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Quantitative Glycomics of Cultured Cells Using Isotopic Detection of Aminosugars with Glutamine (IDAWG)

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Abstract

IDAWG (Isotopic Detection of Aminosugars With Glutamine) is a newly reported, in vivo, stable isotopic labeling strategy for quantitative glycomics of cultured cells. Detailed procedures are provided for glycan analysis using IDAWG including labeling, release of both N- and O-linked glycans, permethylation, and mass spectrometry analysis. The methods for data processing and calculations are also introduced here but have not yet been automated.

Keywords

IDAWG; stable isotopic labeling; quantitative glycomics; cell culture; glycan analysis

INTRODUCTION

Glycomics is the comprehensive study of the entire complement of complex carbohydrates, often called glycans, that are polymers of monosaccharides often found attached to proteins and lipids (Aoki-Kinoshita, 2008). As a post-translational modification, glycosylation plays critical roles in numerous physiological processes including protein folding and cellular signaling, and altered carbohydrate expression is a common feature of many types of cancers (Dube and Bertozzi, 2005). One of the important tasks for glycomics is to develop technologies capable of comparative, relative-quantitative analysis for examining global alterations of glycans between different biological samples. Following the steps of proteomics, several non-isotope-based and stable-isotope-based labeling strategies have recently been developed for quantitative glycomics by mass spectrometry (Alvarez-Manilla et al., 2007; Aoki et al., 2007; Kang et al., 2007).

This unit provides detailed protocols for Isotopic Detection of Aminosugars With Glutamine, termed IDAWG, which is the first in vivo cell culture isotope-labeling strategy for glycan analysis (Orlando et al., 2009). The methods of labeling the aminosugars (GlcNAc, GalNAc, and sialic acid) with amide-¹⁵N-Gln in cell culture is described in Basic Protocol 1, and the detection of these aminosugars in both N- and O-linked glycan structures is described in Basic Protocol 2. The mathematical calculations used to calculate under-incorporation rate and relative ratios of light/heavy species are described in the Support Protocol.

BASIC PROTOCOL 1

CELL CULTURE FOR IDAWG LABELING

This basic protocol is for labeling any cells in culture. Due to large differences in metabolic flux, different cells must be cultured in heavy Gln for different periods of time to achieve a high degree (>95%) of labeling. In our hands, human embryonic stem cells (hESCs) that divide quickly (doubling time of ~30 hr) and are very metabolically active label to

completion in 72 hr (changing the medium every 24 hr). However, we have also labeled differentiated 3T3-L1 adipocytes that are post-mitotic, which required 6 days of labeling to achieve >90% labeling. Thus, the optimum time of labeling must be determined for each individual cell line, but since the label is relatively inexpensive, 1 week of labeling is a good starting point for an unknown cell type, and under-incorporation can be calculated (see Support Protocol). This 1-week labeling is a starting point that can be adjusted based on the metabolic activity and to some extent the doubling time of the cells. In terms of scale, it is recommended to begin with a minimum of 2×10^6 cells per growth condition (scaling up for making protein powder, as described in Basic Protocol 2, is straightforward).

Materials

Cells

Gln-free medium appropriate for cells (Invitrogen)

Amide- ^{15}N -Gln (98%) (Cambridge Isotope Laboratories, Inc.)

Normal-abundance ^{14}N -Gln (L-glutamine; Invitrogen, cat. no. 21051024)

Phosphate-buffered saline (PBS; see recipe)

Appropriate tissue culture equipment

Cell scrapers

1. Grow cells as normal using Gln-free medium supplemented with either amide- ^{15}N -Gln or corresponding normal-abundance ^{14}N -Gln at 2 mM final concentration.

Cells incubated with both the labeled and normal Gln are required for preparing the protein powder in Basic Protocol 2.

2. Change the medium daily and allow the cells to grow for at least 72 hr in heavy label.
3. Do not harvest cells using trypsin, as it will cleave off many of the cell surface glycoproteins. Instead harvest adherent cells by scraping in PBS, and prepare cell pellets.

BASIC PROTOCOL 2

N- AND O-LINKED GLYCAN ANALYSIS WITH IDAWG

This basic protocol describes the detailed procedure for analysis via mass spectrometry of both permethylated N- and O-linked glycans released from cultured cells. The cells are first lysed, then delipidated and turned into protein powders. Both N- and O-linked glycans are released from the protein, and the permethylation of glycans is performed (see Fig. 1). Permethylated glycans are then analyzed using high-resolution mass spectrometry. To compare the quantities of each glycan structures in different samples, the protein powders of normal and ^{15}N -labeled samples are mixed together by weight (see Basic Protocol 2) or by cell number upon culture harvesting. To calculate the under-incorporation rate for each glycan, which is crucial for the calculation of the ratio of light/heavy structures, an extra analysis of pure ^{15}N -labeled sample with exactly the same procedure can be performed (see Support Protocol).

