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The Detection of Glycosaminoglycans in Pancreatic Islets and Lymphoid Tissues

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Summary

In this chapter, we describe the detection of the glycosaminoglycans hyaluronan and heparan sulfate in pancreatic islets and lymphoid tissues. The identification of hyaluronan in tissues is achieved by utilizing a highly specific hyaluronan binding protein (HABP) probe that interacts with hyaluronan in tissue sections. The HABP probe is prepared by enzymatic digestion of the chondroitin sulfate proteoglycan aggrecan which is present in bovine nasal cartilage, and is then biotinylated in the presence of bound hyaluronan and the link protein. Hyaluronan is then removed by gel filtration chromatography. The biotinylated HABP - link protein complex is applied to tissue sections and binding of the complex to tissue hyaluronan is visualized by enzymatic precipitation of chromogenic substrates.

To determine hyaluronan content in tissues, tissues are first proteolytically digested to release hyaluronan from the macromolecular complexes that this molecule forms with other extracellular matrix constituents. Digested tissue is then incubated with HABP. The hyaluronan - HABP complexes are extracted and the hyaluronan concentration in the tissue is determined using an ELISA-like assay.

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[14]The probe is stable and can be stored for about 5 years.

[17]The choice of a particular tissue fixative is determined by the nature of the epitopes of interest to be preserved while ensuring adequate tissue integrity for evaluation of staining patterns. Neutral buffered formalin is routinely used for fixation of human tissue specimens since it permits the successful application of a wide range of special stains. Hyaluronan histochemistry of neutral buffered formalin fixed human tissues generates reproducible patters of intense staining.

[34]The specificity of the Alcian blue staining (0.65 M $MgCl_2$/ pH 5.8) of heparan sulfate in mouse islets has been confirmed by pretreatment of sections with nitrous acid (pH 4.0) which cleaves N-sulfated glucosamines that are present in heparan sulfate but not in other glycosaminoglycans (10).

[35]Routinely, heparan sulfate in small and medium size islets is stained with a very intense blue color, whereas the intensity of blue staining in very large islets can be more variable.

[36]Although heparan sulfate is also localized in the peri-islet basement membrane (9), light microscopic examination of Alcian blue-stained pancreas sections clearly identifies intra-islet heparan sulfate in islet beta cells.

[37]Heparan sulfate is selectively lost from islet beta cells in the T1D mouse pancreas (10).

[43]Test AEC working solution with residual secondary antibody for coloration of chromagen.

Heparan sulfate is identified in mouse tissues by Alcian blue histochemistry and indirect immunohistochemistry. In human tissues, heparan sulfate is best detected by indirect immunohistochemistry using a specific anti-heparan sulfate monoclonal antibody. A biotinylated secondary antibody is then applied in conjunction with streptavidin-peroxidase and its binding to the anti-heparan sulfate antibody is visualized by enzymatic precipitation of chromogenic substrates.

Keywords

Hyaluronan; heparan sulfate; pancreatic islets; lymphoid tissue; hyaluronan binding protein; immunohistochemistry

1. Introduction

Hyaluronan and heparan sulfate are ubiquitous glycosaminoglycans present on cell surfaces and in the extracellular matrix that have been increasingly implicated in various biological processes including cell growth, differentiation and migration, angiogenesis, tissue regeneration, and inflammation (1-6). The distribution and mass of hyaluronan and the cell-associated and extracellular levels of heparan sulfate are crucial for their biological functions (1-3, 6). Therefore identifying the hyaluronan and heparan sulfate morphologic patterns and determining hyaluronan size and abundance are important in addressing questions relating to the role these molecules play in physiologic and pathologic processes affecting different tissues, including pancreatic islets and secondary lymphoid organs. We have found that hyaluronan is located in the extracellular matrix in pancreatic islets and that heparan sulfate is localized at extraordinarily high levels intracellularly in normal insulin-producing islet β cells, as well as in the peri-islet basement membrane (7-10). We and others have also identified hyaluronan and heparan sulfate as components of the extracellular matrix in specialized regions of immune cell activation in the spleen and lymph nodes. The morphologic patterns and abundance of hyaluronan and heparan sulfate are altered in islets and lymphoid tissue in type 1 diabetes, suggesting a potential role for these molecules in the pathogenesis of this disease (8, 10-13). During the course of our studies, we have modified previously developed techniques for hyaluronan and heparan sulfate detection by light microscopy and for determination of hyaluronan content and size by biochemistry, and these modified procedures are described herein.

