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TITLE:

Determination of Glucan Chain Length Distribution of Glycogen Using the Fluorophore-Assisted Carbohydrate Electrophoresis (FACE) Method

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KEYWORDS:

Glycogen, FACE, chain length distribution, average chain length

SUMMARY:

In the present protocol, the Fluorophore-Assisted Carbohydrate Electrophoresis (FACE) technique is used to determine the chain length distribution (CLD) and the average chain length (ACL) of glycogen.

ABSTRACT:

Glycogen particles are branched polysaccharides composed of linear chains of glucosyl units linked by α-1,4 glucoside bonds. The latter are attached to each other by α-1,6 glucoside linkages, referred to as branch points. Among the different forms of carbon storage (*i.e.,* starch, β-glucan), glycogen is probably one of the oldest and most successful storage polysaccharides found across the living world. Glucan chains are organized so that a large amount of glucose can quickly be stored or fueled in a cell when needed. Numerous complementary techniques have been developed over the last decades to solve the fine structure of glycogen particles. This article describes Fluorophore-Assisted Carbohydrate Electrophoresis (FACE). This method quantifies the population of glucan chains that compose a glycogen particle. Also known as chain length distribution (CLD), this parameter mirrors the particle size and the percentage of branching. It is also an essential requirement for the mathematical modeling of glycogen biosynthesis.

INTRODUCTION:

Glycogen, used as carbon and energy storage, is a homopolymer of glucose consisting of linear chains of glucosyl units linked by (1 → 4)-α bonds and attached through (1 → 6)-α glycosidic

bonds or branching points. They appear as β- and α-particles in the cytosol of a wide range of organisms. β-particles are tiny water-soluble particles mainly observed in prokaryotes. Their diameter ranges from 20-40 nm, likely dictated by the glycogen metabolizing enzymes and steric hindrance[1,2].

First described in animal cells, the larger α-particles display up to 300 nm in diameter with a cauliflower-like shape. This particular organization may originate from the aggregation of several β-particles or may arise by budding out of a single β-particle[3]. Interestingly, a recent study has reported the presence of α-particles in *Escherichia coli* [4]. However, unlike α-particles from animal cells, the latter falls apart quickly during the extraction process, which may explain the lack of data in the literature[4]. The appearance of α-particles in eukaryotes and prokaryotes involves phylogenetically unrelated glycogen metabolizing enzymes[5]. This raises questions regarding the function of such particles and the nature of potential cross-linker agents between β-particles[5].

Although two opposing mathematical models were proposed for glycogen molecule formation[6–9], it is generally accepted that β-particles have evolved in response to their metabolic function as a highly efficient fuel reserve for the rapid release of large amounts of glucose. A large body of evidence indicates that glycogen properties such as digestibility and solubility in water are correlated with the average chain length (ACL), which will then dictate the percentage of branching points and the particle size[2,6–8,10,11]. ACL is defined by the ratio between the total number of glucose residues and the number of branching points. Typically, the ACL values vary from 11-14 and 7-23 glucose residues in eukaryotes and prokaryotes, respectively[10]. In humans, several glycogen disorder diseases are due to abnormal glycogen accumulation. For instance, Andersen's disease is associated with the deficient activity of a glycogen branching enzyme, resulting in the accumulation of abnormal glycogen[11]. In prokaryotes, cumulative studies suggest that ACL is a critical factor impacting the degradation rate of glycogen and bacterial survival ability[12,13]. It has been reported that bacteria synthesizing β-particles with a low ACL value degrade more slowly and therefore withstand starvation conditions longer. Thus, knowledge of the architecture of β-particles is essential for understanding the formation of abnormal glycogen particles in human glycogen storage diseases and prokaryotes survival in a nutrient-deficient environment.

Since the first isolation of glycogen from dog liver by the French physiologist Claude Bernard in the late nineteenth century[14], many techniques were developed to characterize glycogen particles in detail. For instance, transmission electron microscopy for glycogen morphology (α- or β-particles)[15], proton-NMR spectrometry for determining the percentage of α-1,6 linkages[16], size exclusion chromatography with multi-detectors for inferring the molecular weight, Fluorophore-Assisted Carbohydrate Electrophoresis (FACE)[17] or High-Performance Anion exchange chromatography with Pulsed Amperometric Detection (HPAEC-PAD) for both chain length distribution (CLD) and ACL determination[18].

This work focuses on the fluorophore-assisted carbohydrate electrophoresis method, which relies on the reductive amination of the hemiacetal group by primary amine function. Historically,

8-amino-1,3,6-naphthalene trisulfonic acid (ANTS) was first used for labeling. Later, it was replaced by the more sensitive fluorophore, 8-amino 1,3,6 pyrene trisulfonic acid (APTS)[19].

[Place **Figure 1** here]

As depicted in **Figure 1**, the hemiacetal function of the reducing end of a glucan chain interacts with the primary amine of APTS under reducing conditions. The sulfonic groups of APTS carry negative charges that enable the separation of glucan chains according to their degree of polymerization (DP). The reductive amine reaction is highly reproducible and efficient. An average efficiency labeling of 80% is obtained for DP3 to DP135 and up to 88% and 97% for maltose (DP2) and glucose, respectively[17,20]. Because one molecule of APTS reacts with the reducing end of each glucan chain, individual chains could be quantified and compared to each other on a molar basis.

PROTOCOL:

1. Incubation with debranching enzymes

1.1 Mix 200 µL of purified glycogen at 0.5-2 mg/mL with 200 µL of 100 mM of acetate buffer (pH 4.8). Add 2 µL of isoamylase (180 U/mg of protein) and 1.5 µL of pullulanase (30 U/mg of protein) (see **Table of Materials**), mix gently by pipetting up and down, and incubate at 42 °C for 16 h in a 1.5 mL tube.

NOTE: Degradation or amylase contamination might occur during the glycogen purification process. To appreciate the presence of free malto-oligosaccharides, samples can be incubated without the debranching enzyme cocktail in parallel. A standard (e.g., maltoheptaose) is included in the analysis to determine the relationship between the elution time and the degree of polymerization.

