, 0.002% bromophenol blue, combined, and then applied to one 3–10NL 11cm IPG strip for 50min of passive rehydration then 12hrs of active rehydration at 50V. Isoelectric focusing was performed as follows: Rapid, 200V, 1hr; Rapid, 500V, 1hr; Rapid, 1000V, 1hr; Linear, 8000V, 2hrs; Rapid, 8000V, 20,000 Vhrs; Rapid, 500V, 30min. The strip was then equilibrated with DTT and iodoacetamide as described [17] then applied to an 8–16% Criterion gel (Bio-Rad). Sequential Cy3 and Cy5 fluorescence imaging was performed as above. The Cy3 and Cy5 fluorescence patterns overlaid each other, even for low mass proteins, indicating that at the resolution level of 2D gel electrophoresis, the alkyne-Cy3 and alkyne-Cy5 labeled protein conjugates are size- and charge-matched (Fig. 2).

As a final test of the utility of our Click-DIGE alkyne-Cy3 and alkyne-Cy5 reagents and the Click-DIGE technique, we tested whether we could use this approach to detect differently glycosylated proteins between two samples with known glycosylation differences. We used Lec8, a CHO cell line with glycosylation defects due to the inability to translocate UDP-galactose into the lumen of the Golgi apparatus [18] and Pro5, the parental cell line. We incubated Lec8 and Pro5 cells with azido-GalNAc and used Click Chemistry to react one aliquot of each lysate with alkyne-Cy3 and a second aliquot of each lysate with alkyne-Cy5. Differences in the fluorescence patterns between the Pro5 and Lec8 samples indicated that the Click-DIGE reagents could indeed detect differences in the azido-modified glycoproteins between the two different cell lines (Fig. 3, *e.g.* Cy3 fluorescence lane 2 vs. 3, Cy5 fluorescence lane 1 vs. 2, as well as red and green bands within lanes 2 and 3 and the all-yellow bands in lanes 1 and 4 of the merged image).

In sum, the Click-DIGE approach differs from traditional DIGE because it enables the specific detection of azide-tagged protein subsets within complex mixtures and thereby facilitates the analysis of differences in specific classes of proteins across multiple complex samples. The alkyne-Cy dye reagents we apply here are specific, spectrally distinct, and well-matched in charge and molecular weight, which makes them ideally suited for use in Click-DIGE experiments. Furthermore, we demonstrate the practical utility of these alkyne-

Cy dyes and the Click-DIGE approach by detecting differences in glycosylation between two biological samples (a glycosylation-defective mammalian cell line vs. its glycosylation-competent parental cell line).

In the future, we envision that Click-DIGE performed with these size- and charge-matched alkyne-Cy dyes and with the diversity of azido-substrates already commercially available will enable the specific detection of multiple classes of post-translationally modified proteins. In addition to glycoproteins, azido-analogs have also been used to label farnesylated proteins [19], proteins modified with ω -azido fatty acids (N-myristoylation and S-acylation) [20], and newly synthesized proteins [21]. Thus, in addition to detecting differences in the glycoprotein profile between two cell lines as we have done, alkyne-Cy dyes and the Click-DIGE technique should enable researchers i) to identify the protein targets of glycosyltransferases or other transferases responsible for adding post-translational modifications (PTMs) to protein targets by metabolically labeling mutant mammalian cell lines or primary cell cultures generated from various mutants (e.g. *C. elegans* glycosylation mutants [7]), ii) to compare the glycoprotein profile of cancerous tissue to healthy tissue, for example by using azido-sugars to metabolically label mice with tumors *in vivo* via established *in vivo* azido-labeling protocols [6, 22] or iii) to detect changes PTMs in response to an environmental stimulus by azido-substrate pulse-chase experiments. Overall, we anticipate that the use of size- and charge-matched alkyne-Cy dyes for Click-DIGE will be broadly useful to the research community for the identification and comparison between samples of specific subsets of proteins or classes of PTMs.

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Abbreviations

Cy	cyanine
GalNAc	N-acetylgalactosamine
PTM	post-translational modification

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Figure 1.

Alkyne-Cy dyes enable specific labeling and detection of azido-labeled proteins. A) Schematic depiction of the Click-DIGE concept. B) Alkyne-Cy3 and alkyne-Cy5 have the same charge and differ in molecular weight by only 2Da. C) Pro5 cells were metabolically labeled for 48hrs with 50 μ M of GalNAc or azido-GalNAc. Lysates were reacted (or not reacted; “NA”) via Click Chemistry with or without copper catalyst in the presence or absence of alkyne-Cy dye. Fluorescence was detected by sequential imaging. Protein

loading was verified by transferring proteins to PVDF and post-staining with Sypro Ruby total protein.

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Figure 2.

Alkyne-Cy3 and alkyne-Cy5 protein conjugates are size- and charge-matched at the resolution of 2DE. Lec8 CHO cells were metabolically labeled with 50 μ M azido-GalNAc for 48hrs. One aliquot of lysate was reacted via Click Chemistry with alkyne-Cy3; another aliquot was reacted with alkyne-Cy5. Both labeled lysates were combined into one 2D gel. Fluorescence was detected by sequential imaging.

Figure 3.

Click-DIGE reagents can detect differences in azido-modified glycoproteins between cell lines with vs. without glycosylation defects. Lec8 (mutant) and Pro5 (parental) cells were metabolically labeled for 48hrs with 50 μ M GalNAc or azido-GalNAc. Lysates were reacted via Click Chemistry with the given alkyne-Cy dye. Equal amounts of Cy3- and Cy5-labeled lysates of the specified genotype were combined and analyzed by SDS-PAGE. Fluorescence was detected by sequential imaging.