2. Materials

2.1. Histochemistry of Hyaluronan

The identification of hyaluronan in tissues is achieved by utilizing a highly specific hyaluronan binding protein (HABP) probe that interacts with hyaluronan in tissue sections (14-16). The HABP probe is prepared by enzymatic digestion of the chondroitin sulfate proteoglycan aggrecan present in bovine nasal cartilage (15, 17-22). The HABP is applied to tissue sections and its binding to tissue hyaluronan is visualized by enzymatic precipitation of chromogenic substrates.

2.1.1. Preparation of Biotinylated Hyaluronan Binding Protein (bHABP)

1. Bovine nasal septum cartilage (Pel-Freez, Rogers, AR).
2. Surform pocket plane (Stanley Tools), cheesecloth, Whatman no. 1filter paper (Whatman, Clinton, NJ).
3. Guanidine buffer: 4.0 *M* Guanidine HCl, 0.5 *M* Na acetate, pH 5.8.
4. HEPES buffer: 0.1 *M* HEPES, 0.1 *M* Na acetate, pH 7.3.
5. Trypsin (type III; Sigma-Aldrich, St. Louis, MO).
6. Soybean trypsin inhibitor (type I-S, Sigma).
7. Coomassie blue staining reagent (Pierce, Rockford, IL).
8. Sulfo-NHS-LC biotin (EZ link; Pierce).
9. Hyaluronan-Sepharose (14, 18, 23): Digest hyaluronan (1 g, Sigma) (*see* **Note 1**) with testicular hyaluronidase (1 mg / mL in 0.02 *M* phosphate buffer, 0.01% BSA, pH 7.0) in 500 mL of 0.15 *M* NaCl / 0.15 *M* Na acetate, pH 5.0 for 3 h at room temperature; boil for 20 min and then centrifuge at 10,000 × g for 15 min; discard supernatant and wash the precipitate in 75% EtOH. Mix the digested hyaluronan with 100 mL of EAH sepharose 4B and 2 g of 1-ethyl-3-(3-dimethylamino-propy)-l carbodiimide. Adjust the mixture pH to 5.0 and incubate for 24 h at room temperature. Add 10 mL of acetic acid to the mixture and incubate for 6 h. Wash the gel with 1 L of 1 *M* NaCl, 1 L of 0.05 *M* formic acid and 1 L of distilled water followed by a wash with 0.5 *M* Na acetate, pH 5.7, 0.02% sodium azide. Store in Corning glass bottles at 4°C.
10. Fraction collector FC-203B (Gilson, Middleton, WI).
11. Dialysis membranes 12-14,000 MWCO (Spectrum Labs, Rancho Dominguez, CA).
12. Econo column (2.5 × 20 cm, Bio-Rad, Hercules, CA).
13. Glycerol.

2.1.2. Tissue preparation for histochemistry and immunohistochemistry

1. Human pancreas, spleen and pancreatic lymph nodes are collected from brain-dead organ donors and procured by the Network of Pancreatic Organ Donors with Diabetes (nPOD) (24, 25) (*see* **Notes 2 and [3]**).

[1]It is important that highly purified hyaluronan be used for preparation of biotinylated hyaluronan.
[2]Donor recovery and tissue procurement are performed by organ procurement organizations in the USA, through subsequent referral to the National Disease Research Interchange or the International Institute for the Advancement of Medicine. The nPOD program provides access to high quality biospecimens from donors selected on the basis of inclusion and exclusion criteria. Donor demographics, laboratory assays and histopathological characterizations of the tissues are available online at the nPOD website http://www.jdrfnpod.org. Pancreas, spleen and non-pancreatic lymph nodes are recovered from cadaveric organ donors, placed in a sterile container with media, submerged in ice and shipped to The Organ Processing and Pathology Core at the University of Florida.
[3]Tissues recovered several hours after the donor has been pronounced dead may be prone to autolytic changes; therefore the level of preservation of tissue integrity should be examined and considered when interpreting histochemical and immunohistochemical staining patterns.

2. Mouse pancreas, spleen and lymph nodes are collected in animals euthanized with carbon dioxide (26, 27).
3. Dissecting instruments: stille straight and tissue serrated forceps, stille and iris straight scissors, dissecting boards.
4. 10% neutral buffered formalin (Thermo Fisher Scientific, Waltham, MA).
5. Methyl Carnoy's fixative: 10% glacial acetic acid, 60% methanol, 30% chloroform.
6. Methyl Carnoy's post-fixative: 42% isopropanol 28% methanol, 30% distilled water.
7. Tissue cassettes (Fisher Scientific).
8. Liquid nitrogen, aluminum foil for tissue snap freezing.
9. Automatic paraffin processor (Sakura VIP300, Sakura Finetek, Torrance, CA).
10. Paraffin (Thermo Fisher Scientific).
11. Paraffin Embedding Station (Fisher Scientific).
12. Superfrost Plus slides (Fisher Scientific).

