Chapter 4

Specific Detection of Neu5Gc in Animal Tissues by Immunohistochemistry

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Abstract

One of the major obstacles in xenotransplantation is tissue expression of the non-human mammalian carbohydrate *N*-glycolylneuraminic acid (Neu5Gc). This 9-carbon backbone acidic sugar is the hydroxylated form of *N*-acetylneuraminic acid (Neu5Ac), and both constitute the two most common sialic acid types in mammals. Loss of CMP-Neu5Ac hydroxylase encoding gene in humans dictates the immunogenic nature of Neu5Gc-containing xenografts. Here we describe an immunohistochemistry method for the detection of Neu5Gc in mammalian-derived tissues using affinity-purified chicken anti-Neu5Gc IgY. Specificity is further demonstrated by competitive inhibition with free Neu5Gc or Neu5Gc-glycoproteins, but not with Neu5Ac or Neu5Ac-glycoproteins. This method can be used to evaluate potential Neu5Gc-immunogenicity of xenografts.

Key words Sialic acid, *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), Anti-carbohydrate antibodies, Xenotransplantation, Immunohistochemistry

1 Introduction

Cross-species transplantation (e.g., pig to humans) had been long envisioned as a potential solution for the limited supply of transplantable cells, tissues, and organs for humans, yet still hampered by adverse immune reactions in the recipient host [1–3]. One of the major obstacles for successful xenotransplantation is the humoral immune response developed against the xenografts [2]. While at first protein antigens seemed to be the main immunological targets, carbohydrate antigens have also been noticed to play a major role in the rejection of xenografts [4]. One of the first targets to be identified is the galactose-α1,3-galactose (αGal) [4–6]; however, the non-human acidic carbohydrate *N*-glycolylneuraminic acid (Neu5Gc) was then discovered as another culprit [7–10]. It differs from the human carbohydrate *N*-acetylneuraminic acid (Neu5Ac) by a single oxygen atom [7]. Humans cannot synthesize Neu5Gc [11], and all have serum anti-Neu5Gc antibodies [12–16] that

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recognize multiple presentations of diverse Neu5Gc-expressing glycans, glycoproteins, and glycolipids [16, 17]. These antibodies have been proposed to mediate the potential deleterious outcomes to the host upon exposure to Neu5Gc-expressing xenograft [4, 9, 10, 17], and recent progress in genetic engineering suggests that elimination of such carbohydrate antigens is a promising approach for reduced immunogenicity [3, 6, 18, 19].

Here we describe a method to assess the expression of Neu5Gc in tissues using a well-characterized anti-Neu5Gc antibody [16, 20, 21]. This mono-specific polyclonal anti-Neu5Gc antibody was produced in chickens, as they do not produce intrinsic Neu5Gc, similar to humans [20]. Developed antibodies were affinity-purified from Neu5Gc-immunized chicken serum to high purity and specificity [20]. While this antibody can be used for a variety of applications (e.g., immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), Western blot, and flow cytometry), here we focus on the immunohistochemistry method. For specific detection of Neu5Gc in mammalian-derived tissues using this affinity-purified chicken anti-Neu5Gc IgY, we incorporated parallel competitive inhibition with the specific inhibitors Neu5Gc or Neu5Gc-glycoproteins and with the non-specific inhibitors Neu5Ac or Neu5Ac-glycoproteins. This method can be used to evaluate potential Neu5Gc-immunogenicity of xenografts.

2 Materials

2.1 Animals

1. C57BL/6 wild-type mice. Mice are bred, maintained, and used according to Animal Care and Use Committee protocols approved by the corresponding ethical committee/s (*see* **Note 1**).