1.2 Stop the reactions by incubating at 95 °C for 5 min.

1.3 Centrifuge at 16,100 x *g* for 5 min at room temperature to pellet and remove any insoluble material.

1.4 Remove the supernatants using a pipette and transfer them to new annotated tubes. Desalt the supernatants by adding the equivalent of 100 µL of anion/cation exchange resin beads (AG-501-X8, see **Table of Materials**) and agitate.

1.4.1. Regularly agitate the beads for 5 min. Collect the samples by pipetting and place them in new annotated tubes.

1.5 Freeze-dry or use a vacuum evaporator set up (see **Table of Materials**) at 30 °C to dry the samples.

1.6 Store dried samples at room temperature or at -20 °C.

NOTE: The samples can be stored for 1 month.

2. Reductive amination

2.1. Mix the dried samples with 2 µL of 1 M sodium cyanoborohydride in tetrahydrofuran (THF) and 2 µL of APTS (5 mg of APTS resuspended in 48 µL of 15% acetic acid) (see **Table of Materials**).

2.2. Incubate at 42 °C for 16 h in the dark.

CAUTION: Sodium cyanoborohydride is handled with adapted personal protection equipment and under a chemical hood. Inhalation and contact with skin are highly toxic and can be fatal, causing severe skin burns and eye damage. Highly toxic gases can be generated when mixing sodium cyanoborohydride with acetic acid. In contact with water, sodium cyanoborohydride releases flammable gases, which may ignite spontaneously. Concentrated samples are handled under a chemical hood and using adapted personal protection equipment starting from this step.

3. FACE analysis

3.1 Add 46 µL of ultrapure water to each sample.

3.2 Dilute the samples directly to 1/50 in micro vials of 100 µL by adding 1 µL of the sample to 49 µL of ultrapure water. Keep the samples in the dark while setting the FACE (5-10 min).

3.3 Perform reverse polarity electrophoresis with a capillary electrophoresis instrument with a laser-induced fluorescence (LIF) detector (see **Table of Materials**). Set the polarity to "reverse mode" for separation, set the LIF at 488 nm emission wavelength and the detector at 512 nm.

3.4 Set the Injection time for 10 s and the injection pressure at 0.5 psi.

3.5 Carry out the APTS-labelled-glucans separation at 30 kV in a bare fused silica capillary of 60.2 cm in length with an inner diameter of 50 µm (375 µm outer diameter) in the N-linked carbohydrate separation buffer diluted to 1/3 in ultrapure water (see **Table of Materials**).

NOTE: The N-linked carbohydrate separation buffer is replaced every 20 runs.

4. DATA processing

4.1 Export the ".ASC "and ".CDF "files containing the electropherogram profile and integration data, respectively.

4.2 Open the **.ASC** file and draw the relative fluorescence unit according to the time chart.

4.3 Open the **.CDF** file, proceed with a first automatic integration and adjust the following parameters: width; valley to valley integration; minimum area.

4.4 Check and correct any improper integration event manually.

REPRESENTATIVE RESULTS:

Determination of the average chain length of glycogen

Figure 2 represents the workflow required to infer the chain length distribution and the average chain length (ACL) of glycogen.

[Place **Figure 2** here]

Figure 3 displays the electropherograms of commercial maltohexaose and debranched bovine liver glycogen. The fluorescence signals observed between 4.13-4.67 min in all experiments originated from the unreacted APTS. The elution time of labeled maltohexaose (DP6) was estimated at 8.49 min (**Figure 3A**). The APTS-labelled glucans of bovine glycogen were identified based on the elution time of DP6 (**Figure 3B**). No trace of free malto-oligosaccharide was detected in the control sample (glycogen not incubated with debranching enzymes) (**Figure 3C**).

[Place **Figure 3** here]

It can be concluded that the release of APTS-labelled glucan is due to the cleavage of branching points by both isoamylase and pullulanase activities. It should be noticed that the capillary electrophoresis profile can be redrawn in a more appropriate format to create a mosaic figure containing several profiles. To do this, DATA files containing fluorescence values are generated with "asc" extension and opened in the spreadsheet program by choosing CSV (comma-separated value) format. Unfortunately, the exported fluorescence values are not associated with the corresponding elution time. Consequently, they must be manually added according to the acquisition frequency setup on the FACE apparatus (4 Hz means one value acquisition every 0.25 s).

Peak areas were then inferred using the FACE instrument's native application or exported as a DATA file with a "cdf" extension to use another application. The area values are exported in a spreadsheet program and normalized by expressing the DP as a percentage of the total surface area (**Figure 4**).

[Place **Figure 4** here]

Finally, the average chain length (ACL) is inferred by calculating the sum of each percentage chain times the corresponding degree of polymerization. Similar experiments were carried out in triplicate on rabbit liver glycogen (**Figure 5A**), on bovine liver glycogen (**Figure 5B**), and oyster glycogen (**Figure 5C**).

[Place **Figure 5** here]

The chain length distribution of rabbit liver glycogen clearly showed a higher content of short malto-oligosaccharide (DP2) than bovine liver glycogen (DP 7) or oyster glycogen (DP6). As a result, the rabbit liver glycogen possesses the lowest average chain length (ACL = 9.8) compared to bovine liver glycogen (ACL = 11.9) and oyster glycogen (ACL = 12.6). It is to be noted that those commercial glycogens are usually used for assaying the glycogen phosphorylase or glycogen synthase activity. This suggests that the determination of kinetic parameters (V_{max} and K_m) of glycogen metabolizing enzyme activities will vary according to the source of glycogen.

Subtractive analyses

The subtractive analysis is a simple method to compare the glucan chains distribution of two samples. For example, CLDs of glycogen produced by the wildtype (WT) *Synechocystis* PCC6803 strain and single isogenic *glgA1* and *glgA2* mutant strains were determined (**Figure 6A**).

