Chapter 11

Characterization and Analysis of Extracellular Matrix in Malignant Brain Tumors and Their Cellular Derivatives

Maksim Sinyuk, Justin D. Lathia, and Mariano S. Viapiano

Abstract

The neural extracellular matrix (ECM) is a key regulator of cellular phenotype in normal and diseased states. Analytical approaches have helped uncover the myriad of roles the ECM plays in a variety of diseases including malignant gliomas, the most prevalent and deadly primary brain tumors. Major components of the glioma ECM include secreted proteoglycans as well as basal lamina proteins such as laminins. Characterization of secreted proteoglycans by protein blotting is an invaluable tool to understand their molecular complexity. To provide complete characterization of these molecules, tissue specimens must be first processed to separate insoluble (structural) from soluble proteoglycans. Here, we describe the procedure to separate subcellular fractions by differential centrifugation and extract chondroitin sulfate proteoglycans. Further treatment of these fractions using glycosidases to remove specific carbohydrates, followed by protein electrophoresis and western blotting, provides rich information about the composition and variability of the glioma matrix. To determine ECM protein localization in vivo, immunofluorescence analysis techniques are required. Here we describe commonly used approaches for protein analysis using fluorescence antibody detection in primary human tumor tissue and patient-derived xenografts. Additionally, the characterization of a cancer stem cell fraction in these tumors has received much attention, and we provide the methodology for the visualization of ECM proteins and carbohydrates by immunofluorescence in three-dimensional tumorspheres. These techniques are particularly relevant to correlate matrix expression and changes with glioma growth and invasion or to follow up potential microenvironmental biomarkers during therapeutic interventions.

Key words Chondroitin sulfate proteoglycan, Chondroitinase, Glycosidase, Immunoblotting, Glycovariants, Immunofluorescence, Integrins, Laminins, Tumorspheres, Cancer stem cell

Abbreviations

AP	Alkaline phosphatase
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CNS	Central nervous system
CSPG	Chondroitin sulfate proteoglycan
DTT	Dithiothreitol
ECM	Extracellular matrix
HA	Hyaluronic acid

Jennie B. Leach and Elizabeth M. Powell (eds.), *Extracellular Matrix*, Neuromethods, vol. 93,
DOI 10.1007/978-1-4939-2083-9_11, © Springer Science+Business Media New York 2015

HABP	Hyaluronic acid-binding protein
NBT	Nitroblue tetrazolium chloride
NEB	New England Biolabs
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde

1 Introduction

Malignant gliomas are the most common and aggressive primary tumors of the central nervous system (CNS) [1]. Analysis of the native extracellular matrix (ECM) of these tumors provides a wealth of information about the composition and organization of the immediate microenvironment surrounding glioma cells [2]. The ECM of normal CNS has a unique composition compared to the ECM of other tissues, with a scaffold of hyaluronic acid (HA) and secreted proteoglycans surrounding neural cells [2, 3], while major fibrillar proteins (such as collagens, laminin, and fibronectin) are largely concentrated in or near vascular structures [4–6]. However, the ECM produced by glioma cells recovers in part the mesenchymal/fibrillar features found in other solid tumors, and, in addition to HA and proteoglycans, the tumor stroma contains fibrillar components absent from normal brain [2, 7–9]. Thus, it is critical to acknowledge this molecular complexity to better understand the role of the glioma ECM in regulating tumor growth and invasion through the CNS parenchyma [2, 4, 10].

Examples of the molecular complexity of the glioma ECM are represented by the major group of secreted chondroitin sulfate proteoglycans (CSPGs) in the matrix, as well as by predominant fibrillar proteins secreted by glioma cells, such as laminins. The major group of CSPGs in the brain and glioma ECM is composed by the HA-binding *lectican* family (aggrecan, versican, neurocan, and brevican) and a soluble form of the receptor phosphatase RPTPζ, known as phosphacan [2, 7, 10]. Although CSPGs are major structural ECM components that limit cell motility in the CNS—including dispersion of metastatic tumors—CSPGs secreted by glioma cells are uniquely glycosylated or cleaved, and these glioma-specific variants facilitate invasion through the CNS parenchyma [10–14]. On the other hand, recent evidence has revealed a key role of laminins and their major receptors (integrins) in glioblastoma tumor progression. For example, laminins expressed by glioblastoma cells and tumor-associated endothelial cells were found to contribute to tumor maintenance by protecting tumor cells from radiation-induced damage [6]. Similarly, integrin α6, a receptor for laminin that is absent in adult neural cells, was nevertheless found in glioma stemlike cells and shown to be critical for glioma cell self-renewal, proliferation, and tumor formation [15].