2.2 Reagents

2. Affinity-purified polyclonal chicken anti-Neu5Gc IgY antibody (Cat. No.: 146903, BioLegend, San Diego, CA, USA).
3. Peroxidase-conjugated AffiniPure donkey-anti-chicken IgY (Cat. No.: 703-035-55, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA).
4. Purified chicken IgY isotype control (Cat. No.: 402101, BioLegend, San Diego, CA, USA).
5. *N*-glycolylneuraminic acid, NGNA (Cat. No.: 1758–9850, Inalco S.P.A., Milano, Italy).
6. *N*-acetylneuraminic acid (NANA, Sialic acid) (Cat. No.: 08371–81, Nacalai Tesque, Kyoto, Japan).
7. Chimpanzee serum–Chimpanzee sera (*see* **Note 2**).
8. Chicken serum (Cat. No.: C5405, Sigma-Aldrich, St. Louis, MO, USA).

9. DAB substrate kit (Cell Marque; Cat. No.: 957D-20, Sigma-Aldrich, St. Louis, MO, USA).
10. Paraformaldehyde, 96% extra pure, ACROS Organics (Cat. No.: 416780010, Fisher Scientific, MA, USA).
11. Sucrose (Cat. No.: S0389, Sigma-Aldrich, St. Louis, MO, USA).
12. Ethylene glycol (Cat. No.: 000518020100, Bio-Lab LTD, Jerusalem, Israel).
13. Tissue Plus liquid Optimal Cutting Temperature Compound (OCT). (Cat. No.: 4586, Scigen, Gardena, CA, USA).
14. Cold-water fish gelatin (Cat. No.: G7765, Sigma, St. Louis MO, USA).
15. Fluoromount-G® (Cat. No.: 0100-01, SouthernBiotech, Birmingham, AL USA).

2.3 Solutions

1. 0.2 M phosphate buffer A: Mix 1 L of double distilled water (DDW) with 24 g of sodium phosphate monobasic anhydrous (NaH_2PO_4 FW 120.0).
2. 0.2 M phosphate buffer B: Mix 1 L of DDW with 28.4 g of sodium phosphate dibasic anhydrous (Na_2HPO_4 FW 141.96).
3. 0.2 M phosphate buffer (1 L) by mixing 230 mL of buffer A with 770 mL of buffer B. If not used immediately, filter solution using 0.22-μm filter.
4. Tissue collecting solution (TCS, 50 mM phosphate buffer containing 28.6% ethylene glycol and 23.8% glycerol): Mix 1 L of 0.1 M phosphate buffer with 600 mL of ethylene glycol and 500 mL of glycerol.
5. 1 M phosphate buffer C: Mix 142 g of sodium phosphate dibasic anhydrous (Na_2HPO_4) with DDW to complete volume to 1 L.
6. 1 M phosphate buffer D: Mix 120 g of sodium phosphate monobasic anhydrous (NaH_2PO_4) with DDW to complete volume to 1 L.
7. 4% paraformaldehyde solution. A series of steps are needed to prepare this solution. First, using a glass beaker, prewarm 1 L of DDW to 60 °C. Mix 900 mL of prewarmed water with 77 mL of phosphate buffer C, and then warm the solution to 60 °C. Add 40 g of paraformaldehyde to yield a 4% solution into the glass beaker on a stir plate in a ventilated hood. The paraformaldehyde will not dissolve unless the pH is basic (*see* **Note 3**). Add 22.6 mL of phosphate buffer D. Slowly adjust to pH 6.9–pH 7.4 by adding 1 N NaOH dropwise using a pipette until the solution clears and all powder had completely dissolved. Once dissolved, the solution should be cooled and

filtered. This solution can be aliquoted and frozen or stored at 2 °C–8 °C for up to one month, although it is best to prepare it fresh for every use.