[Place **Figure 6** here]

To recall, most cyanobacteria strains possess two genes encoding glycogen synthase activities: GlgA1 and GlgA2[21]. Both enzymes transfer the residue glucose of ADP-glucose onto the non-reducing ends of linear glucan chains. As depicted in **Figure 6A**, it is challenging to compare samples by only looking at the chain length distribution profiles. The subtractive analysis consists of subtracting the percentage of each DP between samples (**Figure 6B**). The subtractive analysis of %DP of WT minus % of DP *ΔglgA1* mutant reveals an excess of DP3, 4, and 5 (negative values) and a decreased content of DP 10-20. In contrast, the subtractive analysis between %DP of WT and % of DP *ΔglgA2* mutant indicates an opposite effect. Because the subtractive analysis profiles are different between GlgA1 and GlgA2, this suggests a specific function of each glycogen synthase isoform in glycogen biosynthesis[21].

It is important to note that subtractive analysis is only applicable to experiments that involve a reference sample performed in parallel with the samples. Otherwise, subtractive analysis can be empirical since it lies on the normalized CLD of the reference. In 2015, Deng and collaborators, who investigated stopping mechanisms of glycogen growth in mice and humans, proposed an alternate plotting and interpreting glycogen CLDs to address this issue. This plot uses the maximum peak area to normalize each CLD. The data are then plotted on a logarithmic scale highlighting two components. The latter illustrate two different mechanisms for chain elongation stopping[2]. By drawing lines fitting to the higher DP component, absolute parameters (i.e., slopes and intercepts of the lines) can be used for CLD comparison without normalization to a reference profile. CLDs of the wildtype (WT) *Synechocystis* PCC6803 strain and the single isogenic *glgA1* and *glgA2* mutants were plotted on a log scale, and fitting lines were determined for each profile (**Figure 6C**). The first component was highly similar between samples, peaking with a maximum at DP 6. This illustrates that the branching enzyme explicitly produces a maximum of such chains. The second component appeared as a broad shoulder, which was already described for mice and human glycogens[2]. The inclination of the second component arose at a higher DP in *ΔglgA1* and

the slope of the corresponding fitting line (red line) was lower than the wildtype profile. Thus, the lack of GlgA1 slows down the arrest of chain elongation during biosynthesis that was proposed to occur by steric hindrance for mice and human glycogen[2]. These data suggest that the remaining elongating enzyme (*i.e*., GlgA2) produces longer chains before chain crowding. In *ΔglgA2*, the opposite effect was observed with a more dramatic drop of the fitting line, corroborating that the chains produced by the remaining GlgA1 are overall shorter than those synthesized by GlgA2 alone before steric hindrance. This analysis suggests that both isoforms possess distinct kinetics and/or that their respective concertation with branching enzyme activity differ.

FIGURE LEGENDS:

Figure 1: The reductive amination reaction with 8-amino 1,3,6 pyrene trisulfonic acid (APTS). Reductive amination reaction of the hemiacetal group by primary amine function of 8-amino 1,3,6 pyrene trisulfonic acid (APTS) under reductive conditions

Figure 2: Workflow to determine the chain length distribution (CLD) and average chain length.

Figure 3: Electropherograms of a standard and bovine liver glycogen. (**A**) The time elution (8.49 min) of a glucan standard**,** maltohexaose (DP6), was used as a reference to determine the degree of polymerization (DP) of APTS-labelled glucans released from bovine liver glycogen after the action of debranching enzyme activities (**B**). The inset panel shows a separation of glucan chains up to 44 DP. In parallel, untreated bovine glycogen was labeled with APTS to detect possible traces of free malto-oligosaccharides in sample (**C**).

Figure 4: Data normalization, chain length distribution, and average chain length value. Fluorescence peak areas were imported and normalized in a spreadsheet. The chain length distribution is shown as the percentage of DP for each DP. The average chain length (ACL) is calculated by summing each percentage chain times the corresponding degree of polymerization.

Figure 5: Chain length distribution of commercial glycogen. The rabbit liver (**A**), bovine liver (**B**), and oyster glycogen (**C**) were incubated in the presence of debranching enzymes (isoamylase and pullulanase). The APTS-labelled glucans were then separated according to their degree of polymerization (DP) using FACE analysis. Maltose (DP2), maltohexaose (DP6) maltoheptaose (DP7) represent the most abundant glucans in rabbit liver, oyster, and bovine liver glycogen, respectively. The Standard Error of Mean (SEM) was inferred from three independent experiments.

Figure 6: Comparison of chain length distributions using subtractive analysis. (**A**) The chain length distributions of glycogen purified from cyanobacterial strains: wildtype (WT) *Synechocystis* PCC6803 and single isogenic *glgA1* and *glgA2* mutant strains were determined using FACE analysis. The Standard Error of Mean (SEM) was inferred from three independent experiments. (**B**) Subtractive analyses were performed by subtracting the % of each DP of WT to the % of each DP of *ΔglgA1* and subtracting the % of each DP of WT to the % of each DP of DP *ΔglgA2*. This

straightforward mathematical manipulation displays the alteration of glucan chains in mutant strains (black lines). (**C**) The average chain length distributions of glycogen from wildtype and mutants of *Synechocystis* were normalized according to the maximum peak observed for each CLD (DP6 for all samples). Two components are evidenced by plotting the normalized CLD on a logarithmic scale (N_{de} (DP)). Each component indicates a different mechanism of growth stoppage (for more information, see Reference[2]).

DISCUSSION:

The physicochemical properties of glycogen particles (e.g., size, morphology, solubility) are directly associated with the length of glucans composing the particles. Any imbalance between biosynthetic and catabolic enzymes results in the alteration of the chain length distribution and, *per se,* the accumulation of abnormal glycogen that can be hazardous for the cell[11]. The FACE analysis is a method of choice to determine glycogen's chain length distribution (CLD). As depicted in **Figure 2**, the determination of CLD allows the inference of the average chain length value of glycogen (ACL), which mirrors the structure of glycogen particles. Animal glycogens with high ACL values are associated with the appearance of abnormal particles. The subtractive analysis is a helpful method for comparing two glycogen samples from different genetic backgrounds (mutant versus wildtype). By plotting on a logarithmical scale CLDs normalized to the maximum peak, on the other hand, has the advantage of comparing CLD independently of a reference and gave us information on the growing glycogen mechanism.