2.1.3. Histochemical Localization of Hyaluronan using bHABP

1. 5 μm paraffin-embedded tissue sections mounted on Superfrost Plus slides, baked 1 h at 50°C.
2. Graded EtOH series for rehydration: 100, 95, 70, and 50%.
3. Xylene.
4. Phosphate buffered saline (PBS): 8 g NaCl, 0.22 g KCl, 1.15 g Na_2HPO_4, 0. 2 g KH_2PO_4, 800 mL distilled H_2O. Adjust pH to 7.4 with HCl and bring volume to 1 L with distilled H_2O.
5. Calcium and magnesium-free phosphate buffer saline (PBS-A).
6. Tris-HCl buffer (TB): 0.05 *M*, pH 7.6 with 1 *N* HCl.
7. Acetate buffer (50 m*M* NaOAc, 0.15 *M* NaCl, pH 5.2).
8. 0.7% H_2O_2 in absolute methanol.
9. Blocking solution: 10% normal goat serum (NGS) in PBS.
10. PBS / 0.1% BSA: Add 1 mg of bovine serum albumin, globulin-free (BSA) / mL of PBS.
11. Biotinylated hyaluronan binding protein (bHABP) 100 μg / mL in PBS-A; bHABP stock solution 5 mg / mL in distilled water, stored at −20°C.
12. Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA).
13. DAB substrate kit (Vector Laboratories).
14. 2% methyl green in sodium acetate buffer, pH 4.2.

15. *Streptomyces* hyaluronidase (1 U / μL dissolved in 10% calf serum in PBS-A).
16. High molecular weight hyaluronan (>1000 kDa).
17. Humidified chamber with lid.

2.2. Biochemical Determination of Hyaluronan Content in Tissues

The determination of hyaluronan content in tissues requires the release of hyaluronan from its complexes with other extracellular matrix molecules. Tissues are first proteolytically digested. The digested tissue is then incubated with the HABP; hyaluronan - HABP complexes are extracted and the hyaluronan concentration in the tissue is determined using an ELISA-like assay (28).

1. Proteinase K (Sigma).
2. 100 m*M* ammonium acetate pH 7.0.
3. Lyophilizer (VirTis, Warminster, PA).
4. Calcium and magnesium-free phosphate buffer saline (PBS-A).
5. 10% calf serum (Irvine Scientific, Santa Ana, CA) in PBS.
6. Hyaluronan-BSA: Dissolve 100 mg hyaluronan (Sigma, St Louis, MO) in 500 mL of 0.2 *M* NaCl. Adjust the pH to 4.7. Add 100 mg of BSA followed by 20 mg of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (Sigma). Dialyze extensively against PBS with 0.02% sodium azide. Aliquot and store at −20°C.
7. Hyaluronan standards at the concentrations of 0, 50, 100, 200, 400, 600, 800, 1000 ng / mL in PBS.
8. 96-well plate (Thermo Scientific).
9. Peroxidase-labeled streptavidin (Sigma).
10. Peroxidase substrate (0.03% H_2O_2 in 3-ethylbenzthiazoline-6-sulfonic acid; Sigma).
11. 0.1 *M* sodium citrate, pH 4.2.
12. 2 m*M* sodium azide.
13. OPTImax microplate reader (Molecular Devices, Sunnyvale, CA).

2.3. Determination of Hyaluronan Size Distribution in Tissues

To determine size distribution of hyaluronan in tissue samples, the tissue extract obtained from proteolytic digestion is first enriched for hyaluronan using anion-exchange chromatography. The enriched product is then fractionated using gel-filtration chromatography. The hyaluronan concentration in each fraction is then determined by an ELISA-like assay.

1. Diethylaminoethyl-Sephacel (DEAE; Sigma) equilibrated with 8 *M* urea buffer.
2. Poly-prep chromatography columns, 0.8 × 4 cm (Bio-Rad).

3. 8 *M* urea buffer: 480.48 g urea, 0.59 g EDTA, 6.06 g Tris Base, 800 mL distilled H_2O. Adjust the pH to 7.5 and bring the volume to 1 L with distilled H_2O.
4. 0.7 × 30 cm chromatography column (Bio-Rad) packed with Sephacryl S-1000 (GE Life Sciences) in PBS with 0.02% sodium azide. Fill column with a thick slurry of gel suspension (*see* **Note 4**) and equilibrate with PBS containing 0.02% sodium azide. Connect the inflow tubing to buffer reservoir and the outflow tubing to a fraction collector (FC-203B, Gilson, Middleton, WI).
5. Hyaluronan standards at the following molecular weights: 30 kDa, 200 kDa, 1500 kDa (Lifecore Biomedical, Chaska, MN).