Materials

Cell pellets from cell culture (see Basic Protocol 1)

Phosphate-buffered saline (PBS; see recipe)

Methanol, HPLC grade, ice cold

Chloroform, HPLC grade

18.2 MΩ (Milli-Q) water

Acetone, HPLC grade, ice cold

Source of dry N₂

40 mM NH₄HCO₃, pH8

2 mg/ml trypsin (Sigma-Aldrich, cat. no. T8003) in 40 mM NH₄HCO₃, pH8 (store at −20°C)

2 mg/ml chymotrypsin (Sigma-Aldrich, cat. no. C4129) in 40 mM NH₄HCO₃, pH 8 (store at −20°C)

2 M urea in 40 mM NH₄HCO₃ (prepare fresh)

Acetonitrile, HPLC grade

5% and 10% (v/v) acetic acid (HPLC grade)

20% (v/v) isopropanol (HPLC grade) in 5% acetic acid

40% (v/v) isopropanol (HPLC grade) in 5% acetic acid

Isopropanol, HPLC grade

100 mM sodium phosphate, pH 7.5

7.5 μg/ml Peptide-N-glycosidase F (PNGase F), store at 4°C (ProZyme, <http://www.prozyme.com/>)

1 M sodium borohydride (prepare fresh)

AG 50W-X8 resin stock (see recipe)

1 M HCl

9:1 methanol:glacial acetic acid (HPLC grade)

50% w/w sodium hydroxide solution

Anhydrous methanol (99.8%, Sigma)

Anhydrous dimethylsulfoxide (DMSO; 99.9%, Sigma)

Iodomethane (99.5%, Aldrich)

Dichloromethane, HPLC grade

1 mM NaOH in 50% methanol

10-ml Dounce homogenizer

8-ml screw-top glass tubes, precleaned with methanol

End-over-end rotator

VWR Clinical 50 centrifuge or equivalent

Pierce Reacti-Vap Evaporating Unit and Reacti-Therm Heating/Stirring Module

Heating block (e.g., Fisher Isotemp 125D)

Bakerbond SPE Octadecyl (C₁₈) disposable extraction columns (J.T. Baker)

Speed-Vac evaporator

Bath sonicator (Branson Ultrasonic Cleaner; Model 1510R-MT)

500- μ l microsyringe

Fused-silica emitter ($360 \times 75 \times 30 \mu\text{m}$, SilicaTip; New Objective, <http://www.newobjective.com/>)

LTQ-Orbitrap XL mass spectrometer with nano ESI source (ThermoFisher) or equivalent

NOTE: In this protocol, we introduce the method of mixing samples by weighing protein powder (in step 15). Alternatively, cells labeled light and heavy can be combined before step 1, based on accurate equal cell numbers.

Perform cell lysis and delipidation

1. Wash cell pellet ($\sim 2 \times 10^6$ cells) by adding 5 to 10 ml PBS, centrifuging 5 min at $1200 \times g$, 4°C , and then removing the supernatant. To the pellet, add water to 100 μ l, and transfer the suspension to a 1.5-ml microcentrifuge tube.

Cells grown in media with the light and heavy label, respectively, are required.

2. Add 500 μ l ice-cold methanol to the tube and move the cells to a 10-ml Dounce homogenizer.
3. Disrupt the cells well on ice by Dounce homogenization (6 to 8 strokes), then transfer the mixture to a tared 8-ml glass tube.
4. Add 3.5 ml methanol, 1.5 ml water, and 2 ml chloroform to yield a final ratio of 4:8:3 chloroform:methanol:water.
5. Incubate the mixture for 3 hr at room temperature with end-over-end rotation to extract lipids.
6. Centrifuge 30 min at $3300 \times g$, 4°C .
7. Remove the supernatant, add 4 ml methanol, 1.5 ml water, and 2 ml chloroform to the insoluble materials, and incubate for 2 more hr at room temperature to extract the lipids again.

The supernatant can be discarded or stored for analysis of glycolipids.