In addition, FACE analysis is a powerful technique for characterizing the catalytic properties of glycogen metabolizing enzymes. For instance, all glycogen branching enzymes cleave (1 → 4)-α linkages and transfer oligomaltosyl groups onto a (1 → 6)-α position or branching points. Branching enzymes are distinguishable due to their affinity for polysaccharides (e.g., amylopectin, glycogen) and the length of transferred glucans despite the similar catalytic mechanism[22]. Thus, various sources of branching enzymes (human, plant, bacteria) can be characterized and classified through a series of incubation experiments and CLD comparisons using FACE analysis[23].

As mentioned in the introduction, HPAEC-PAD is also an alternative method for determining the chain length distribution[18]. Both techniques require the complete hydrolysis of (1 → 6)-α linkages or branching points by isoamylase-type debranching enzymes before separating the pool of linear glucans according to their degree of polymerization (DP). However, the HPAEC-PAD method harbors two disadvantages compared to FACE: (1) the amperometric pulse response decreases as the glucan chains increase, which does not provide quantitative information. This mass bias issue can be circumvented using HPAEC-ENZ-PAD that involves a post-column enzyme reactor between the anion exchange column and PAD[24]. The column enzyme reactor hydrolyzes malto-oligosaccharides into glucose residues allowing a constant pulse amperometric response. (2) The HPAEC-PAD allows the separation of glucan chains with a degree of polymerization up to 70. Although the resolution is enough for determining the CLD of glycogen samples, FACE separates chains with DPs up to 150, suitable for starch samples[17]. It is essential to keep in mind that both HPAEC-PAD and FACE analysis have pros and cons. For instance, the amination reaction requires a free hemiacetal group to react with the primary amine function of the APTS. This implies that

APTS labeling cannot be used for glucan chains lacking reducing ends (e.g., inulin). The HPAEC-PAD method does not require the presence of reducing ends. A second interesting aspect of the HPAEC-PAD method is that the anion exchange column can be loaded with a few milligrams of linear glucans, allowing the purification of malto-oligosaccharide with a specific DP or ^{14}C-radiolabeled malto-oligosaccharide for enzymatic assay[18,25]. Finally, although mass spectrometry (e.g., MALDI-TOF) is a fast and sensitive technic to determine chain length distribution, this technic appears to be less reproducible. It overestimates the amount of long glucan chains[26]. Nevertheless, the latter can be used for a specific application such as MALDI imaging to map the presence of glycogen across cellular tissue[27].

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DISCLOSURES:

The authors have no conflicts of interest associated with this work.

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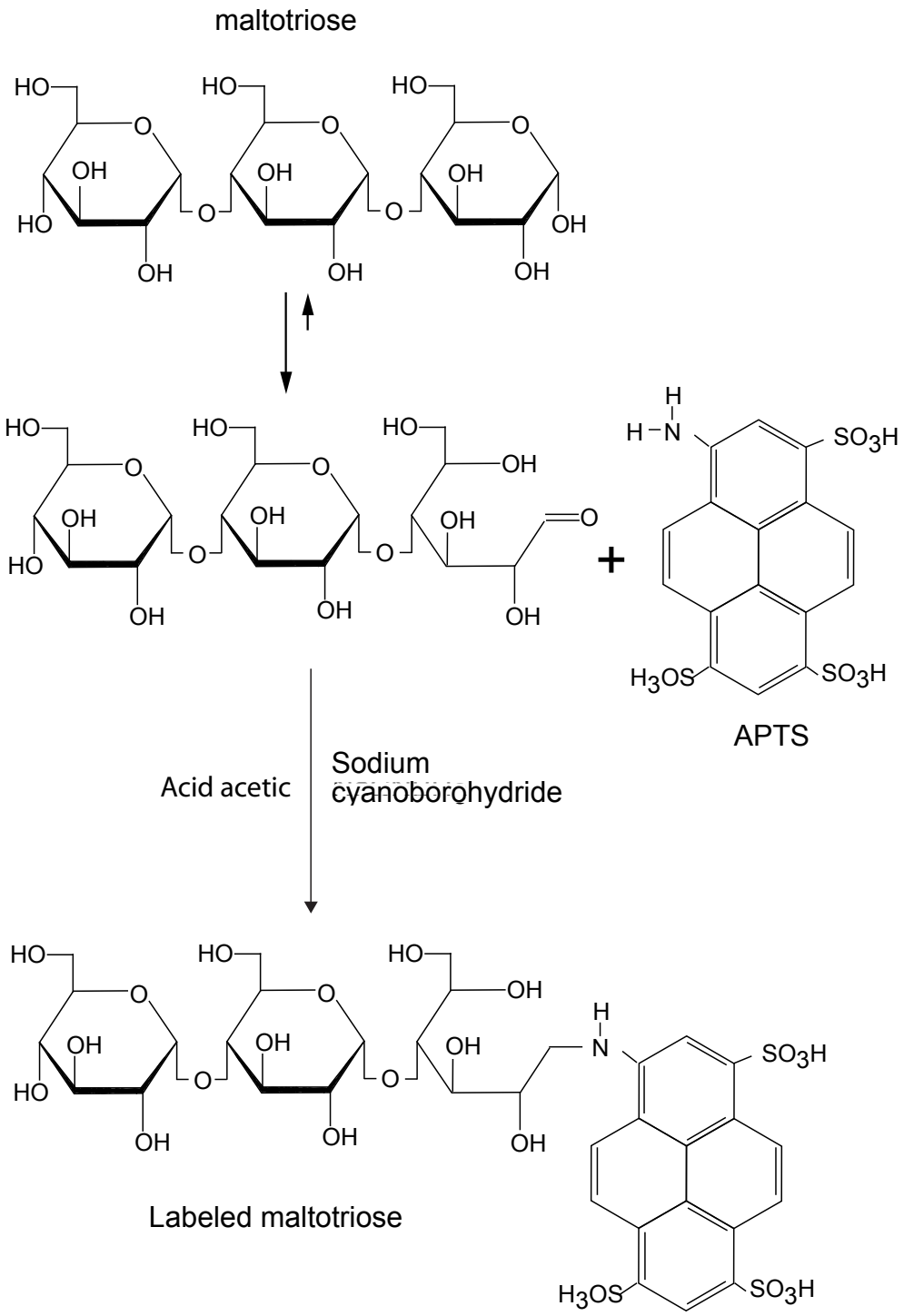