A major challenge to analyzing, and potentially targeting, ECM components is their high degree of molecular variability.

CSPGs in particular exhibit multiple splice variants, differentially glycosylated forms (*glycovariants*), and stable proteolytic fragments that have as much—if not more—functional relevance as the full-length molecules [2, 10, 16]. Analysis of CSPG mRNA and conventional immunohistochemistry are usually insufficient to reveal CSPG posttranslational variability, and only in a few cases CSPG variants can be detected by these techniques [17]. Therefore, conventional polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting have largely been adopted as the best option to provide accurate information about expression and structural variability of brain- and glioma-derived CSPGs. Other ECM components such as HA and fibrillar proteins exhibit less molecular complexity than CSPGs; therefore, immunohistochemical methodologies have proven extremely versatile to investigate these ECM components. However, proper cell and tissue preparation prior to staining is in many cases nontrivial and paramount to garner optimal signal.

Both technical approaches (biochemistry to analyze CSPGs, immunochemistry to analyze HA and laminins) require very specific steps to minimize damage to samples and ensure that the epitopes of importance are preserved. When properly used, they become particularly powerful to reveal the complexity of ECM molecules and demonstrate how different ECM components co-localize with specific cell types in the CNS and brain tumor stroma. Here, we describe first a procedure to prepare subcellular fractions from fresh tissue by differential centrifugation [18, 19], followed by characterization of CSPGs in the different fractions by selective deglycosylation, SDS-PAGE, and western blotting [20, 21]. Next, we describe a procedure to prepare sections derived from tumor tissues or their cellular derivatives, for optimal detection of ECM components and their receptors. Since the procedures for tumor xenografting and protein electrophoresis are fairly conventional, they will only be briefly mentioned, with most attention paid to procedures for subcellular fractionation, protein deglycosylation, and tumorsphere immunochemistry.

2 Materials

All solutions should be prepared in ultrapure deionized or double-distilled water, with electrical resistance of 18 MΩ at 25 °C. All reagents should be of analytical grade. Stock solutions must be prepared at room temperature and, unless indicated otherwise, filtered through Whatman filter paper of grades 2–3 (6–8 μm pore). Working solutions should be prepared no more than 1 or 2 days before the experiments and stored at the indicated temperatures. No preservatives should be added to the solutions, in particular those that will be used with enzymes. All enzymes should be aliquoted in sterile microcentrifuge tubes and used no longer than

6 months after the expiration date indicated in their label. Any waste solutions that have contacted brain or tumor tissue should be disposed according to institutional biosafety regulations. All work with animals must follow American Veterinary Medical Association guidelines and must be approved by the Institutional Animal Care and Use committee.

2.1 General Stock Solutions and Materials for Subcellular Fractionation

1. *Stock Tris–HCl buffer*: 1 M Tris–HCl, pH 7.6. Dissolve 121.14 g Tris base in 900 mL water. Adjust the pH to 7.6 with HCl and complete volume to 1 L. Store at room temperature.
2. *Stock sucrose solution for protein analysis*: 1 M sucrose. Prepare 342.30 g sucrose in 1 L water as indicated in step 1; do not adjust pH. The stock sucrose solution can be aliquoted in 50 mL aliquots and frozen at −20 °C. Thaw the aliquots at room temperature and keep leftovers at 4 °C (do not refreeze) for up to a week.
3. *Stock NaCl solution*: 2.5 M NaCl. Weigh 146.13 g NaCl and prepare a 1 L solution as indicated in step 1. Store at room temperature.
4. *Stock guanidine solution*: 8 M guanidine hydrochloride. Weigh 191.06 g guanidine hydrochloride and dissolve it in 250 mL water as indicated in step 1. Do not adjust pH. Store at room temperature without filtration.
5. *Stock acetate/acetic buffer*: 200 mM sodium acetate—acetic acid—pH 5. Weigh 4.10 g sodium acetate anhydrous (or 6.80 g sodium acetate trihydrate) and dissolve in 200 mL. Adjust the pH with acetic acid and complete the volume to 250 mL. Filter through Whatman paper and store at room temperature.
6. *Stock Triton solution*: Triton X-100 20 % w/v. Weight 20 g of pure Triton X-100 (Sigma-Aldrich X100) in a graduated cylinder and complete with water to 100 mL. Mix well, filter through 0.45 μm-pore filter, and store at room temperature. Discard this stock solution after 6 months.
7. *Protease inhibitors*: There is a large variety of commercial protease inhibitor cocktails that have better shelf life, reproducibility, and safety than "homemade" preparations, which are not recommended (*see* **Note 1**). The composition of most of these cocktails is well known, and they can be purchased as water-soluble tablets or 100× stock solutions. When using tablets, we recommend preparing a 50× solution in water (e.g., if a tablet is rated for 50 mL solution, dissolve it in 1 mL water and add the necessary volume to the tissue homogenization solution). Protease inhibitors should be added to the working solutions just before use. Use inhibitor cocktails *without EDTA* (*see* **Note 2**). If the absence of EDTA creates a concern about residual metalloprotease