8. Centrifuge 30 min at $3300 \times g$, 4°C , and remove the supernatant.
9. Add 1 ml water to the insoluble materials in glass tube and vortex well.
10. Add 6 ml ice-cold acetone to the glass tube and vortex well to precipitate the proteins.
11. Incubate for 10 min on ice and then centrifuge 30 min at $3300 \times g$, 4°C , and remove the supernatant.
12. Repeat steps 9 to 11 two more times.
13. Dry the insoluble protein powder under a stream of nitrogen at 40°C using the Pierce Reacti-Vap Evaporating Unit and Reacti-Therm Heating/Stirring Module.
14. Weigh the protein powders in the tared 8-ml glass tubes.
15. Take the same amount of protein powder (3 to 5 mg each) of normal and ^{15}N -labeled cell populations and mix together. Prepare one sample of mixed protein

powder to prepare N-linked glycans and another sample to prepare O-linked glycans. Proceed to release N-linked glycans (step 16) and O-linked glycans.

Release N-linked glycans

16. Resuspend the mixed protein powder in an 8-ml glass tube with 200 μ l of 40 mM NH_4HCO_3 , seal the tube, then place in a heating block with temperature set at 100°C for 5 min.
17. After cooling to room temperature, centrifuge briefly to collect the solution at the bottom of the tube, then add 25 μ l of 2 mg/ml trypsin solution in 40 mM NH_4HCO_3 and 25 μ l of 2 mg/ml chymotrypsin in 40 mM NH_4HCO_3 . Finally, add 250 μ l of 2 M urea (in 40 mM NH_4HCO_3 to yield a final concentration of 1 M urea. Seal the tube.
18. Incubate the solution for 18 hr at 37°C to digest the proteins. After incubation, place the tube in the 100°C heating block for 5 min to deactivate the enzymes. Allow to cool to room temperature.
19. While the solution is cooling, equilibrate a Sep-Pak C_{18} cartridge column by washing three times with 100% acetonitrile followed by three washes with 5% acetic acid.
20. Add 500 μ l of 10% acetic acid to the cooled solution, then load the solution onto the equilibrated Sep-Pak C_{18} cartridge column. After loading all the sample, wash the column with 1 ml of 5% acetic acid three times. Next, put a glass collection tube under the column and elute the peptides stepwise, first with 1 ml of 20% isopropanol in 5% acetic acid, then with 1 ml of 40% isopropanol in 5% acetic acid, and finally with 1 ml of 100% isopropanol. Combine the elutions and dry down in a Speed-Vac evaporator to remove solvents.

It is possible either to use stepwise elutions or simply to use 100% isopropanol for all three washes.
21. Resuspend the peptides with 45 μ l of 100 mM sodium phosphate (pH 7.5) and then add 5 μ l PNGase F stock to release the N-linked glycans from the peptides. Incubate for 20 hr at 37°C.
22. After PNGase F digestion, add 450 μ l of 5% acetic acid to solution, then the load solution onto an equilibrated Sep-Pac C_{18} column (see step 19 for equilibration). Elute the N-linked glycans with three 1-ml aliquots of 5% acetic acid and collect them in a glass tube. Dry down in Speed-Vac evaporator for permethylation (proceed to step 28).

If desired, peptides can be eluted with a high organic solvent percentage in the 5% acetic acid.

Release O-linked glycans

23. In an 8-ml glass tube, add 1 ml of freshly prepared 1 M sodium borohydride to the mixed protein powder made in step 15, vortex, and sonicate the sample tube quickly in a Branson Ultrasonic Cleaner at room temperature using the default settings. Incubate for 18 hr at 45°C in a heating block.
24. After incubation, cool the sample to room temperature. Add 10% acetic acid dropwise to the tube while vortexing, until bubbling stops.

25. Pack a 1-ml bed volume of AG 50W–X8 resin stock into a Pasteur pipet to make the cation-exchange column and wash the column sequentially with 2 ml methanol, 1 ml of 1 M hydrochloric acid and 2 ml 5% acetic acid.
26. Load sample onto equilibrated column, elute the O-linked glycans with 7 ml of 5% acetic acid, and collect in an 8-ml glass tube. Dry down in a Speed-Vac evaporator.
- If desired, peptides can be recovered with high salt or high pH wash. To recover the peptides, wash three times using 1 M NaCl in 40 mM NH_4HCO_3 , pH 8.
27. Add 1.5 ml of a 9:1 methanol:glacial acetic acid mixture and then dry the sample under a stream of dry nitrogen using the Pierce Reacti-Vap Evaporating Unit and Reacti-Therm Heating/Stirring Module. Repeat this step two more times to remove the borate. Proceed to step 28 for permethylation.