Figure 2

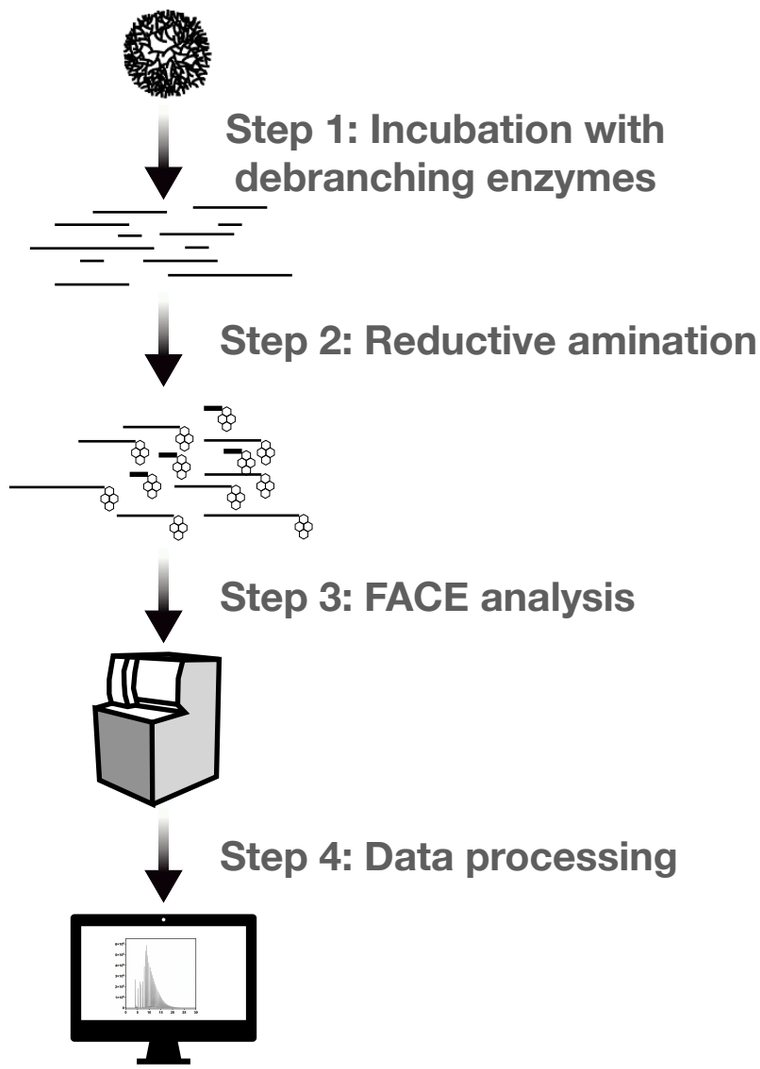

Figure 3

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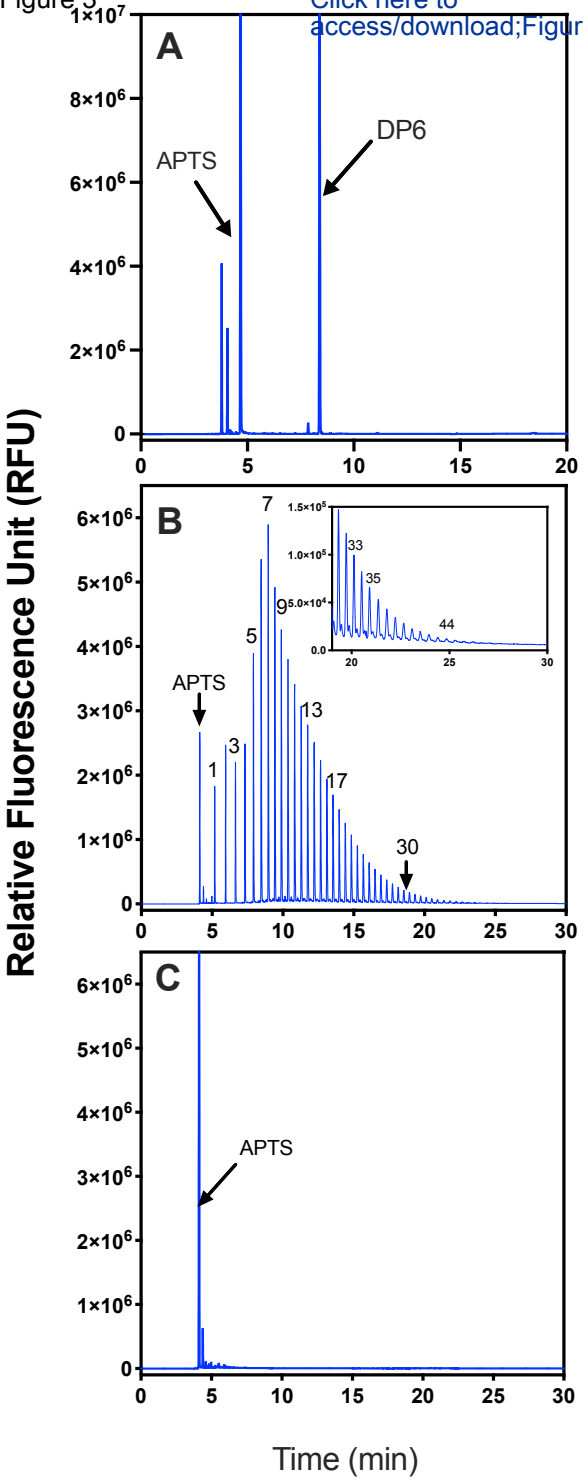