2.4. Histochemistry and Immunohistochemistry of Heparan Sulfate

Heparan sulfate can be localized in mouse pancreas by Alcian blue histochemistry. Heparan sulfate in human pancreas sections is routinely detected by immunohistochemistry, using anti-heparan sulfate monoclonal antibodies.

2.4.1 Histochemical Localization of Heparan Sulfate in Mouse Pancreatic Islets—

The selective staining of heparan sulfate by Alcian blue, a cationic dye, requires the stringent conditions of 0.65 *M* $MgCl_2$ at pH 5.8, as specified by the Critical Electrolyte Concentration (CEC) principle of differential staining of glycosaminoglycans using salt solutions (29). The staining procedure below is a modification of the method published by Calvitti et al. (30).

2.4.1.1 Preparation of Alcian Blue Stain

1. 1% Alcian blue 8GX (Sigma) in deionized H_2O.
2. 1 *M* $MgCl_2$ (in 100 mL deoinized H_2O, *see* **Note 5)**.
3. 1 *M* acetate buffer: 1 *M* glacial acetic acid (19 mL), 1*M* sodium acetate trihydrate (181 mL). Adjust the pH to 5.8 with glacial acetic acid (*see* **Note 5)**.
4. Alcian blue working solution: 1% Alcian blue (0.5 mL), 1 *M* acetate buffer (5 mL), 1 *M* $MgCl_2$ (3.25 mL), deionized water (41.25 mL) i.e., total volume of 50 mL (*see* **Note 6**).

2.4.1.2 Histochemical Localization of Heparan Sulfate

1. 4-μm thick formalin-fixed paraffin-embedded unstained mouse pancreas sections (*see* **Subheading 2.1.2)** mounted on uncoated Superfrost Plus slides, baked for approximately 1 h at 70° C.
2. 0.1 *M* acetate buffer.
3. Xylene.

[4]Allow gel to pack and make sure it settles without visible interfaces. Equilibrate gel with two or three column volumes of eluent buffer.
[5]1 *M* $MgCl_2$ and 1 *M* acetate buffer can be stored at 4°C for up to 2 months.
[6]Alcian blue working solution is made freshly on the day of staining.

4. Graded ethanol series for rehydration: 2 × 100%, 2 × 90%, 70% and tap water.
5. Coplin or Heyerdahl glass staining jars (50 mL).
6. 0.01% Safranin O in deionized H_2O as counterstain.
7. Micromount mounting medium (Leica, GmbH, Wetzlar, Germany).

2.4.2. Immunohistochemical Localization of Heparan Sulfate in Human Pancreatic Islets

1. 5-μm thick formalin-fixed paraffin-embedded human pancreas sections provided by nPOD (*see* **Subheading 2.1.2**).
2. Xylene.
3. Graded EtOH series for rehydration: 2 × 100%, 1 × 90%, 1 × 70%, tap water.
4. 30% H_2O_2, 3% H_2O_2 in methanol, 3% H_2O_2 in deionized H_2O.
5. 0.5 mg / mL (0.05%) Pronase (Calbiochem, San Diego, CA) in deionized H_2O.
6. Animal Free Block (Vector Laboratories) diluted to 20% in deionized H_2O.
7. Phosphate-buffered saline (PBS): 8 g NaCl / L, 1.25 g $Na_2HPO_4.2H_2O$ / L, 0.35 g $NaH_2PO_4.H_2O$ / L in deionized H_2O.
8. Protein concentrate from M.O.M.-peroxidase kit (PK-2200, Vector Laboratories) diluted 1/13.5 in PBS.
9. Mouse anti-human anti-heparan sulfate monoclonal antibody 10E4 mAb, 1 mg / mL (Chuo-Ku, Tokyo, or Amsbio, Lake Forest, CA) (*see* **Notes 7 and [8]**).
10. Isotype control mouse IgM (0.25 mg / mL, BD Biosciences, San Jose, CA).
11. Polyclonal rabbit anti-mouse Ig- horseradish peroxidase (HRP) secondary antibody (DAKO, Carpentaria, CA).
12. 3-amino-9-ethylcarbazole (AEC chromagen, Sigma), 8 mg / mL in N-N-dimethyl formamide (Sigma).
13. 0.2 μm chemically resistant filter (CR, Minisart).
14. Acetate buffer, 0.1 *M*, pH 5.2: 0.1 N acetic acid (10.5 mL), 0.1 M sodium acetate (39.5 mL).
15. Gill's hematoxylin.
16. Ammonium H_2O: 100 μL ammonia in 250 mL deionized H_2O.
17. Glycergel mounting medium (DAKO).
18. 37°C incubator.

[7]10E4 mAb recognizes N-sulfated/N-acetylated glucosamine in disaccharides in heparan sulfate (32, 33).
[8]Heparatinase treatment of tissue sections abolishes 10E4 immunohistochemical detection of heparan sulfate, confirming the specificity of the 10E4 mAb for heparan sulfate (32).

