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Fluorescent Lectin Staining of *Drosophila* Embryos and Tissues to Detect the Spatial Distribution of Glycans During Development

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Abstract

Glycans are the result of the coordinated activities of glycosyltransferases responsible for specific sugar additions. Glycans present on proteins can influence protein stability, transport, function, and recognition, and thus can have profound effects on cell–cell interactions, adhesion, and signaling events occurring during eukaryotic development. Lectin staining provides a useful tool to detect the spatial distribution of specific glycans in developing tissues in situ. Here we describe a method to detect diverse glycans present in developing *Drosophila* tissues and organs using fluorescently labeled lectins.

Keywords

Glycosylation; Glycans; Lectins; Confocal microscopy; *Drosophila*; Development

1 Introduction

Glycosyltransferases are responsible for the formation of specific glycan structures that can have diverse biological effects. Lectins are sugar-binding proteins that have been used to detect a variety of glycan structures present during eukaryotic development [1, 2]. In an effort to determine the spatial distribution of glycans during *Drosophila* development, we have stained *Drosophila* embryos and tissues with fluorescently labeled lectins and performed confocal microscopy [3]. This method is compatible with whole-mount staining, allowing one to image glycan expression in a 3D embryo or tissue at specific developmental stages.

2 Materials

Prepare all solutions using distilled water. Strictly follow all storage and waste disposal procedures.

1. Bovine serum albumin (BSA): Albumin bovine fraction V (MP Biomedicals LLC., Solon, OH, USA). Store at 4 °C.
2. Dechoriation solution: Germicidal bleach, active ingredient is 6.15 % sodium hypochlorite (Clorox® Professional Products Company, Oakland, CA, USA). Store at room temperature.

3. Heptane (Sigma-Aldrich, St. Louis, MO, USA). Store at room temperature in flame resistant cabinet.
4. Glycerol (Life Technologies, Carlsbad, CA, USA). Make 70 % glycerol solution with 1× PBS. Store at room temperature.
5. Methanol: Methyl alcohol, anhydrous (Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.). Store at room temperature in flame resistant cabinet.
6. Paraformaldehyde: 10 % stock solution, EM grade, methanol and RNase free (Electron Microscopy Science, Hatfield, PA, USA). Store at room temperature.
7. Phosphate Buffered Saline (PBS), 10× (Quality Biological, Inc., Gaithersburg, MD, USA). Dilute to 1× with distilled water. Store at room temperature.
8. Triton[®]X-100 (Sigma-Aldrich). Store at room temperature.
9. Embryo Fixation Stock Buffer: 800 mM KCl, 200 mM NaCl, 150 mM PIPES (1,4-Piperazinediethanesulfonic acid, Sigma-Aldrich), and 20 mM EGTA (ethylene glycol-bis (2-aminoethylether)-*N, N, N', N'*-tetraacetic acid, Sigma-Aldrich) in 400 mL. Adjust pH to 7.4 with 5 N NaOH (*see* Note 1) and bring to final volume of 500 mL. Autoclave to sterilize. Store at 4 °C.
10. Embryo Fixation Working Solution: Combine 2 parts distilled water, 2 parts 10 % paraformaldehyde and 1 part Embryo Fixation Stock Buffer. Make fresh before each use.
11. Larval Fixation Solution: 4 % paraformaldehyde in 1× PBS. Make fresh before each use.
12. Lectins: Fluorescent lectins purchased from EY Laboratories (San Mateo, CA, USA) included tetramethylrhodamine isothiocyanate (TRITC)-conjugated *Canavalia ensiformis* (Con A); TRITC-conjugated *Dolichos biflorus* (DBA); fluorescein isothiocyanate (FITC)-conjugated *Artocarpus integrifolia* (Jacalin); FITC-conjugated *Vicia villosa* (VVA); and FITC-conjugated *Triticum vulgare* (WGA). Lectins purchased from Molecular Probes (Eugene, OR, USA) included Alexa Fluor 488-conjugated *Arachis hypogaea* (PNA) and Alexa Fluor 568-conjugated *Glycine max* (SBA). FITC-conjugated *Helix pomatia* (HPA) (Sigma-Aldrich). Lectins were reconstituted at 1 mg/mL in distilled water and stored as 20 µL aliquots at −20 °C (*see* Note 2). All fluorescent lectins should be protected from light.
13. Sugars (Sigma-Aldrich) for inhibition of lectin binding: *N*-Acetyl-*D*-galactosamine (GalNAc), for inhibition of HPA, DBA, SBA and VVA (store at 4 °C); *N*-Acetyl-*D*-glucosamine (GlcNAc) for inhibition of WGA (store at −20 °C); *D*-(+)-Galactose (Gal) for inhibition of Jacalin and PNA (store at room temperature); *D*-(+)-Mannose (Man) for inhibition of Con A (store at room

¹PIPES does not dissolve completely until the pH approaches 7.4.