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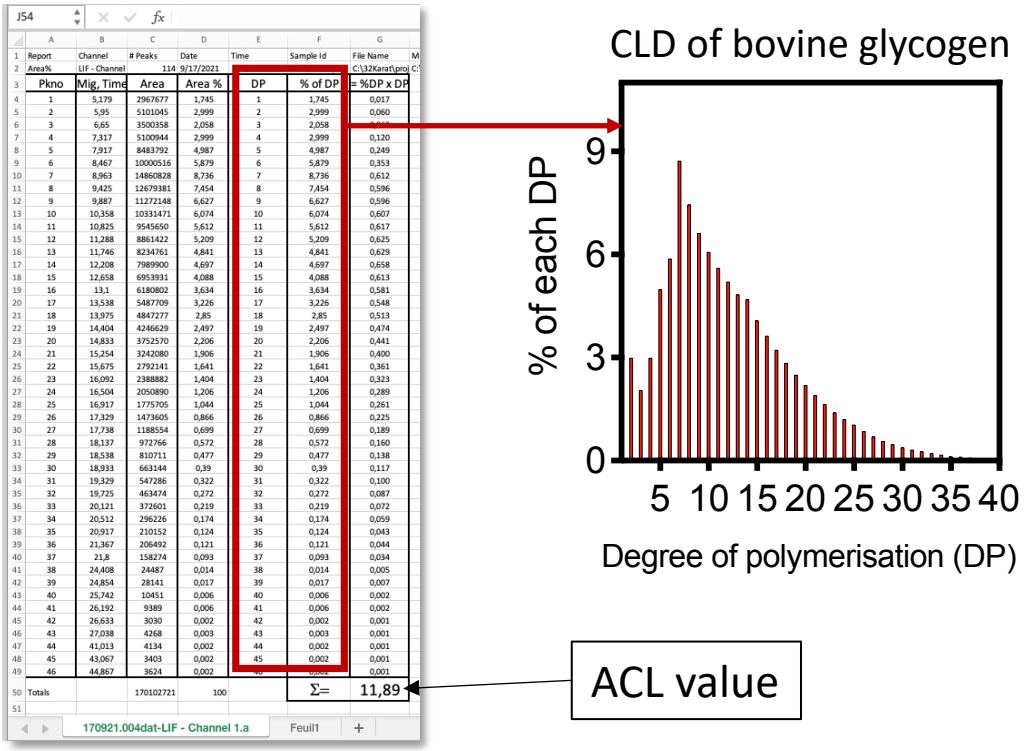