8. 30% sucrose solution: Add 30 g of sucrose to DDW and complete volume to 100 mL.
9. PBS × 1: Mix 10 mL of PBS × 10 with 90 mL of DDW.
10. Blocking buffer (0.5% fish gelatin in PBST): Mix 49.45 mL of PBS pH 7.4, 50 μL of Tween-20 (0.1%), 500 μL of cold-water fish gelatin (fish gelatin stock is 40%–50%). Both Tween-20 and fish gelatin are viscous solutions; hence, cut the edge of the pipettor tip before collecting the solutions, and then wash the tip thoroughly with the PBS solution for proper application of these materials.
11. 40 mM Neu5Ac (MW = 309.273 g/mol) in PBS at pH 7 (12.37 mg/mL, ×2 solution): Dissolve 61.85 mg of Neu5Ac with 4.95 mL of DDW, adjust to pH 7 with NaOH (confirm pH with pH paper), and then Q.S. to 5 mL with DDW (*see* **Notes 4–6**).
12. 40 mM Neu5Gc (MW = 325.2693 g/mol) in PBS at pH 7 (13 mg/mL; ×2 solution): Dissolve 65 mg of Neu5Gc with 4.95 mL of DDW, adjust to pH 7 with NaOH (confirm pH with pH paper), and then Q.S. to 5 mL with DDW (*see* **Notes 4–6**).

2.4 Materials and Equipment

1. Dry ice.
2. 1.5-mL microtubes.
3. Peel-away disposable plastic tissue-embedding molds truncated size 12 × 12 mm.
4. Super frost PLUS slides white 76 × 26 mm 90 ground 90 corners (Cat. No.: BN9308C, BAR-NAOR Ltd., Ramat-Gan, Israel).
5. Microscope cover glass, thickness #1.0, 0.13–0.17 mm, 24 × 60 mm, 10 × 100 pcs. (Cat. No.: BN1052441C, BAR-NAOR Ltd., Ramat-Gan, Israel).
6. Microtome such as MICROM HM 450 (Cat. No.: 22-050-856, Thermo Scientific, MA, USA).
7. 96-well cell culture cluster, flat bottom with lid, polystyrene (Cat. No.: 3596, Corning Incorporated, NY, USA).
8. Disposable wipers such as Kimwipes.
9. Petri dish plastic 90 mm.
10. Hirschmann Pasteur pipettes 150 mm.
11. Costar® 48-well clear TC-treated multiple well plates (Cat. No.: CC-3548, Corning Incorporated, NY, USA).

12. *da Vinci* painting brush, series 373, size 2.
13. Blunt-nosed thumb forceps with serrated tips for increased grip.
14. Blunt-end forceps.

3 Methods

3.1 Preparation of Mouse Liver Tissues for Analysis (See Note 1)

1. Prepare 100 mL of PBS × 1 from the ×10 solution and cool it on ice.
2. Sacrifice C57BL/6 mouse in a CO_2 chamber, and excise the liver out.
3. Wash liver three times in 30 mL cold PBS in a 100 mL glass beaker. Cut the liver into small pieces (~10 × 10 × 2 mm).
4. Fix cut pieces of mouse liver tissues in 4% paraformaldehyde. Put each tissue section in 1 mL of 4% paraformaldehyde in a 1.5-mL microtube and keep overnight at 4 °C.
5. Using forceps (blunt-nosed thumb forceps with serrated tips for increased grip), transfer tissue into a new Eppendorf containing 1 mL 30% sucrose, and incubate for 2–3 h at 4 °C, until tissue settles down at the bottom of the tube (*see* **Note 7**).

3.2 Tissue Sectioning with Microtome

3.2.1 Prepare Microtome for Tissue Sectioning

1. Sectioning of tissues is done in a microtome instrument. The microtome tissue-holding platform must be leveled and adjusted before each sectioning (use bubble level to adjust). After leveling the surface, set the microtome to cut tissues into 30-μm-thick sections.
2. On the tissue mounting stand, set a square block of liquid OCT compound for each tissue to be sectioned (*see* **Note 8**).
3. Place crushed dry ice around the mounting stand to maintain cold temperature on the stand and allow OCT squares to freeze until it becomes opaque (*see* **Note 9**).
4. Replenish dry ice around the mounting stand throughout the tissue sectioning procedure (*see* **Note 10**).