19. Custom-made covered large staining tray with lid (humidified) and a small immunostaining tray for incubation at 37°C.

20. Diamond pen (ProSciTech, Thuringowa, Qld, Australia).

3. Methods

3.1. Histochemistry of Hyaluronan

3.1.1. Preparation of Biotinylated Hyaluronan Binding Protein (bHABP)

1. Shred the bovine nasal cartilage into fine pieces using a Surform pocket plane. Add 10 mL of guanidine buffer for each gram of cartilage and incubate overnight at 4°C *(see* **Note 9**).

2. Pour mixture through several layers of cheese cloth, then centrifuge at 10,000g for 45 min at 4°C. Filter supernatant through Whatman filter paper.

3. Dialyze supernatant against distilled water (water volume is 200 times the sample volume, *see* **Note 10**). Lyophilize the extract, aliquot and store at −20°C.

4. Dissolve 3 g of lyophilized extract in 100 mL of HEPES buffer, incubate overnight at 4°C.

5. Add 1.6 mg of trypsin and incubate for 2 h at 37°C.

6. Add 3.2 mg of soybean trypsin inhibitor and adjust the pH to 8.0.

7. Determine the protein content using Coomassie blue staining reagent.

8. Add 0.1 mg sulfo-NHS-LC biotin per 1 mg protein. Allow the coupling reaction to proceed for 1-2 h at room temperature to form bHABP.

9. Dialyze the mixture against 3 changes of guanidine buffer.

10. Wash 100 mL of hyaluronan-sepharose in a Buchner funnel with fritted disc with 4 *M* guanidine buffer. Transfer hyaluronan-sepharose and the bHABP mixture into a large dialysis bag and dialyze against 10 volumes of distilled water, overnight at 4°C (*see* **Note 11**).

11. Pour the mixture into a glass column.

12. Wash the column with 1 *M* NaCl and then with 3 *M* NaCl.

13. Connect the column to a fraction collector and elute bHABP with guanidine buffer. Each fraction is assayed for protein concentration. Pool the bHABP-containing fractions and dialyze against 0.15 *M* NaCl (*see* **Note 12**).

14. Mix bHABP with glycerol (1:1 vol/vol), aliquot and store at −20°C (*see* **Notes 13-15**).

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[9]The mixture is poured into a large beaker placed on a shaking table. The solution is too thick to use a stirring bar.
[10]Dialyze against several changes of distilled H_2O to ensure complete removal of guanidine HCl buffer.
[11]For the first 4 h it is important to re-suspend the gel by turning the dialysis bag upside down every 30-40 min.
[12]SDS-PAGE analysis of the final product shows two bands, one at ~ 70-80 kDa (the hyaluronan-binding domain of aggrecan) and one at ~ 43 kDa (link protein).

3.1.2. Tissue Preparation for Histochemistry and Immunohistochemistry

HUMAN TISSUES

1. Tissue slicing (24, 25)

   Pancreas: Divide the pancreas into 3 regions of head, body, and tail. Slice each pancreas region in a transverse “bread loaf” manner and prepare slices that are ~ 1.5 × 1.5 × 0.5 cm. *Spleen*: Slice splenic tissue in pieces of ~ 1.5 × 1.5 × 0.5 cm.

   Pancreatic lymph node (PLN): Isolate PLN from fat and trim tissue surrounding the PLN capsule. Divide PLN in half.

2. Fix tissue pieces in neutral buffered formalin for 16-24 h (*see* **Notes 16-**[18]). Fixed tissues are then paraffin embedded and sectioned for histochemistry and immunohistochemistry (*see* **Note 19**).

3. Wrap tissue pieces in aluminum foil, snap freeze in liquid nitrogen and store at −80°C for biochemical analysis of hyaluronan.

MOUSE TISSUES

1. Anesthetize and euthanize the mouse according to the institution's requirements for animal care and use. Pin the mouse down on a dissection board with the abdomen facing up and wipe down fur with 70% EtOH. Open the abdomen cutting first along the ventral midline and continue through the sternum to open the thorax, and then laterally and down on both sides to create 2 flaps of skin. Pin the 2 skin flaps down on the dissection board.

2. Dissect out pancreas first and then the spleen and lymph nodes (*see* **Notes 20 and 21**).

3. Fix tissues in methyl Carnoy's solution for 1-2 h at 4°C (*see* **Note 22**). Store tissues in methyl Carnoy's post-fixation solution at 4°C until processed for paraffin embedding for hyaluronan histochemistry (*see* **Note 23**).