Permethylate glycans

28. Prepare the dry sodium hydroxide solution for permethylation:
- a. In a glass tube, combine 100 μl of 50% w/w sodium hydroxide solution and 200 μl anhydrous methanol and vortex briefly.
 - b. Add 4 ml anhydrous dimethylsulfoxide (DMSO) and vortex.
 - c. Centrifuge the tube quickly (30 sec) at $1068 \times g$, room temperature, and pipet off dry DMSO, salts, and white residue, leaving clean sodium hydroxide solution in the tube.
 - d. Repeat steps 2 and 3 four to five more times to remove all of the white residue in the tube.
 - e. Once the tube is clean, add 2 ml dry DMSO and pipet up and down gently.
29. Add 200 μl dry DMSO to the released N- or O-linked glycan samples (from step 22 or 27) and purge the tube with dry N_2 to remove air. Next, sonicate 2 min in a Branson Ultrasonic Cleaner at room temperature using the default settings and vortex quickly to dissolve sample.
30. Add 250 μl of the prepared sodium hydroxide solution (from step 28) to sample tube, purge with dry N_2 , and sonicate quickly in a Branson Ultrasonic Cleaner at room temperature using the default settings.
31. Add 100 μl iodomethane with a 500- μl microsyringe to the sample, purge with dry N_2 , and vortex vigorously for 5 min.
32. Add 2 ml water and bubble off iodomethane with dry N_2 gently. After the solution becomes clear, add 2 ml dichloromethane and vortex.
33. Centrifuge the tube quickly (30 sec) at $1068 \times g$, room temperature, and then remove the top aqueous layer.
34. Add 2 ml water, vortex, and centrifuge quickly (30 sec) at $1068 \times g$, room temperature, and then remove the aqueous layer.
35. Repeat steps 33 and 34 four more times, then dry the sample under a gentle stream of dry N_2 using the Pierce Reacti-Vap Evaporating Unit and Reacti-Therm Heating/Stirring Module.

Analyze glycans by mass spectrometry

36. Dissolve the permethylated N- or O-linked glycans in 30 μ l of 100% methanol. Place 15 μ l of the solution into a 1.5-ml microcentrifuge tube, then add 35 μ l of 1 mM NaOH in 50% methanol to achieve a final volume of 50 μ l.
37. Infuse the solution directly into the mass spectrometer using a nanospray ion source with a fused-silica emitter ($360 \times 75 \times 30 \mu\text{m}$, SilicaTip) at 2.0 kV capillary voltage, 240°C capillary temperature, and a syringe flow rate of 0.4 μ l/min.
38. Acquire the full FTMS (Fourier Transform Mass Spectrometry) spectra at 400 to 2000 m/z in positive ion and profile mode with two microscans and 1000 maximum injection time (msec).

This step must be optimized for the particular instrument and can be combined with tandem mass spectrometry analysis of the analytes.

39. Calculate the ratios of the same glycan structures in each sample (normal and ^{15}N -labeled) according to Support Protocol 1.

N- or O-linked glycan structures can be manually interpreted using GlycoWorkbench software (Ceroni et al., 2008), for example, but it is outside the scope of this article to describe this interpretation.

SUPPORT PROTOCOL

CALCULATING RELATIVE RATIOS OF GLYCANS

The IDAWG technology is designed to compare the quantities of each glycan structure in different samples. The glycan structures can be manually interpreted before the calculations (as mentioned in the annotation under step 39 of Basic Protocol 2). Thus, the number of nitrogens can be easily determined based on the total number of GlcNAc, GalNAc, and sialic acid in the structure. Because the reagent with amide- ^{15}N -Gln used in cell culture to label the glycans (see Basic Protocol 1) is not 100% pure, there will be under-incorporation of ^{15}N for each glycan structure labeled. The under-incorporation rate could be different from one structure to another, and this value is needed when calculating the relative ratios. Thus, it is useful to perform an extra procedure of glycan analysis with the ^{15}N -labeled sample alone, instead of the mixture of both light and heavy. This protocol describes the mathematical calculations of under-incorporation rate and relative ratios of light/heavy species after we get the N- and O-linked glycan data in separate analyses of the permethylated sugars (see Basic Protocol 1). The discussions in this section will be based on an example spectrum of a ^{15}N -labeled N-linked glycan structure (Fig. 2A) and a mixture of a light and heavy O-linked glycan structure (Fig. 2B). It should be noted that a software package to automate the calculations is currently under development.