3.2.2 Prepare Tissues for Cutting

1. Place 3–5 mL of PBS in a sterile Petri dish.
2. Place one drop of liquid OCT per tissue on another sterile Petri dish.
3. In order to prepare tissue for sectioning on OCT blocks, use blunt-end forceps (blunt-nosed thumb forceps with serrated tips for increased grip) and gently lift one fixed tissue piece (after overnight incubation in sucrose; *see* Subheading 3.1, **Step 5**) to gently dry the tissue with a Kimwipe.

4. Using a Pasteur pipet, place one drop of PBS on the center of one frozen OCT block on the microtome mounting stand. Then simultaneously take the dried tissue with forceps, dip it quickly and gently into the liquid OCT drop, and then immediately place it on top of the PBS drop on the frozen OCT block with the dipped OCT tissue facing down toward the PBS drop (*see* **Note 11**).
5. Flatten the tissue on the frozen OCT block using blunt-end forceps and then cover it locally with finely crushed dry ice (*see* **Note 12**).
6. Allow tissue to completely freeze before sectioning.

3.2.3 Sectioning of Tissue

1. Prepare a 96-well plate with TCS solution for collecting sections of tissues: place 200 μL of TCS per well in 96-well plates.
2. Place 3–5 mL of PBS in a sterile Petri dish.
3. Using a brush, remove the dry ice from the tissue to be sectioned, and then cut the OCT-embedded tissue sections into 30-μm-thick sections using the microtome.
4. Using a brush, pick up each cut section and place it in PBS in the Petri dish (*see* **Note 13**).
5. Using a brush, pick up each cut section from the PBS and place it individually into a well in the 96-well plate containing TCS solution.
6. Repeat **Steps 3–5** for each tissue block to be sectioned.
7. Properly label the 96 wells to identify the tissue sections.
8. Cover the sectioned-tissue plates with a cling film or plastic nylon cover.
9. Store at −20 °C until further use (up to 2 weeks; *see* **Note 14**).

3.3 Immuno-spi1; histochemistry Protocol

Tissue immunohistochemistry staining protocols involve the following steps: fixed tissue sections are washed, blocked, exposed to primary antibody, washed again, exposed to labeled secondary antibody, then washed, and staining allowed to develop with substrate. For tissue Neu5Gc-staining and specificity assessment, the primary antibody chicken anti-Neu5Gc IgY is used and pre-complexed with selected specific (Neu5Gc or Neu5Gc-glycoproteins) or non-specific (Neu5Ac or Neu5Ac-glycoproteins) inhibitors, as detailed in Table 1 (*see* **Note 15**).

3.3.1 Washing of Tissues

1. For tissue staining, wash the tissue sections with PBS as detailed below.
2. Prepare a 48-well plate with 300 μL of PBS pH 7.4 per well.
3. Using a brush, pick up each cut tissue section from the 96-well plate (tissues that were stored in TCS in −20 °C) and transfer it gently into the PBS in the 48-well plate.

Table 1
Description of tissue staining scheme

No.	Primary antibody	Inhibitor	Secondary antibody	Expected reactivity on a Neu5Gc-positive tissue
1.	Control IgY	–	HRP-donkey anti-chicken IgY	Negative
2.	Anti-Neu5Gc IgY	–	HRP-donkey anti-chicken IgY	Positive
3.	Anti-Neu5Gc IgY	Neu5Ac	HRP-donkey anti-chicken IgY	Positive
4.	Anti-Neu5Gc IgY	Neu5Gc	HRP-donkey anti-chicken IgY	Negative
5.	Anti-Neu5Gc IgY	Neu5Ac-glycoproteins	HRP-donkey anti-chicken IgY	Positive
6.	Anti-Neu5Gc IgY	Neu5Gc-glycoproteins	HRP-donkey anti-chicken IgY	Negative

4. Place the 48-well plate with tissue sections on top of horizontal shaker for 5 min.
5. Repeat the PBS wash two more times by aspirating the PBS with a pipette and re-adding 300 µL of PBS pH 7.4 per well (*see* **Notes 16** and **17**).