4. For heparan sulfate histochemistry and immunohistochemistry, fix tissues in 10% neutral buffered formalin for at least 2 days at room temperature until processing and paraffin embedding.

5. Wrap tissues in aluminum foil, snap freeze in liquid nitrogen and store at −80°C for biochemical analysis of hyaluronan.

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[13]The HABP concentration is about 100 μg / mL.
[15]Purified bHABP is also commercially available at EMD Millipore (Billerica, MA).
[16]Tissues should be immersed immediately into fixative.
[18]Formalin fixed tissues are stored in 70% EtOH if not processed immediately for paraffin-embedding for analysis of hyaluronan only. Formalin-fixed tissues for heparan sulfate histochemistry are not to be stored in 70% ethanol prior to processing, as this method of storage appears to interfere with heparan sulfate detection by Alcian blue staining.
[19]Other methods of tissue embedding (cryostat, plastic) may be used.
[20]Pancreas should be fixed whole.
[21]The spleen is cut lengthwise with a scalpel prior to fixation. The cut surface is embedded face down prior to sectioning.
[22]Mouse pancreas fixation in methyl Carnoy's solution should not exceed 2 h.
[23]Application of the same concentration of bHABP on mouse tissues fixed in neutral buffered formalin generates a less intense hyaluronan staining than in tissues fixed in Carnoy's solution without altering the hyaluronan staining pattern.

3.1.3. Histochemical Localization of Hyaluronan using bHABP

1. Cut 5-μm thick tissue sections and mount sections on Superfrost Plus slides.
2. Deparaffinize tissue sections in three changes of xylene, 5 min each (*see* **Note 24**).
3. Rinse tissue in two changes of 100% EtOH, 2 min each.
4. Quench endogenous tissue peroxidase by incubating tissue in 0.7% H_2O_2 in absolute methanol for 20 min.
5. Hydrate tissue sections in graded EtOH series.
6. Rinse for 10 min in PBS.
7. Incubate sections in 10% NGS in PBS for 30 min to block nonspecific binding (*see* **Notes 25 and [26]**).
8. Apply bHABP (5 μg / mL in PBS-A) diluted in PBS with 0.1% BSA. Incubate overnight at 4°C (*see* **Note 27**).
9. Rinse tissue sections in three changes of PBS, 10 min each.
10. Prepare Vectastain Elite avidin biotin complex (ABC): add two drops of reagent A to 5 mL of buffer in the ABC reagent mixing bottle; add two drops of reagent B to the same mixing bottle, mix immediately. Allow the ABC reagent to incubate for 30 min before applying to tissue.
11. Apply the ABC reagent to sections for 1 h at room temperature.
12. Rinse sections in three changes of PBS, 5 min each
13. Prepare the DAB substrate solution: Add 2 drops of buffer stock solution to 5.0 mL of distilled water; add 4 drops of the DAB stock solution; add 2 drops of the H_2O_2 solution. If a gray-black reaction product is desired add 2 drops of the nickel solution. Mix well before use (*see* **Note 28**).
14. Incubate sections with the DAB substrate solution for 10 min at 37°C. Stop reaction by washing sections in PBS.
15. Rinse sections with H_2O for 20 min.
16. Counterstain with methyl green for 5 min (*see* **Note 29**). Dehydrate through 95% and 100% EtOH (2 changes, 1 min each), clear in xylene (three times, 5 min each) and cover slip.
17. Examine slides under a light microscope (*see* **Notes 30 and [31]**).

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[24]Never allow sections to dry out.
[25]All incubation steps are performed in humidified chambers.
[26]Bovine serum albumin should be globulin-free (immunohistochemical grade) when used to block nonspecific protein interactions.
[27]To detect hyaluronan by fluorescent microscopy, fluorescent HABP (16) can be applied instead of bHABP. Sections are rinsed in PBS, cover slipped and examined under a fluorescent microscope.
[28]Sodium azide is an inhibitor of peroxidase activity and should not be included in buffers used to make peroxidase substrate or the ABC reagent.
[29]Counterstaining with Harris haematoxylin can also be used.

18. Controls for hyaluronan staining and specificity include digestion with hyaluronidase and preincubation of the bHABP with excess hyaluronan prior to bHABP application to the tissue section. Sections are digested with *Streptomyces* hyaluronidase (20 U / mL in sodium acetate buffer) at 37°C for 1 h. Undigested control sections are also stained in parallel and incubated in sodium acetate buffer only. For the preincubation experiments, bHABP (at working concentration of 5 μg / mL) is mixed with 100 μg / mL hyaluronan (>1000 kDa) in order to block the hyaluronan-binding sites. Slides are then processed as described in **Steps 6-16**.