activity use 1,10-phenantroline as alternative metalloprotease inhibitor (*see* **Note 3**).

8. *Tissue homogenization buffer*: 25 mM Tris–HCl, 320 mM sucrose, 1× protease inhibitors, pH 7.6. Prepare 50 mL for every 1.5 g of tissue to process (1.25 mL stock Tris–HCl buffer, 16 mL stock sucrose solution, protease inhibitors as indicated by the manufacturer and water to a final volume of 50 mL). Filter through 0.45 μm-pore filter and store at 4 °C.
9. *Proteoglycan extraction solution*: 4 M guanidine chloride, 0.5 % v/v Triton X-100, pH 5.8. Mix 50 mL stock guanidine solution, 2.5 mL stock Triton solution and 25 mL water. Measure the pH and add stock acetate/acetic buffer until the pH is 5.8. Complete with water to 100 mL, filter through 0.45 μm-pore filter, and store at 4 °C.
10. *Acid ethanol for precipitation*: Mix 1 volume of stock acetate/acetic buffer (pH 5) with 12 volumes of absolute ethanol (final concentration of sodium acetate: 15–16 mM).
11. *Tissue homogenizer apparatus*: Use a Potter-Elvehjem or Dounce tissue grinder (Corning or Wheaton, Fisher Scientific, Waltham MA) (*see* **Note 4**). The Dounce grinder must be operated manually while the Potter-Elvehjem can be connected to a vertical-standing stirrer motor (e.g., T-Line stirrer from Troemner, Thorofare, NJ, maximum speed 7,500 rpm).
12. *Centrifuge*: An appropriate centrifuge and rotor will be needed to centrifuge the samples at forces ranging from 900 × *g* up to 100,000 × *g*. We recommend floor models even for small-volume samples. Benchtop minicentrifuges are appropriate to sediment the highest-density fraction (nuclei-enriched fraction), but lower-density membrane fractions and soluble proteins will not be fully separated in these units.
13. *Protein measurement*: We recommend a Bradford assay-based commercial reagent (e.g., Protein Assay Dye Reagent Concentrate, Bio-Rad, Hercules, CA) with a suitable bovine serum albumin standard. Albumin can be purchased from Bio-Rad (#500-0002) or Sigma-Aldrich (A7906).

2.2 Deglycosylation Components

1. *Stock SDS solution*: Sodium dodecyl sulfate (SDS) 20 % w/v. Dissolve 100 g SDS in 500 mL water prewarmed at 40–45 °C. Once the solution is completely clear, filter through 0.45 μm-pore filter and store at room temperature. Discard this stock solution after 6 months.
2. *Stock Nonidet P-40 solution*: Nonidet P-40 10 % w/v (*see* **Note 5**). Weight 1 g of Nonidet P-40 in a graduated conical tube and complete with water to 10 mL. Mix well, filter through 0.45 μm-pore filter, and store at room temperature. Discard this stock solution after 6 months.