3.3.2 Blocking of Tissues

1. Prepare blocking buffer (*see* **Note 18**).
2. Aspirate PBS from tissue wells, and then add 300 µL of blocking buffer and incubate at room temperature for 1.5 h with shaking (*see* **Note 19**).

3.3.3 Preparation of Primary Antibody Solutions

Primary antibody is pre-complexed with the specific/non-specific inhibitors, and then incubated on ice for 1.5 h during the tissues blocking step. To prepare primary antibody–inhibitor solutions, the primary antibody and the inhibitors are first prepared at ×2 of their final concentration, and then mixed 1:1 to reach the appropriate concentration, as detailed in Table 2.

1. Prepare control primary antibody at 1:500 dilution in blocking buffer (×2 solution): mix 0.3 µL of control chicken IgY in 150 µL of blocking buffer.
2. Prepare primary antibody at 1:500 dilution in blocking buffer (×2 solution): mix 1.6 µL of chicken anti-Neu5Gc IgY in 800 µL of blocking buffer.
3. Prepare the non-specific inhibitor, Neu5Ac-glycoproteins (human serum or chicken serum), at 20% in blocking buffer (×2 solution): add 40 µL of human serum into 160 µL of blocking buffer (*see* **Note 20**).

Table 2
Description of tissue staining scheme

No.	Compound	Final concentration	Working concentration	Buffer
1.	Control IgY	1:1000	1:500	[a]Blocking
2.	Anti-Neu5Gc IgY	1:1000	1:500	Blocking
3.	Neu5Ac	20 mM	[b]40 mM	PBS
4.	Neu5Gc	20 mM	[b]40 mM	PBS
5.	Neu5Ac-glycoproteins (Human or chicken serum)	10%	20%	Blocking
6.	Neu5Gc-glycoproteins (chimpanzee serum)	10%	20%	Blocking

[a]Blocking buffer (0.5% fish gelatin in PBST)
[b]The sialic acid solutions must be titrated to pH 7 (*see* Note 4)

4. Prepare the specific inhibitor, Neu5Gc-glycoproteins (chimpanzee serum), at 20% in blocking buffer (×2 solution): add 40 μL of chimpanzee serum into 160 μL of blocking buffer (*see* **Note 21**).
5. Prepare as described or have ready after thawing the carbohydrate solutions (40 mM Neu5Ac as non-specific inhibitor and 40 mM Neu5Gc as specific inhibitor).
6. Then, label 1.5-mL microtubes as in Table 2 and prepare the antibody–inhibitor (×1) solutions as described below. All solutions are used at 300 μL per well for each tissue section.
7. Tube No. 1: add 150 μL of blocking buffer into 150 μL of control IgY diluted 1:500.
8. Add 150 μL of 1:500 diluted chicken anti-Neu5Gc IgY into five 1.5-mL microtubes (tubes no. 2–6).
9. Tube no. 2: add 150 μL of blocking buffer.
10. Tube no. 3: add 150 μL of 40 mM Neu5Ac in PBS pH 7.
11. Tube no. 4: add 150 μL of 40 mM Neu5Gc in PBS pH 7.
12. Tube no. 5: add 150 μL of 20% human serum in blocking buffer.
13. Tube no. 6: add 150 μL of 20% chimpanzee serum in blocking buffer.
14. Gently vortex all primary antibody tubes and then incubate on ice (without additional mixing) for 1.5 h during the tissues blocking (*see* **Note 22**).
15. Aspirate the blocking buffer from the 48-well plate with tissues, then add primary antibody solutions (tubes no. 1–6), and properly label the 48-well plate for the different tubes/treatments.