Figure 5

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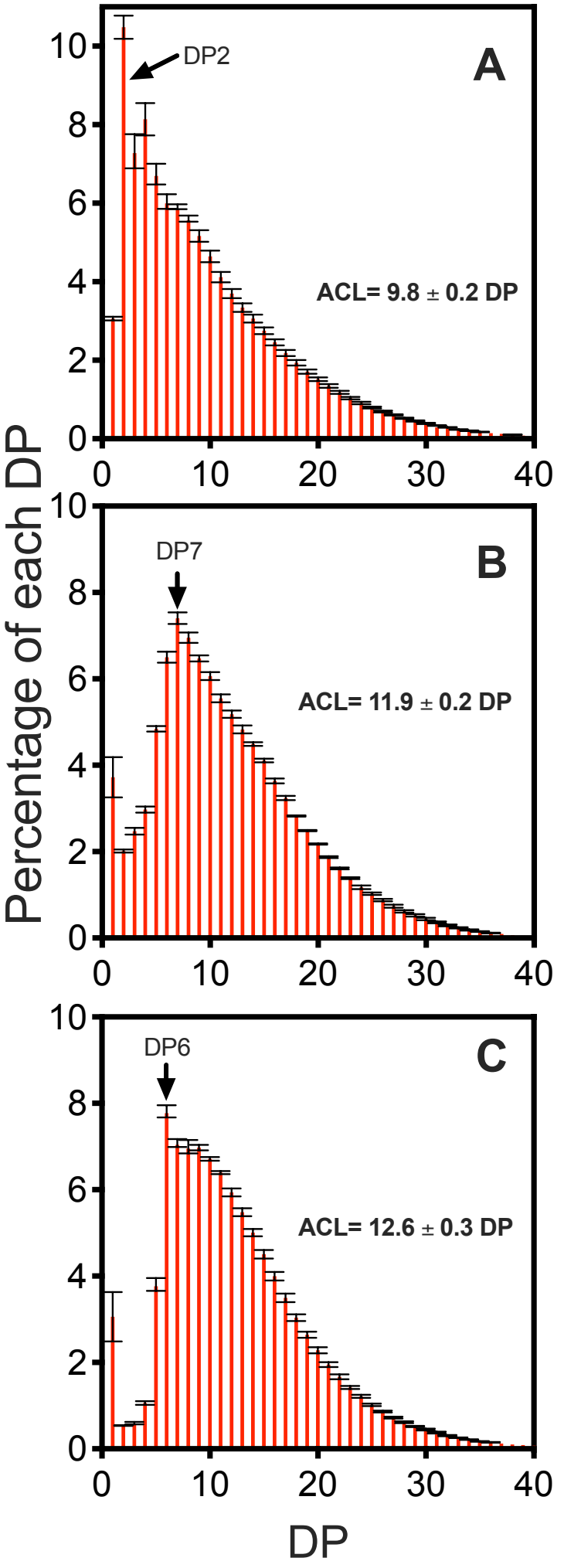
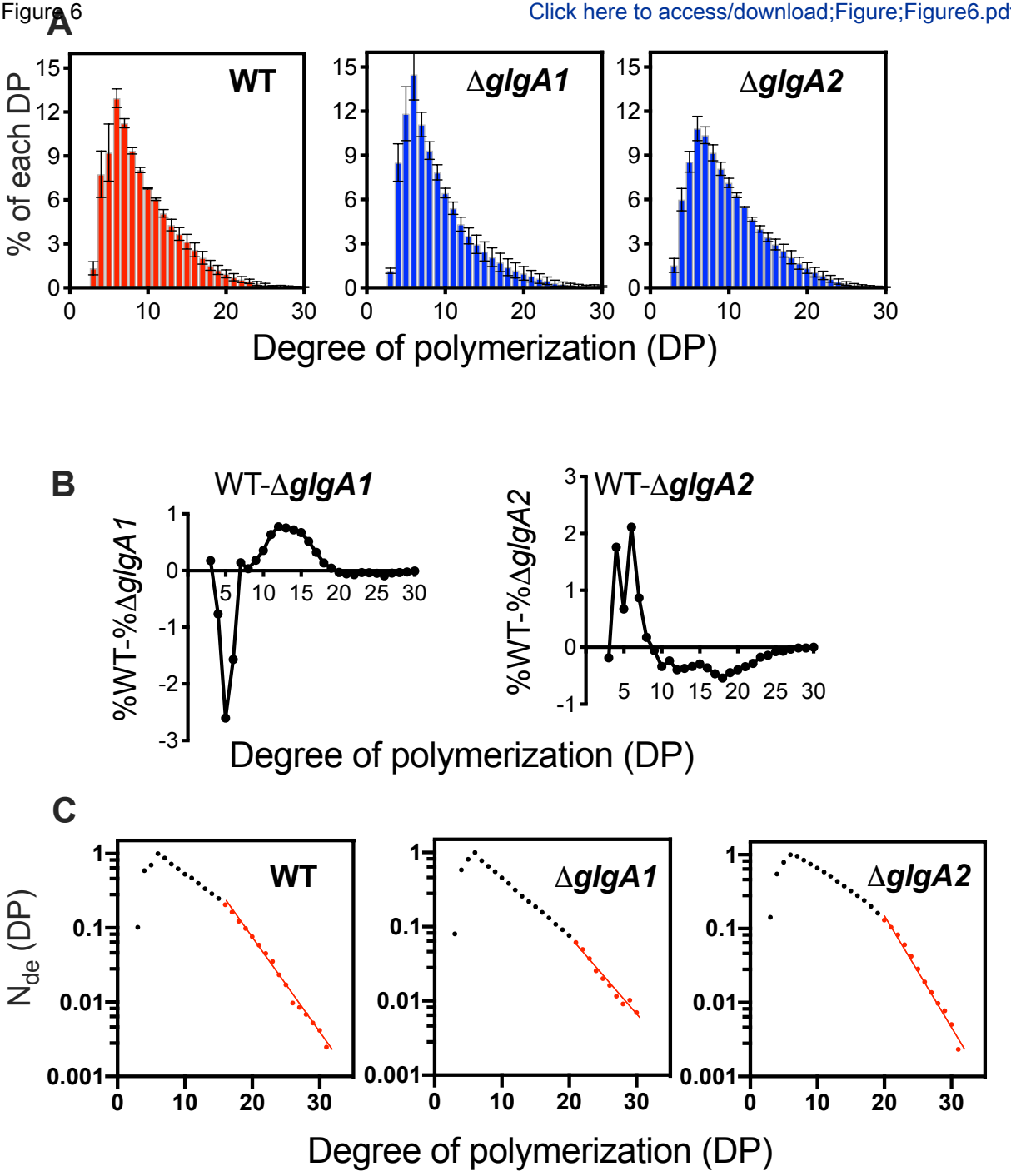


Figure 6



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November 29, 2021

Dear Reviewers,

We are thankful for your comments on our manuscript. We have revised the manuscript and changed in line with comments. The edited manuscript contains the changes (in red) that we have made. Once more thank you for the comments that will improve the original manuscript. We believe that the manuscript is now ready for publishing.
Sincerely,
Professor Christophe Colleoni
On behalf of all authors

Editorial comments:

Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
Check
2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Beckman Coulter PA800 plus instrument; AG-501-X8 resin; Beckman Coulter; Excel, Libre office (even if Libre office is open-source); 32 Karats (AB Sciex); chromeleon software etc

All commercial products have been withdrawn from the manuscript body and have been listed in the table of materials and reagents.
Line 118 "AG-501-X8 resin" has been replaced by: " anion / cation exchange resin beads"
Line 150: "Beckman Coulter PA800 plus" has been replaced by "capillary electrophoresis instrument equipped "
Line 158: "Beckman Coulter" has been replaced by "see table of Materials and Reagents"
Line 178: "(e.g. Excel, Libre office)" has been deleted
Line 183: "32 Karats (AB Sciex)" has been replaced by " the native program implemented with FACE instrument"
Line 185: (e.g chromeleon software) has been deleted

3. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be CONCISE and used sparingly (ONLY ONE CONCISE NOTE allowed between steps/substeps). Please include all safety procedures and use of hoods, etc.
The protocol section is now written in the imperative tense.

4. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please ensure the inclusion of specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

5. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
6. Please add all items (plasticware, glassware, buffers, solvents, equipment, software etc) in the Table of Materials so that it serves as a handy reference for users to get everything ready for the protocol. Please sort the Materials Table alphabetically by the name of the material.
Check
7. Please refer to the Table of Materials as the Table of Materials (in the text). If you want to separate solution compositions into a separate table called Table 1, then please have separate tables--Table 1 for solution compositions; Table of Materials for ALL materials (equipment, software, plasticware etc) used in this protocol.

Check

Point-by-point response to reviewers

Reviewer #1:
Manuscript Summary:
The manuscript by Christophe Colleoni et al. reported how to dissect glycogen primary structure via FACE in a clear and concise manner. Both eukaryotic and prokaryotic glycogen samples were used for the analysis. Indeed, FACE is an important technique nowadays for understanding glycogen (and also starch) structure. The information obtained from chain length distributions and average chain lengths could provide insights into not only glycogen structure and physiological functions, but also potential mechanisms for glycogen particle assembly. After going through the manuscript, the reviewer found that the manuscript was well written and organized and would like to recommend the publication of this study once the authors revise the minor issues listed below.