3.2. Biochemical Determination of Hyaluronan Content in Tissues

3.2.1. Extraction of Hyaluronan from Pancreas and Lymphoid Tissue

1. Lyophilize frozen tissue and measure dry weight.
2. Digest tissue with proteinase K (250 μg / mL) in 100 m*M* ammonium acetate pH 7.0 overnight at 60°C.
3. Stop the reaction by heating the tissue digest to 100°C for 20 min.

3.2.2. Quantitative Evaluation of Extracted Hyaluronan from Pancreas and Lymphoid Tissue (20)

1. Coat each well (96-well plate) with 100 μL hyaluronan-BSA and incubate for 1h at room temperature.
2. Wash wells with PBS (3 times, 200 μL each).
3. Block with 100 μL of 10% bovine calf serum in PBS for 1h at room temperature.
4. Add an equal volume of 3 mg / mL bHABP in 10% calf serum in PBS to proteinase K-digested tissue samples and to the hyaluronan standards. Incubate for 1 h at room temperature.
5. Rinse the plate with 100 μL PBS after blocking. Add 70 μL aliquot of sample to each well and incubate for 1 h at room temperature.
6. Wash thoroughly with distilled water (4 times, 200 μL each).
7. Incubate for 20 min with 100 μL / well of peroxidase labeled streptavidin (1mg / mL in 50% glycerol, diluted 1:500 in 10% calf serum in PBS).
8. Wash plates with distilled water (4 times, 200 μL each). Add 100 μL of peroxidase substrate consisting of 0.03% H_2O_2, 0.5 mg / mL 3-ethylbenzthiazoline-6-sulfonic acid in 0.1 *M* $C_2H_3NaO_2$, pH 4.2.
9. Terminate the reaction after 30 min by adding 25 μL / well of 2 m*M* sodium azide.

[30]Intense hyaluronan staining has been observed in different human tissues fixed in neutral buffered formalin (34-47). The hyaluronan staining pattern of normal human pancreatic islets does not change with aging. Hyaluronan distribution and abundance are altered in human pancreatic islets in type 1 diabetes, the degree of alteration varying with disease duration and severity of islet inflammation. Increased hyaluronan deposits also occur in human spleen and pancreatic lymph nodes in type 1 diabetes.

[31]The intensity of hyaluronan staining in tissue sections may vary as a function of fixation techniques (48). The bHABP probe generates a more intense hyaluronan staining in mouse tissues fixed in Carnoy's solution than in tissues fixed in neutral buffered formalin. However, the patterns of hyaluronan staining in the mouse tissues are not influenced by the type of the fixative used.

10. Use an ELISA plate reader to determine the OD_{405} reading (*see* **Note 32**).
11. Plot standard curve on semi-log graph. The curve is linear over the hyaluronan concentration range of 50 ng / mL to 1000 ng / mL.

3.3. Determination of Hyaluronan Size in Pancreas and Lymphoid Tissues

3.3.1. Concentration and Purification of Proteinase K-Digested Samples by DEAE Micro-chromatography

1. Digest tissues as described **under Subheading 3.2.1.**
2. Equilibrate DEAE-Sephacrel resins with 8 *M* urea buffer. Pack 300 μL matrix bed by adding 600 μL of DEAE Sephacrel slurry to an Econo column. Wash off any excess resins on the side of the column with 5-10 mL of 8 *M* urea buffer.
3. Spin down the proteinase K-digested sample. Collect supernatant and pour it onto the column. Wait until the sample goes completely into the column.
4. Wash the column with 8 *M* urea buffer (4 times, 10 mL each).
5. Elute hyaluronan with urea buffer with 0.25 *M* NaCl (3 times, 300 μL each)
6. Store eluents at −20°C.

3.3.2. Gel Filtration Chromatography (31)

1. Set the fraction collector to collect fractions every 1.5 min (fraction volume is 0.3 mL).
2. Apply an aliquot (200 μL containing about 6-7 μg of hyaluronan) of DEAE purified sample onto an analytical Sephacryl S-1000 column. Add 10 μL of Vitamin B12 (10 mg / mL, red color) to mark the end of the column.
3. Run the hyaluronan standards to calibrate the column.
4. Elute column with PBS at a flow rate of 12-15 mL / h. The column is completed when the red color is eluted off the column.
5. Subject an aliquot of each fraction to ELISA (*see* **Subheading 3.2.2**.) to generate a hyaluronan profile.

3.4. Histochemical Localization of Heparan Sulfate

1. Cut paraffin sections of mouse pancreas at 4μm and mount sections on untreated Superfrost slides.
2. Dewax in xylene, 2 × 5 min.
3. Rehydrate sections in graded EtOH series (2 × 90%, 2 × 95%, 1 × 70% to tap water).