²To avoid freeze and thaw cycles, 50 % glycerol can be added to lectin stock solutions and stored at −20 °C for up to 1 year.

temperature). Make 1 M stock solution of each sugar in 1× PBS. Store stock solutions at −20 °C.

14. Hoechst 33342 nuclear counterstain, 10 mg/mL stock solution (Molecular Probes, Eugene, OR, USA). Store at 4 °C.
15. Mounting Solution: 2 % 1,4-Diazabicyclo[2.2.2]octane (Dabco, Sigma-Aldrich) in 70 % glycerol. Store at −20 °C.
16. Glass slides: 75 × 25 mm Superfrost®/Plus Microslides (Daigger, Vernon Hills, IL, USA).
17. Cover slips: 24 × 50mm microscope cover glass, No. 1 (Thermo Scientific, Portsmouth, NH, USA).
18. Cell strainer, 70 µm nylon mesh (BD Biosciences, San Jose, CA, USA).
19. Red sable paintbrush, size 2 (Thomas Scientific, Swedesboro, NJ, USA).
20. 7 mL scintillation vials with unattached cap (Daigger).
21. GyroMini™ Nutating Mixer (Labnet International, Woodbridge, NJ, USA).
22. Pyrex® Spot Test Plates, 9 well, 85 × 100 mm (Thomas Scientific).
23. Dissecting Dish with Plastic Dish (Electron Microscopy Science).

3 Methods

3.1 Drosophila Embryo Collection and Fixation

Carry out all procedures at room temperature unless specified otherwise.

1. Collect embryos at desired developmental stage from juice agar plates (*see ref. [4]* for details of *Drosophila* embryo collection). Transfer embryos from plate into a cell strainer using a paintbrush.
2. Wash embryos with distilled water thoroughly to remove all debris from agar plates. Immerse cell strainer containing embryos in 100 % Clorox bleach for 1–3 min to dechorionate. Dechoriation is complete when embryos become shiny and dorsal appendages are detached (*see ref. [5]* for details on dechoriation).
3. Rinse embryos thoroughly with distilled water. Transfer embryos to a 7 mL scintillation vial using a glass Pasteur pipette. Rinse embryos with water again. Then remove as much water as possible (*see Note 3*).
4. Add 2.5 mL freshly made Embryo Fixation Working Solution to the vial. Then add 2.5 mL heptane. Vortex vial containing embryos at medium speed for 20 min. Embryos will then be present at the interface of the two solutions.
5. Remove all of the lower aqueous phase from vial with a glass Pasteur pipette.

³The dechorionated embryos can stick to the inside of transfer pipettes. Caution should be taken to avoid aspiration of embryos far up into the pipette. Pipettes can be repeatedly rinsed with water to dislodge trapped embryos.

6. Add 4 mL methanol and shake back and forth by hand for 30 s to 2 min to break the vitelline membrane. Embryos will then sink to the bottom of the vial. Remove heptane and most of the methanol with a glass Pasteur pipette, being careful not to aspirate embryos.
7. Rinse embryos with 100 % methanol. Transfer embryos to a 1.5 mL Eppendorf tube and rinse with methanol again. Embryos can be stored in methanol at -20°C for several months.

3.2 *Drosophila* Larval Tissue Collection and Fixation

Carry out all steps at room temperature unless specified otherwise.

1. Collect desired larval tissues from staged larvae.
2. Immediately put the dissected larval tissues in Larval Fixation Solution on ice. Use 1 mL of Larval Fixation Solution for tissues from 4 to 5 larvae (in a 1.5 mL Eppendorf tube). Put the tube on a nutating mixer and rotate 20–30 min.
3. If larval tissues are not used immediately for lectin staining, they should be washed in methanol five times (2–5 min each) and then washed in ethanol five times (2–5 min each). Imaginal disks can then be stored in ethanol at -20°C for 1–2 months.

3.3 Whole-Mount Lectin Staining

Carry out all steps at room temperature and on a nutating mixer.