Calculations of under-incorporation rate—To calculate the under-incorporation rate, determine the ratio of the area of peaks that correspond to under-labeling to the total area of all isotopic peaks resulting from one glycan structure. For example, in Fig. 2A, the area of isotopic peak (labeled as $^{13}\text{C}_0\ ^{15}\text{N}_0$, $^{13}\text{C}_0\ ^{15}\text{N}_1$, $^{13}\text{C}_0\ ^{15}\text{N}_2$, $^{13}\text{C}_1\ ^{15}\text{N}_2$, $^{13}\text{C}_2\ ^{15}\text{N}_2$, $^{13}\text{C}_3\ ^{15}\text{N}_2$, $^{13}\text{C}_4\ ^{15}\text{N}_2$) is A_i ($i = 1, 2, 3, 4, 5, 6, 7$), so the overall under-incorporation rate (UI) can be calculated as:

$$\text{UI} = \frac{\sum_{i=1}^7 A_i}{\sum_{i=1}^7 A_i} \quad \text{Equation 1}$$

For any glycan structure, if the number of nitrogens in the molecule is K and the number of isotopic peaks shown in the spectrum is M:

$$UI = \frac{\sum_{i=1}^K A_i}{\sum_{i=1}^M A_i} \quad \text{Equation 2}$$

The incorporation rate for each nitrogen can be calculated as:

$$\text{incorporation} = \sqrt[K]{1 - UI} \quad \text{Equation 3}$$

Calculations of relative ratios

1. Generate the theoretical isotopic patterns for the light structure by software called “emass” written by the Somerharju Lipid Group, University of Helsinki (<http://www.helsinki.fi/science/lipids/software.html>).
2. In Fig. 2B, there are seven major isotopic peaks observed in the spectrum. Calculate the area of each peak (A_1 through A_7) using peak list and intensities extracted from the spectrum. Due to the peak overlap and under-incorporation, both the light and heavy structures can contribute to the area of each peak. So, if the actual area resulting from light structure is L_1 through L_7 , and from heavy structure is H_1 through H_7 , then we have:

$$\begin{aligned} L_1 + H_1 &= A_1 \\ L_2 + H_2 &= A_2 \\ L_3 + H_3 &= A_3 \\ L_4 + H_4 &= A_4 \\ L_5 + H_5 &= A_5 \\ L_6 + H_6 &= A_6 \\ L_7 + H_7 &= A_7 \end{aligned}$$

3. If the ratio of theoretical isotopic pattern for the seven peaks from the light structure is $P_1(=1):P_2:P_3:P_4:P_5:P_6:P_7$, which can be generated using the software “emass,” we can have:

$$\begin{aligned} L_1 &= L_1 \times P_1 \quad (P_1=1) \\ L_2 &= L_1 \times P_2 \\ L_3 &= L_1 \times P_3 \\ L_4 &= L_1 \times P_4 \\ L_5 &= L_1 \times P_5 \\ L_6 &= L_1 \times P_6 \\ L_7 &= L_1 \times P_7 \end{aligned}$$

4. By the definition of under-incorporation rate shown before:

$$UI = \frac{\sum_{i=1}^3 H_i}{\sum_{i=1}^7 H_i} \quad \text{Equation 4}$$

Determine the value of UI based on the spectrum for heavy labeled glycans. Given that $H_i = A_i - L_i$, rearrange Equation 4, with a substitution of H_i into A_i and L_i , according to the equations above, to yield Equation 5:

$$L_1 = \frac{A_1 + A_2 + A_3 - UI \times \sum_{i=1}^7 A_i}{P_1 + P_2 + P_3 - UI \times \sum_{i=1}^7 P_i} \quad \text{Equation 5}$$

5. After calculation of L_1 , calculate L_i ($i = 2$ to 7) and H_i ($i = 1$ to 7) based on the above equations. The equation for heavy/light ratio will be:

$$\frac{\text{Amide-}^{15}\text{N-Gln}}{\text{Amide-}^{14}\text{N-Gln}} = \frac{\sum_{i=1}^7 H_i}{\sum_{i=1}^7 L_i} \quad \text{Equation 6}$$

6. For any glycan structure, if the number of nitrogens in the molecule is K and the number of isotopic peaks shown in the spectrum of light/heavy mixture is M , Equations 5 and 6 can be rewritten as Equations 7 and 8, respectively:

$$L_1 = \frac{\sum_{i=1}^K A_i - UI \times \sum_{i=1}^M A_i}{\sum_{i=1}^K P_i - UI \times \sum_{i=1}^M P_i} \quad \text{Equation 7}$$

$$\frac{\text{Amide-}^{15}\text{N-Gln}}{\text{Amide-}^{14}\text{N-Gln}} = \frac{\sum_{i=1}^M H_i}{\sum_{i=1}^M L_i} \quad \text{Equation 8}$$