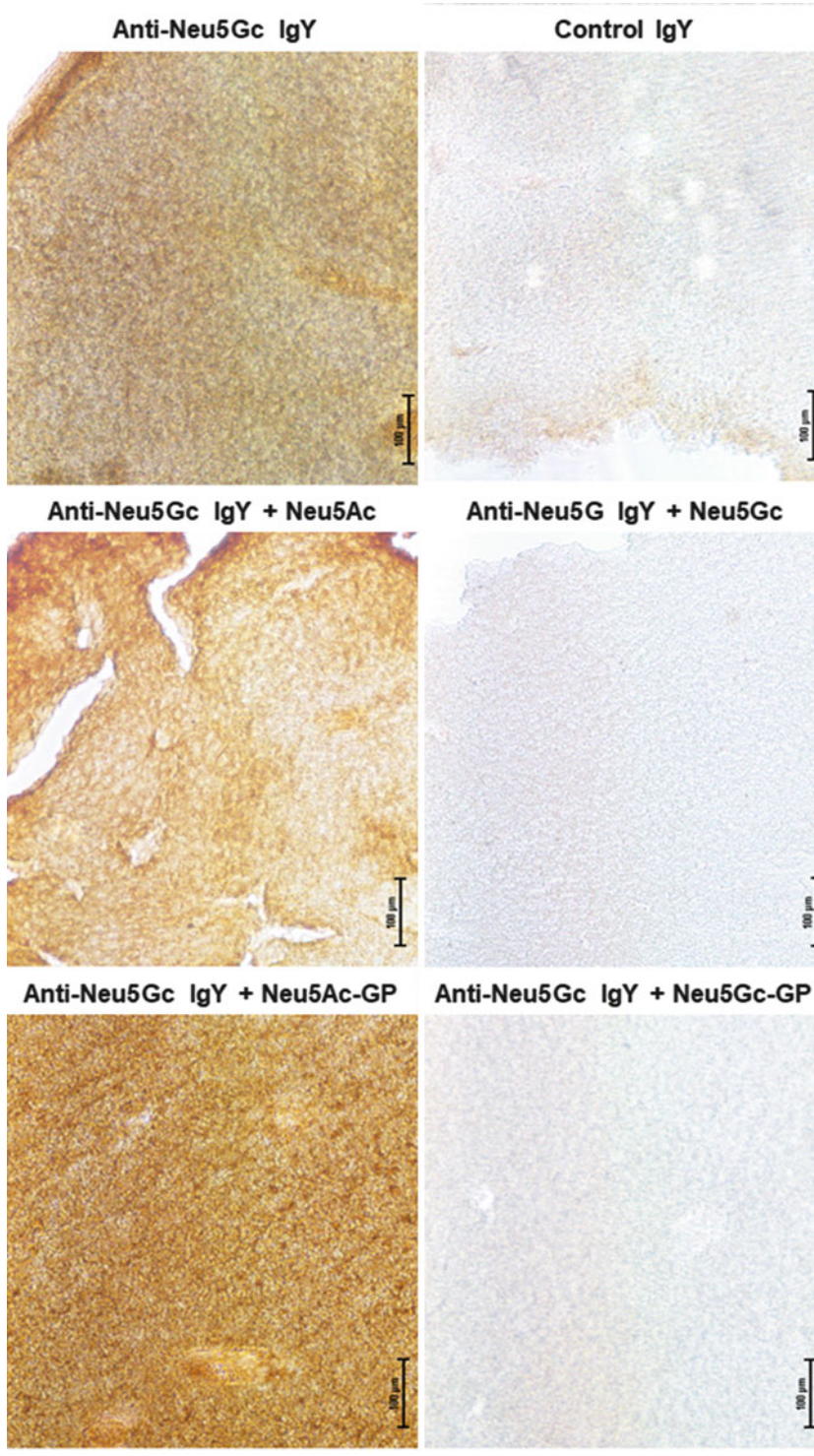

Fig. 1 Immunohistochemical determination of Neu5Gc in wild-type mouse liver tissue. Mouse liver tissue sections stored in −20 °C in 96-well plates in TCS were washed in PBS, and then incubated in blocking buffer (0.1% fish gelatin in PBST) at room temperature for 1.5 h. Subsequently, pre-complexed primary antibody