Major Concerns:
N/A

Minor Concerns:
1. Line 51-52, please delete the comma
Done
2. Line 54, Line 56: β-particles, not b-particles.
Done
3. Line 80: the lower case for 'fluorophore'
Done
4. Line 100: acetate buffer (pH 4.8)
Done
5. Line 131: Incubation at
Done
6. Line 134-135: be fatal, causing...
Done
7. Line 153: Injection for 10 seconds
Done
8. 160: NOTE: The N-linked...
Done
9. Please consider re-formatting Table 1 (center all the text to make the table look tidier)
Done
10. glgA should be in italics.
Line 215-216; Line 267;
11. Synechocystis should be in italic.
Line 215; Line 267
12. Line 207: DglgA1 should be ΔglgA1 to avoid confusion. Apply to other gene names in the manuscript.
Done
13. Figure 1: for each chemical, please label the corresponding name for it in the figure.
Done
14. Please consider improving figure 2 to make it look better. Instead of a flow chart, an illustrative graph might be more suitable for the demonstration.
Done
15. Line 236: ...and average chain length
Done

Reviewer #2:
Manuscript Summary:
This is a good description of a useful technique. While the technical methods are well described, there are major problems with the subsequent data interpretation.

Major Concerns:
1. Line 48: while it is possible that α particles arise from the aggregation of β particles, it is likely that under many circumstances, they may arise by "budding" out of a single β particle. An important effect, which the authors do not discuss, is that there is evidence that β particles can only grow to a maximum average size, of which the size distribution is dictated by the kinetic parameters for crowding, propagation, etc.) and that the glycogen particles have a density distribution which decreases towards the perimeter over most of the molecules (e.g. D. Konkolewicz, O. Thorn-Seshold, A. Gray-Weale, Models for randomly hyperbranched polymers: theory and simulation, J. Chem. Phys. 129 (5) (2008), 054901; B Deng, MA Sullivan, AC Wu, J Li, C Chen, RG Gilbert. Biomacromolecules 16 1870-2 (2015).
The remarks were incorporated in the manuscript: line 47-51 as well as the references.

2. Line 198. A subtractive analysis is entirely empirical and the inferences can depend on the "reference" distribution. Although no biosynthetic models are available for parameterization, the paper by Deng et al. shows that a log plot of the CLD can reasonably be represented as one of two straight lines. The slopes of these and differences between their intercepts and normalization-independent parameters can be used to parameterize the CLD. The subtractive analysis used in this paper can depend on the normalization of the CLD.
This subtractive analysis is usually performed when isogenic mutants are compared to wildtype strain from the same experiment. Nonetheless, we agree with the reviewer that this method can be entirely empirical if the "reference" was not prepared in parallel with the samples.
A method that does not rely on a reference is more suitable to compare samples either from the same experiment or from different sources. As suggested by the reviewer and based on the reference Deng et al 2015, we have included additional figure (figure 6C) and a new paragraph that tends to interpret the Synechocystis glycogen data set.
Minor concerns:
3. Line 287. It is essential to state that HPAEC suffers from mass bias, which is laborious to correct; Wong, K. S., & Jane, J. (1997). Quantitative analysis of debranched amylopectin by HPAEC-PAD with a postcolumn enzyme reactor. Journal of Liquid Chromatography & Related Technologies, 20, 297.

Line 44: the preferred notation is (1→4)-α and (1→6)-α
Done
4. Line 46: "hydro-soluble" is the French term; the preferred English one is "water-soluble"
Done

Reviewer #3:
Manuscript Summary:
The manuscript by Fremont and colleagues describes a novel technique that they first utilized a few years ago called fluorophore-assistsed carbohydrate electrophoresis (FACE). I don't see a recent method paper on this technique to quantify chain length distribution for glycogen. The method is a rigorous one that provides important information regarding glucans. It will be of interest to many in the carbohydrate research community. The manuscript is well written and the detailed method and video will be an outstanding resource to the community.

Major Concerns:
There are no major concerns.

Minor Concerns:
* Some a, b or D instead of α, β and Δ.
Check
* Line 51: eucaryote / line 62: eukaryote → Need to choose which want they want to use
Done
* Line 113: replace rpm value with rcf value
Done
* Line 117: name of the brand is missing
The brand of resin is listed in the in the Table of Materials and Reagents.
* Line 170: which type of bovine glycogen?
Done
* glgA1 glgA2 genes of S. PCC6803, as well as Synechocystis should be written in italic (bacteria nomenclature)
Done
* FIG4: bovine instead of "bovin"
Done
* FIG 5&6: SD or SEM?
The sentence "The Standard Error of Mean (SEM) was inferred from three independent experiments" has been added in the figure legends.

* Line 167-168: "The fluorescence signals at 4.13 min, observed in all experiments, derives from unreacted APTS". However, APTS peak doesn't seem to be at 4.13 min for fig3A.
The peaks and retention times of unreacted APTS can vary from APTS stock solution. This is particularly visible for the DP6 standard, which has been labeled using another APTS stock solution. To avoid any confusion, the sentence has been modified: "The fluorescence signals observed between 4.13 to 4.67 min in all experiments derive from unreacted APTS."

* The authors do a good job of comparing FACE and its benefits with HPAEC-PAD. There are two recent papers that utilize mass spect based approaches to quantify aspects of glucans:
1) Young et al., 2020 Carbohydrate Polymers: Quantifies glucose amounts and glucose phosphorylation
2) Sun et al., 2021 Cell Metabolism: Uses MALDI imaging to quantify glycogen and glucose chain length
To be complete, the authors could also mention these two methods if they wish. There are clear benefits to each method and these mass spec methods do not reduce the importance of FACE.

We added a new comment about the use of MALDI in chain length distribution analysis