Example calculation—Taking the structures in Fig. 2B as an example, the ratio of theoretical isotopic pattern for the seven peaks from the light structure generated by using the software “emass” is $P_1(=1):P_2:P_3:P_4:P_5:P_6:P_7 = 1:0.6517:0.264:0.0798:0.0198:0.0042:0.0008$, and the total is:

$$\sum_{i=1}^7 P_i = 2.0203 \quad \text{Equation 9}$$

The peak areas can be calculated by extracting the peak list and peak intensities from raw data and using the software “OriginPro 8” (<http://www.originlab.com/>) and the areas are:

$$A_1=288182.7; \quad A_2=180298.2; \quad A_3=91459.51; \quad A_4=276748.8; \quad A_5=153348.9; \quad A_6=54793.07; \quad A_7=10981.6.$$

The total is:

$$\sum_{i=1}^7 A_i = 1055813 \quad \text{Equation 10}$$

UI (under-incorporation rate) of this structure is 0.17 (using the calculating method introduced before). Taking all the values into Equation 5, then we have:

$$L_1 = \frac{A_1 + A_2 + A_3 - UI \times \sum_{i=1}^7 A_i}{P_1 + P_2 + P_3 - UI \times \sum_{i=1}^7 P_i} = \frac{288182.7 + 180298.2 + 91459.51 - 0.17 \times 1055813}{1 + 0.6517 + 0.264 - 0.17 \times 2.0203} = 241979.6 \quad \text{Equation 11}$$

After the calculation of L_1 , based on the series of equations under step 3, we can calculate L_2 through L_7 . Based on the series of equations under step 1, we can then calculate H_1 through H_7 . The totals are:

$$\sum_{i=1}^7 L_i = L_1 + L_2 + L_3 + L_4 + L_5 + L_6 + L_7 = 488872 \quad \text{Equation 12}$$

$$\sum_{i=1}^7 H_i = H_1 + H_2 + H_3 + H_4 + H_5 + H_6 + H_7 = 566941 \quad \text{Equation 13}$$

Based on Equation 8:

$$\frac{\text{Amide-}^{15}\text{N-Gln}}{\text{Amide-}^{14}\text{N-Gln}} = \frac{\sum_{i=1}^M H_i}{\sum_{i=1}^M L_i} = \frac{566941}{488872} = 1.16 \quad \text{Equation 14}$$

The value obtained (1.16) represents the relative ratio of this very glycan structure in two samples from different stages of stem cell differentiation. Upon verification by duplicated experiments, we can conclude based on the value 1.16 that the abundance of this glycan structure does not change dramatically between these two stages of stem cell differentiation, since we mix the two samples in a 1:1 ratio.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps.

AG 50W-X8 resin

To prepare the resin stock, add the AG 50W-X8 resin (BioRad) to HPLC-grade methanol, stir well, and decant the methanol. Repeat this batch washing procedure twice more, suspend the resin in methanol, and incubate overnight at room temperature. After this, put the resin in a column and wash the resin successively with methanol, 1 M HCl, and 5% acetic acid by pushing the solvents through the column with air. Keep the resin in methanol at 4°C as the stock.

Phosphate-buffered saline (PBS)

80.0 g NaCl

2.0 g KCl

14.4 g Na₂HPO₄

2.4 g KH₂PO₄

Milli-Q water (18.2 MΩ) to 1 liter

COMMENTARY

Background Information

Glycomics comprehensively studies all the glycan structures in a given biological system. The glycans are complex carbohydrates which are oligosaccharide chains usually linked to proteins and lipids (Aoki-Kinoshita, 2008). Glycosylation is a process of covalent attachment of glycan structures on protein backbones, which is one of the most common post-translational modifications of proteins (Morelle et al., 2009). It has been estimated that glycosylated proteins account for ~60% to 80% of all mammalian proteins at some point during their existence and nearly 100% of all membrane and secreted proteins (Atwood et al., 2008). There are two main ways that carbohydrate chains are linked to protein backbones: N-linked glycan is linked through the side chain of an asparagine residue present in the tripeptide consensus sequence, Asn-X-Thr/Ser (where X can be any amino acid except proline); O-linked glycan is attached to the oxygen on the side chain of a serine or threonine (Morelle et al., 2009). Each of these glycosylation sites can be attached with many different glycan structures.

Glycans often play critical roles in various physiological processes. The important functions of glycans include but are not limited to: modulation of biological activity, cell-cell recognition and interaction, and distribution in tissues and signal transduction (Lowe and Marth, 2003). As affected by different factors, the expression of glycans in a biological system can vary with species, tissue, developmental stage, and even the genetic and physiological state (Atwood et al., 2008). It has also been noticed that altered carbohydrate expression is a common feature of many types of cancers (Dube and Bertozzi, 2005). Moreover, particular protein glycosylations may be altered more specifically or frequently than their underlying core protein in certain disease states, which is a potential advantage of using glycans for diagnostics (Yue et al., 2009). Given all the important roles of glycans in physiological processes, considerable effort has been taken to develop technologies to identify and quantify glycan structures in various environments.