16. Incubate while shaking on a horizontal shaker for 4 h at room temperature (*see* **Note 23**).
17. Aspirate the primary antibody, then wash with 300 μL of PBST per well, twice, then finally wash with 300 μL of PBS per well.
18. Prepare secondary antibody at 1:500 dilution: 4 μL of HRP-conjugated donkey anti-chicken IgY-HRP in 2 mL of PBS.
19. Aspirate PBS from tissue wells and add diluted secondary antibody at 300 μL per well, and then incubate at room temperature for 1 h.
20. Aspirate then secondary antibody and wash with 300 μL of PBS per well. To this end, incubate while shaking for 5 min. Repeat the washing step two more times.
21. For generating the read out of the reactions, prepare substrate developing solution: use DAB substrate kit (*see* **Note 24**) according to manufacturer instructions: 1 drop of DAB chromogen substrate in 1 mL of kit buffer (prepare 2 mL), cover to keep away from light.
22. Aspirate PBS from all wells and then add 300 μL of DAB reagent to each well.
23. Allow DAB staining to develop for 1 min, and tissue becomes brown (allow 2 min if staining is faint). Once brown precipitate is seen on the tissues, aspirate the substrate solution from all the wells.
24. Wash stained tissue sections in 1 mL of PBS per well, three times, with intermittent shaking for 5 min per wash.
25. Place stained tissues on slides. To this end, use positively charged adhesion slides (adhesion microscope slides). To place tissue sections without folding, first place a drop of DDW (~50 μL) on the glass slide, and then, using a brush, place the tissue section gently on top of the water drop. Flatten tissue section on the slide by tilting the slide until water slides out and tissue sticks flat on the slide slightly dried.
26. Once the sections are slightly dried (up to 3 min, don't leave for over drying), place ~100 μL of Fluoromount-G (*see* **Note 25**). Then, gently place a microscope cover glass over the slide using forceps. Make sure that there are no bubbles near the tissue sections once the coverslip is placed.

Fig. 1 (continued) solutions were added (control IgY or chicken anti-Neu5Gc IgY in blocking buffer, in the presence of specific/non-specific inhibitors, as described in Table 2), and tissues incubated on for 4 h at room temperature. Binding was probed with HRP-donkey-anti-chicken IgY in PBS incubated at room temperature for 1 h. Tissue staining was developed with DAB reagent for 3 min, followed by PBS washing, and then observed in a Nikon Eclipse T*i*-S microscope under ×10 bright field objective. Images were analyzed using the imaging software NIS-Elements version 4.0 (representative of at least two independent experiments)

27. Observe stained tissue section in a microscope such as a Nikon Eclipse T*i*-S under 10× Bright field objective. The refractive index of 10× objective (MRL00042) is 0.30.
28. Capture images using a camera such as a Nikon DS-U3 DS-Filc-U3.
29. Analyze images using an imaging software such as NIS-Elements version 4.0.