3. *Stock deglycosylation buffer* (*10×*): 200 mM Tris–HCl, 250 mM NaCl, 200 mM sodium acetate, pH 7.5. Dissolve 4.1 g sodium acetate anhydrous in 150 mL water. Add 50 mL stock Tris–HCl buffer (1 M, pH 7.6) and 25 mL stock NaCl solution (2.5 M) and carefully adjust the pH to 7.5 using diluted HCl (0.05 N). Complete the volume to 250 mL with water, filter through 0.45 μm-pore filter, and store at room temperature.
4. *Deglycosylating enzymes*: Table 1 lists common glycosidases used to selectively remove carbohydrates from CSPGs (*see* **Note 6**). Aliquot and store according to the manufacturer's instructions. Avoid multiple freeze-thawing of aliquots.

Table 1
Common enzymes used to selectively deglycosylated CSPGs

Enzyme	Activity	Limitations	Sources
N-glycosidase F (PNGase F) EC 3.5.1.52 [30]	Removes most types of N-linked oligosaccharides by cleaving between the innermost GlcNAc and the Asn residue in the protein	Cannot remove fucosylated GlcNAc (GlcNAc-α(1, 3)-Fuc)[a]. Complete removal of *N*-glycans requires protein denaturation	*Elizabethkingia meningosepticum* NEB (P0704, 500 U/mL) Sigma (G1549, 300 U/mL)
N-glycosidase A EC 3.5.1.52	Same as *N*-glycosidase F but can also remove fucosylated GlcNAc	Strict optimum pH around 5.0 makes it difficult to combine with other glycosidases. Requires protein denaturation	Almond extract Roche (11642995001, 50 U/mL)
O-Glycosidase (endo-α-*N*-acetyl-galactosaminidase) EC 3.2.1.97 [31]	Removes O-linked disaccharides by cleaving between their innermost GalNAc and the Ser or Thr residue in the protein	Only removes *core 1* (Gal-β(1,3)-GalNAc) and *core 3* (GlcNAc-β(1,3)-GalNAc) disaccharides. Other glycosidases are first required to remove residues from extended O-linked glycans[b]. Cannot remove O-linked mannosylation that is common in neural CSPGs	*Diplococcus pneumoniae* (Roche 11347101001, 500 mU/mL) *Enterococcus faecalis* (NEB P0733, 40,000 mU/mL)
Sialidase (neuraminidase) EC 3.2.1.18	Removes most types of terminal sialic acid residues linked to N-linked and O-linked glycans	Optimum pH around 5.0–5.5 results in reduced activity when combined with other glycosidases at pH 7.0–7.5	*Arthrobacter ureafaciens* (Roche 10269611001, 10 U/mL) *Clostridium perfringens* (NEB P0720, 50 U/mL)

(continued)

Table 1 (continued)

Enzyme	Activity	Limitations	Sources
Chondroitinase ABC (chondroitin ABC lyase) EC 4.2.2.4	Removes chondroitin-4-sulfate (CS-A), dermatan sulfate (CS-B), chondroitin-6-sulfate (CS-C), and chondroitin. It can also degrade HA slowly	Optimum conditions require pH 7.5–8.0 and 25–50 mM acetate in the reaction buffer	*Proteus vulgaris* Sigma (C3667, 5 U/vial, 10 U/mL)
Chondroitinase AC (chondroitin AC lyase) EC 4.2.2.5	Similar to chondroitinase ABC but specific for chondroitin CS-A and CS-C	Available formulation contains albumin as stabilizer, which may cause difficult detection of some proteins by western blotting	*Flavobacterium heparinum* Sigma (C2780, 5 U/vial, 10 U/mL)
β (1–4) galactosidase EC 3.2.1.23	Removes terminal β(1–4) linked Gal residues from N- and O-linked glycans	Optimum pH 5.5–6.0. When combined with other glycosidases, more enzyme or longer incubation times are needed	*Bacteroides fragilis* (NEB 8,000 mU/mL) *Diplococcus pneumoniae* (Sigma, 6 U/mg)
β-*N*-acetyl-glucosaminidase EC 3.2.1.52	Removes terminal β linked GlcNAc residues from N- and O-linked glycans	Optimum pH around 5.0 requires more enzyme and longer incubation times when combined with other glycosidases. With sialylated glycans, sialidase must be added to remove terminal sialic acid	*Xanthomonas manihotis* (NEB 4,000 mU/mL)