[32]Hyaluronan ELISA-like assay kits are commercially available for purchase at Echelon Bioscience (Salt Lake City, UT), Corgenix (Broomfield, CO) and R&D Systems (Minneapolis, MN) (28).

4. Treat sections with working buffer: 1 *M* acetate buffer (5 mL), 1*M* $MgCl_2$ (3.25 mL), deionized H_2O (41.75 mL) for 10 min (use 50 mL staining jar).
5. Transfer sections to Alcian blue working solution for 40 min.
6. Wash sections in running tap water for 2 min and flick off excess water.
7. Counterstain in 0.01% safranin for 5 min.
8. Blot sections and air dry completely.
9. Mount in micromount mounting medium and coverslip.
10. Examine slides under a light microscope (*see* **Notes 33-**[38]).

3.5 Immunohistochemical Localization of Heparan Sulfate

1. Paraffin sections of formalin-fixed human pancreas provided by nPOD (*see* **Subheading 2.1.2**).
2. Prepare 0.1 *N* acetic acid and 0.1 *M* sodium acetate, store at 4°C (*see* **Note 39**).
3. Deparaffinize tissue sections in xylene, 2 × 1 min.
4. Rehydrate tissue sections in graded EtOH series (2 × 100%, 1 × 90%, 1 × 70%, tap water (5 min each)).
5. Block endogenous peroxidase activity by incubating sections in 3% H_2O_2 for 10 min.
6. Rinse in PBS, 2 × 2 min, and then in running tap H_2O, 2 × 2 min.
7. Prepare 0.5 mg / mL (0.05%) pronase for antigen retrieval.
8. Transfer the slides to fresh tap H_2O.
9. Remove excess H_2O from sections.
10. Incubate sections with 0.05% pronase in a small humidified immunostaining tray placed in a 37°C incubator for 10 min.
11. Rinse sections in PBS, 2 × 2 min.
12. Prepare 20% Animal Free Block (*see* **Subheading 2.4.2.1**).
13. Prepare 1/13.5 ml dilution of Protein Concentrate (M.O.M. diluent, *see* **Subheading 2.4.2.1**).
14. Dilute the anti-heparan sulfate 10E4 mAb to 0.2 mg / mL in M.O.M. diluent.
15. Dilute isotype control IgM to 0.2 mg / mL in M.O.M. diluent.

[33]Islets in normal mouse pancreas are distinguished from surrounding exocrine pancreas tissue by their heparan sulfate positive staining. Heparan sulfate is localized in insulin-producing islet beta cells (10).
[38]Histochemical staining of heparan sulfate using Alcian blue has also been successfully demonstrated on surgically-resected 10% formalin-fixed human pancreas specimens, using a staining time of 45 min with Alcian blue working solution.
[39]Diluted acetic acid and sodium acetate are prepared immediately prior to the assay.

16. Remove excess PBS from sections and incubate with 20% Animal Free Block for 5 min at room temperature.
17. Tip off Animal Free Block and remove excess from the sections.
18. Incubate sections with 0.2 mg / mL 10E4 mAb or mouse IgM for 30 min at room temperature.
19. Prepare 8 mg / mL AEC stock solution (*see* **Note 40**).
20. Rinse sections with PBS and wash 2 × 2 min in PBS.
21. Dilute secondary rabbit anti-mouse Ig-HRP antibody to 0.03 mg / mL in M.O.M. diluent.
22. Remove excess PBS from sections and incubate in secondary antibody for 30 min at room temperature.
23. Prepare 0.1 *M* acetate buffer (*see* **Subheading 2.4.2.1** and **Note 41**).
24. Prepare AEC working solution: 0.1 *M* acetate buffer (4.75 mL), 8 mg / mL AEC stock solution (0.25 mL), 3% H_2O_2 in deionized H_2O (25 μL). Sterile filter using a CR filter (*see* **Notes 42-44**).
25. Rinse sections with PBS and then wash 2 × 2min in PBS.
26. Remove excess PBS from sections and incubate with AEC working solution for 30 min at room temperature.
27. Rinse sections with deionized H_2O and then wash 3 × in deionized H_2O over 10 min (total wash time).
28. Counterstain in Gill's hematoxylin.
29. Wash 2 × in deionized H_2O, 2 × brief immersion in diluted ammonium H_2O, wash 2 × in deionized H_2O.
30. Mount in liquid Glycergel mounting medium and coverslip.
31. Examine slides under a light microscope.

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[40]Stock AEC solution is prepared during incubation of sections with primary antibody (or isotype control Ig), and then stored at 4°C until the preparation of the AEC working solution.
[41]0.1 *M* acetate buffer is prepared during the secondary antibody incubation.
[42]AEC working solution is protected from light and used within 2 h of preparation.
[44]Prepare liquid Glycergel by warming stock bottle in a beaker of hot tap water

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