4 Notes

1. Any other wild-type mouse strain can be used as a source for -Neu5Gc-positive tissues. In fact, any other non-human mammal can be used as a source for Neu5Gc-positive tissues, and any tissue can be used except for brain that lacks Neu5Gc [22–24].
2. The sera were obtained in our case from the local zoo only during routine maintenance procedures and kindly provided by Dr. Gillad Goldstein, curator of the Zoological Center Tel Aviv, Safari Park (Israel), and Dr. Nili Avni-Magen, Head Veterinarian and Zoological Director of The Tisch Family Zoological Gardens in Jerusalem (Israel).
3. Read the MSDS before working with paraformaldehyde. This chemical is toxic, flammable, and health hazardous; hence, prepare paraformaldehyde solution in a fume hood. (Use personal protective equipment. Ensure adequate ventilation. Avoid dust formation. Remove all sources of ignition. Take precautionary measures against static discharges). Also note that immunohistochemistry stains will be compromised by over-fixation.
4. Sialic acids have a net negative charge at physiological pH (pKa of 2.6) because their carboxylate group is deprotonated, a property that dominates their physiochemical properties. Therefore, the initial pH of 40 mM Neu5Ac/Neu5Gc in PBS is around pH2 and must be titrated with NaOH to reach ~ pH7. Without titration, the non-specific inhibitor Neu5Ac solution will also inhibit anti-Neu5Gc IgY binding, just due to the low pH of the solution.
5. 40 mM Neu5Ac/Neu5Gc pH adjustment: Add small drops of NaOH, mix the tube, allow pH to adjust, and then measure pH with a pH paper. Repeat until pH7 is reached.
6. The 40 mM Neu5Ac/Neu5Gc solutions can be aliquoted and stored in −20 °C.

7. Settling of tissue in the sucrose solution will occur within 1–2 h and up to overnight incubation. It is critical that tissue is settled down before starting the staining protocol.
8. In the microtome tissue mounting stand, while preparing for tissue sectioning, up to 6 OCT blocks can be made at a time.
9. OCT becomes opaque in nature once it is cooled. Dry ice must be put *after* arranging the OCT blocks in the microtome tissue mounting stand.
10. Throughout the tissue sectioning procedure, it is critical to make sure the dry ice is kept around the mounting stand.
11. To glue the tissue on top of the OCT frozen block, it is important to dip one side of the tissue in OCT drop within the Petri dish, and then immediately place it with OCT-laden side on the PBS on the frozen OCT block.
12. It is important that the dry ice is finely crushed.
13. Expect 15–20 tissue sections per tissue block.
14. Tissue sections in TCS do not freeze and remain in liquid solution and can be stored for up to two weeks before staining.
15. Specificity is demonstrated by competitive inhibition with free Neu5Gc or Neu5Gc-glycoproteins that is expected to inhibit anti-Neu5Gc IgY reactivity and with Neu5Ac or Neu5Ac-glycoproteins as controls that are not expected to inhibit anti-Neu5Gc IgY reactivity if the antigen is indeed Neu5Gc.
16. To aspirate the PBS, place the 48-well plate on top of a dark surface to visualize the tissue, tilt the plate, and aspirate the PBS with a pipet (make sure the tissue remains in the well), and gently add 300 μL of PBS.
17. If tissue is sturdy, it is possible to complete the whole tissue washing and staining procedures in a 96-well plate, with 100 μL of washing/blocking solution volumes (enough to completely cover the tissue).
18. Note that both Tween-20 and fish gelatin are viscous solutions.
19. Tissue can be incubated with blocking solution overnight (1.5 h at the minimum).
20. Humans and chicken have a dysfunctional *CMAH* encoding gene; hence, serum glycoproteins are decorated only with the Neu5Ac type of sialic acids and lack expression of Neu5Gc [11, 20, 25, 26].
21. Chimpanzee serum contains both Neu5Gc-conjugated glycoproteins (~14%) and Neu5Ac-glycoproteins (~86%) [13, 20].
22. After antibody–inhibitor solutions are placed on ice for incubation, do not mix the tubes again to avoid disruption of proper pre-complexing.

23. Incubation with primary antibody can be done at room temperature for 4 h or alternatively at 4 °C overnight while shaking.
24. Adjust DAB reagent to room temperature during the incubation with the secondary antibody for about 30 min.
25. Fluoromount-G is a viscous solution; hence, cut the edge of the pipettor 200 μL tip before collecting the solutions. Mounting media volume needs to be sufficient to cover the slides and coverslip, use ~100 μL. Place the mounting media around the tissues, not directly on the tissue. While placing the cover slip, it will cover the top of tissue as well.

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