GlcNAc N-acetylglucosamine, *GalNAc N*-acetylgalactosamine, *Gal* galactose, *Fuc* fucose, *NEB* New England Biolabs (Ipswich, MA), *Sigma* Sigma-Aldrich, *Roche* Roche Applied Biosciences

[a]Fucosylated GlcNAc is found in N-linked carbohydrates in plants and insects but not in mammalian cells

[b]*O*-glycosidase is most commonly used together with sialidase

5. *Enzymatic deglycosylation cocktail*: In a microcentrifuge tube combine the following (*see* **Note 7**):

Water	32 μL
10× deglycosylation buffer	10 μL
50× protease inhibitor cocktail	2 μL
Chondroitinase ABC (Sigma)	1 μL (final conc. 100 mU/mL)
Sialidase (Roche)	1 μL (final conc. 100 mU/mL)
O-glycosidase (Roche)	2 μL (final conc. 10 mU/mL)

(continued)

(continued)

N-glycosidase F (Sigma)	1 μL (final conc. 5 U/mL)
β(1–4)-galactosidase (NEB)	0.5 μL (final conc. 40 mU/mL)
β-*N*-acetylglucosaminidase (NEB)	0.5 μL (final conc. 20 mU/mL)
Total volume	50 μL
If used for denaturing deglycosylation, reduce water in the cocktail from 32 to 22 μL, for a final volume of 40 μL (*see* Sect. 3.3.4)	

2.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Components

For complete details and procedures to prepare, run, and transfer SDS-polyacrylamide gels, we recommend the excellent guide from Davis et al. [22]. Only major reagents are listed here:

1. *Stacking gel buffer*: 0.5 M Tris–HCl, pH 6.8. Prepare 30.29 g Tris base in 500 mL water, adjusting pH to 6.8 with HCl.
2. *Resolving gel buffer*: 1.5 M Tris–HCl, pH 8.8. Prepare 90.86 g Tris base in 500 mL water, adjusting pH to 8.8 with HCl.
3. *Running buffer*: 25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.0–8.3. Dissolve 3.03 g Tris base, 14.43 g glycine, and 5 mL SDS stock solution in 1 L water. Measure pH but do not adjust; discard the solution if pH is not in the expected range. Use this solution immediately.
4. *Stock Laemmli buffer* (*4*×): Combine 8 mL stacking gel buffer, 8 mL pure glycerol, 1.6 g SDS and complete to 20 mL with water. Let the SDS dissolve and all components mix thoroughly. Finally add 5 μL of bromophenol blue (Sigma-Aldrich B0216, dissolved 2 % w/v in water). Aliquot in 750 μL aliquots and store at −20 °C.
5. *Reducing Laemmli buffer* (*3*×): Mix 750 μL of 4× Stock Laemmli buffer with 250 μL pure 2-mercaptoethanol (Bio-Rad, 14.3 M). Store at −20 °C and use within a week.
6. *Reducing Laemmli buffer* (*3*× alternative, *see* **Note 8**): Dissolve 0.19 g dl-dithiothreitol (DTT, Sigma-Aldrich D0632) in 1 mL water prewarmed at 40 °C to make a 1.2 M DTT solution. Mix 750 μL of 4× stock Laemmli buffer with 250 μL DTT solution. Use this 3× reducing Laemmli buffer immediately and discard leftovers. Store the DTT solution at −20 °C and use within a week.
7. Acrylamide/Bis-acrylamide solution: Use a 30 % acrylamide solution with acrylamide:bis-acrylamide ratio 37.5:1 (30 g acrylamide + 0.8 g N′N′-bis-methylene-acrylamide in 100 mL water) (*see* **Note 9**).
8. *Vertical electrophoresis equipment*: There are many commercial options available. One of the most popular systems is the Mini-Protean® cell from Bio-Rad, used with 1.5 mm-thick gels.

9. *Gradient maker*: Required to prepare gradient acrylamide gels for CSPGs. Each side of the gradient maker must hold half the volume of the final gel. The model GM-20 from CBS Scientific (San Diego, CA) is suitable for a single gel 8 cm tall × 10 cm wide × 1.5 mm thick (Bio-Rad Mini-Protean standard).
10. *Molecular weight (MW) markers*: We recommend two pre-stained standards: Precision Plus Kaleidoscope (Bio-Rad, range 10–250 kDa) and HiMark high molecular weight standard (Invitrogen, approximate range 35–500 kDa when run in Tris–Glycine gels).