1. Remove storage solution from fixed embryos and/or larval tissues.
2. Wash embryos and/or larval tissues with $1\times$ PBS containing 0.1 % Triton®X-100 for 3–5 min. Remove PBS solution and repeat washing step two additional times.
3. Remove final PBS wash solution and add 0.1 % BSA in PBS containing 0.1 % Triton®X-100 for 1 h to block.
4. While blocking, dilute lectins to be used and prepare sugar inhibitor control by pre-incubating lectins with appropriate inhibitory sugars for 30 min to 1 h. Briefly, dilute lectins to 10 $\mu\text{g/mL}$ in a solution of 0.1 % BSA in PBS containing 0.1 % Triton®X-100. Prepare sugar inhibitor control by incubating an aliquot of diluted lectin with 0.1–0.2 M of the appropriate inhibitory sugar. The exact concentration of sugar needed to specifically block the binding of each lectin should be determined empirically.
5. Remove the blocking solution and add 200 μL to 1 mL of diluted lectin (depending on sample size) or 200 μL to 1 mL of diluted lectin pre-incubated with sugar to samples. Incubate in the dark for 2–3 h.
6. Remove lectin solution and wash samples with 1 mL PBS containing 0.1 % Triton X-100 for 20–30 min in the dark. Repeat washing step two additional times.

7. Add 1:20,000 dilution of 10 mg/mL Hoechst 33342 nuclear counterstain to Mounting Solution.
8. Remove washing buffer and equilibrate the samples with 500 μ L Mounting Solution containing nuclear counterstain overnight at 4 °C in the dark. Mount samples on glass slide with coverslip the following day (see Note 4). Use approximately 200 μ L Mounting Solution to mount samples on slide. Place coverslip on the sample by holding one edge of the cover slip with forceps and letting the other edge touch the mounting media on the surface of the slide. Gently lower the cover slip to avoid trapping air bubbles beneath it. Before sealing the slide, remove any excess mounting medium at the edges of the cover slip using the edge of a paper towel. Glycerol based mounting solutions will not polymerize, so the edges of slides should be sealed with nail polish. To preserve the fluorescent signal, mounted slides should be stored at 4 °C or –20 °C and protected from light.
9. Image samples using confocal microscopy according to standard procedures (see Note 5).

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⁴Embryos can be directly transferred in Mounting Solution to a glass slide for mounting. Larval tissues of interest can be dissected away from other tissues by carefully transferring the larval samples (using a plastic 1 mL pipette tip with the end cut off to enable aspiration of the tissues of interest) in a small amount of Mounting Solution to a Pyrex® Spot Test Plate or a Dissecting Dish. After dissection, tissues can then be transferred with dissection forceps to a glass slide and additional Mounting Solution can be added (if needed) prior to mounting.

⁵Zeiss LSM 510 confocal laser scanning microscope was used for sample analysis. Stacked confocal images were captured. Images were processed by either Zeiss LSM Image Browser or NIH ImageJ software and assembled in Photoshop (examples are shown in Fig. 1).

Fig. 1.

Glycan expression in *Drosophila* embryos and larval tissues as detected by fluorescently labeled lectins. Fluorescent confocal stacked images are shown. Controls incubated with the competing sugar (Gal, GalNAc, or GlcNAc) to demonstrate staining specificity are shown to the *right* of each panel. **(a)** PNA detects mucin-type Core 1 O-linked glycans (Gal β 1-3GalNAc α -serine/threonine) in the central nervous system (CNS) of stage 17 embryos. **(b)** VVA detects mucin-type O-linked glycans (GalNAc α -serine/threonine) in the foregut (fg), salivary glands (sg), tracheal system (ts), and hindgut (hg) in stage 15 embryos. *Inset* shows a magnified view of the salivary gland (outlined with *white dashed line*) with

abundant O-linked glycans present along the apical and luminal surfaces. **(c)** Jacalin detects mucin-type Core 1 O-linked glycans in the amnioserosa (as) and tracheal placodes (tp) in stage 10 embryos. **(d)** WGA detects *N*-acetylglucosamine (GlcNAc)-containing glycans in the foregut, salivary glands, tracheal system, and hindgut in stage 15 embryos. **(e)** VVA detects mucin-type O-linked glycans (GalNAc α -serine/threonine) in the larval wing (wd), leg (ld), and haltere (hd) imaginal disks. **(f)** Jacalin stains larval wing and leg imaginal disks. Nuclear counterstaining is shown in *blue*. Scale bars = 100 μ m