It is one of the major challenges in the fields of -omics to develop relative-quantitative analysis technologies that are able to generate meaningful data to compare the expression levels of targeted molecules in different biological samples or developmental states. For proteomics, there are already some powerful tools available. Since mass spectrometry has fulfilled its role as a rapid and reliable method for proteomics studies, MS signal intensities, ion chromatograms, spectral counts, and accurate mass retention time pairs have been used as label-free methods to quantify changes in protein abundances (Wang et al., 2003; Liu et al., 2004; Radulovic et al., 2004; Silva et al., 2005). In addition to label-free methods, stable isotopic labeling methods have become more popular in recent years. Among these labeling methods, ICAT (Isotope-Coded Affinity Tags) as an *in vitro* labeling strategy and SILAC (Stable Isotope Labeling with Amino acids in Cell culture) as an *in vivo* labeling strategy are broadly applied and have been commercialized (Gygi et al., 1999; Ong et al., 2002).

Analytical technologies for glycomics are not as mature as those for proteomics. However, the field of glycomics has followed in the steps of proteomics and successfully adapted some of the quantitative proteomics tools for glycomic analysis. For example, total ion mapping, a label-free strategy, allows identification of glycan structures based on fragmentation information from tandem MS, and permits quantification of the prevalence of each glycan structure by normalizing each ion intensity on the mass spectrum to the total (Aoki et al. 2007). As for *in vitro* isotopic labeling methods, several groups have used heavy methyl iodide ($^{13}\text{CH}_3\text{I}$, $^{12}\text{CDH}_2\text{I}$, $^{12}\text{CHD}_2\text{I}$, and/or $^{12}\text{CD}_3\text{I}$) to label glycans during the permethylation procedure, which is normally performed before MS analysis for both N- and O-linked glycans. The glycan samples containing heavy isotopes are then mixed with samples permethylated using light methyl iodide ($^{12}\text{CH}_3\text{I}$) prior to analysis (Alvarez-Manilla et al., 2007; Aoki et al., 2007; Kang et al., 2007).

In 2009, IDAWG (Isotopic Detection of Aminosugars With Glutamine) was reported as the first *in vivo*-stable isotopic labeling strategy for quantitative glycomics (Orlando et al., 2009). The IDAWG methodology takes advantage of the hexosamine biosynthetic pathway, which uses the side chain of glutamine as the only source of nitrogen when producing aminosugars. As a result, if the cells are fed Gln-free media and glutamine with a ^{15}N -labeled side chain, all the aminosugars (GlcNAc, GalNAc, and sialic acids) produced in the cells will be labeled with ^{15}N , and thus the mass of all glycan structures will be shifted by +1 dalton per aminosugar (see Fig. 3). As an *in vivo* labeling strategy, IDAWG shares some advantages with SILAC over *in vitro* labeling methods. After the labeling, the ^{15}N -labeled sample can be mixed with the normal sample immediately after the cell harvest or the cell lysis. Thus, the glycans from two cell types are subjected to the same experimental conditions for glycan release and permethylation until they are analyzed by MS, which will dramatically reduce technical variability. The IDAWG technology has been successfully used to analyze both N- and O-linked glycans released from murine embryonic stem cells,

and is predicted to be useful for various comparative glycomic studies in the future (Orlando et al., 2009).

Critical Parameters

The method of quantitative glycomics of cultured cells using IDAWG consists of six steps:

1. Cell culture: cells are either fed amide- ^{14}N -Gln or amide- ^{15}N -Gln.
2. Cell lysis: differently labeled cell populations are lysed, delipidated, and then combined.
3. Release of glycans: N- and O-linked glycans are released from glycopeptides enzymatically or chemically.
4. Permethylated: released glycans are permethylated prior to mass spectrometry analysis.
5. Mass spectrometry analysis and data collection.
6. Calculations of relative ratios.

For the cell culture step, labeling using amide- ^{15}N -Gln for 3 days should be sufficient for embryonic stem cells to achieve a good degree of incorporation. However, for those cells with a relatively slower metabolic rate, longer labeling times may be required to attain a high degree of labeling. A recommendation of 7 days is advised for new cell lines.

For the cell lysis step, there are three parameters to consider. First, the delipidating solvent should have the ratio of chloroform:methanol:water equal to 4:8:3, or it will introduce layers into the solvent and impair delipidation. Second, washing with ice-cold acetone and water after delipidation is crucial because acetone helps precipitate proteins and water can dissolve oligomeric hexose ladders, which are common contaminants in glycan analyses. Third, if it is possible to count the cell number, mixing equal amounts of the two cell populations together immediately after cell culture based on cell number instead of by protein weight is also an option when performing IDAWG experiments.

For releasing O-linked glycans, an extra cleanup via C_{18} columns may be added to the protocol to help remove the borate. The sample can be dissolved in 5% acetic acid, loaded onto equilibrated columns, and eluted with 5% acetic acid. Throughout all the procedures, when drying the sample under a stream of nitrogen gas, always keep the drying time as short as possible. Overdrying will likely reduce the yield of the experiments because some product could be volatilized or displaced from the tube by the nitrogen gas after the solution is dried.

Troubleshooting

Low ^{15}N incorporation rate—The ^{15}N labeling time in cell culture may be too short. Label embryonic stem cells for 3 days and label differentiated cell types for at least 7 days to obtain sufficiently high (>90%) incorporation rates.

No glycans detected in mass spectrum

1. The enzymatic digestion or chemical reaction may be incomplete. Make sure the correct amounts of enzyme or chemical reagents are used and that appropriate conditions are applied (see Basic Protocol 2). Multiple methods exist, including commercial glycoprotein stains or lectin blotting of proteins, that can be used to confirm if deglycosylation has been successful.

2. Excessive contamination might have been introduced throughout the procedures. Make sure the protein powder is washed with acetone and water properly. All the micro-centrifuge tubes, glass tubes, and pipets must be precleaned with methanol, or the polymer peaks can dominate the spectra.

Anticipated Results

Example spectra are shown in Figure 2. The relative ratio of any glycan structure with a similarly complete isotopic pattern (typically with more than two isotopic peaks showing up) can be calculated using IDAWG. For example, more than 125 N-linked glycans and 35 O-linked glycans have been identified by the authors' group for mouse embryonic stem cells using these protocols. Among these glycan structures, those that could be reliably relatively quantified by IDAWG were approximately half (about 65 structures for N-linked glycans and 20 for O-linked glycans; these glycans have more than two isotopic peaks in the spectra, which make the calculation reliable). Of course, some of these quantified glycans are in fact isobaric mixtures, and this should be taken into account when interpreting results. IDAWG is not able to separately quantify the isobaric structures, and thus a ratio is calculated based on changes in a set of isobaric structures.

Time Considerations

At least 72 hr (and sometimes longer than a week) are needed to label the cells. The cell lysis and delipidation require incubation of 5 hr to overnight. The release of N-linked glycans requires two overnight incubations for digestion, and the release of O-linked glycans requires one overnight incubation for the chemical reaction. The time for drying samples in the Speed-Vac evaporator is variable depending on the efficiency of the Speed-Vac and the solvent volume used. It takes less than 5 hr to perform permethylation and mass spectrometry analysis. Thus, the entire procedure, excluding the initial labeling and data interpretation, takes 3 to 4 days. The authors of this unit, working with others in the field, are currently attempting to automate the calculations for under-incorporation and relative ratios. Furthermore, the introduction of spiked standard glycans is being explored so that IDAWG labeling can be used to follow glycan turnover, remodeling, and synthesis.

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1. Combine the cells before homogenization and delipidation to get mixed protein powder.
2. Separate homogenization and delipidation before mixing the protein powder.

Figure 1.

Schematic workflow of IDAWG labeling strategy for comparative glycomics. It is possible either to combine equal cell numbers before homogenization and delipidation to obtain the mixed protein powder, or to perform the homogenization and delipidation before mixing the protein powder by weight.

Figure 2.

Example mass spectra of IDAWG glycans. **(A)** Isotopic pattern of ^{15}N -labeled N-linked glycan structure ($\text{Man}_8\text{GluNAc}_2$). **(B)** Isotopic pattern of mixture of normal and ^{15}N -labeled O-linked glycan structure. Green circle: mannose; blue square: GlcNAc; purple diamond: Neu5Ac; yellow circle: galactose; yellow square: GalNAc.

Figure 3.

Schematic of the hexosamine biosynthetic pathway demonstrating that the side chain of glutamine is the only source for nitrogen in the production of aminosugars. If the cells are fed with Gln-free medium and glutamine with a ^{15}N -labeled side chain, all the aminosugars including GlcNAc (blue square), GalNAc (yellow square), and sialic acids (purple diamond) produced in cells will be labeled with ^{15}N , and thus the mass of all glycan structures will be shifted by +1 dalton per aminosugar (Orlando et al., 2009).