2.4 Western Blotting Components

1. *Towbin buffer*: 25 mM Tris, 192 mM glycine, 20 % v/v methanol, pH 8.0–8.3. Dissolve Tris base and glycine in 800 mL water and complete to 1 L using methanol (*see* **Note 10**). Do not adjust pH. Prepare fresh, keep at 4 °C until the moment of use, and discard leftover buffer.
2. *Transfer assembly for vertical electrophoresis*: The Trans-Blot® module from Bio-Rad can be inserted in the Mini-Protean® cell to transfer proteins to nitrocellulose or PVDF.
3. *Western blotting membrane*: Unsupported nitrocellulose membranes with 0.2 μm pore size (Bio-Rad #162-0112) are excellent for everyday use (*see* **Note 11**). PVDF membranes (Bio-Rad #162-0177) are useful for multiple re-probing or specific procedures (*see* nonenzymatic deglycosylation in membranes, in Sect. 3.4).
4. *Protein detection solution*: 0.1 % w/v amido black, 10 % v/v acetic acid, 25 % v/v isopropanol. In a graduated cylinder, combine 0.25 g amido black (Sigma-Aldrich Naphthol Blue Black N3393), 62.5 mL isopropanol, 25 mL glacial acetic acid and complete to 250 mL with water. Mix well, filter through Whatman paper, and store at room temperature (*see* **Note 12**).
5. *Low-salt washing buffer*: 50 mM Tris–HCl, 150 mM NaCl, 0.2 % v/v Tween-20. Combine 50 mL stock Tris–HCl solution (1 M), 60 mL stock NaCl solution (2.5 M), and 2 mL pure Tween-20 (Sigma-Aldrich) and complete to 1 L. Store at 4 °C.
6. *High-salt washing buffer*: Prepare as low-salt washing buffer, but use 200 mL stock NaCl solution for a final concentration of 500 mM NaCl.
7. *Alkaline phosphatase (AP) buffer*: 100 mM Tris–HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 9. Dissolve 12.11 g Tris base, 5.84 g NaCl, and 1.02 g magnesium chloride hexahydrate in 900 mL water. Adjust pH to 9.0 with HCl, complete to 1 L with water, filter, and store at room temperature.

8. *Nitroblue tetrazolium chloride* (*NBT*): Prepare a 70 % v/v solution of *N*,*N*-dimethylformamide (Sigma-Aldrich D4551) by mixing 14 mL dimethylformamide and 6 mL water. Dissolve 1 g *p*-NBT (Sigma-Aldrich N6639) in 20 mL 70 % dimethylformamide, aliquot in 1 mL aliquots, and store at −20 °C (*see* **Note 13**).
9. *5-Bromo-4-chloro-3-indolyl phosphate* (*BCIP*): Dissolve 500 mg of BCIP *p*-toluidine (Sigma-Aldrich B8503) in 10 mL dimethylformamide. Aliquot and store in the same manner as NBT (*see* **Notes 13** and **14**).
10. *Antibodies*: Table 2 lists some of the most common antibodies described in the literature to detect ECM CSPGs and carbohydrates. Aliquot and store according to the manufacturer's instructions. Avoid multiple freeze-thawing of aliquots.

Table 2
Common antibodies used to detect CSPGs and glycosaminoglycans

Antigen	Major isoforms and fragments	Suggested antibodies	Epitope	Species[a]
Aggrecan 480 kDa ~100 CS	• Multiple glycovariants with different O-linked glycans and CS chains • Splice variants with and without EGF motif (rat)	Cat-301 (Millipore MAB5284)	Core protein [17]	r, m, h
		Cat-315 (Millipore MAB1581)	O-linked mannosyl glycan [32]	r, m, h
		Cat-316 (Millipore MAB1582)	CS motif [17]	r, m, h
Versican 600 kDa ~ 20 CS	• Four major splice variants: *V0* (600-kDa, 20 CS), *V1* (480 kDa, 14 CS), *V2* (380 kDa, 7 CS), *V3* (90 kDa, no CS). One N-terminal fragment (*GHAP*) originated by ADAMTS cleavage	12C5 (DSHB)	Core protein (N-terminal HA-binding domain). *Detection is strongly diminished in reduced samples*	r, h
Neurocan 250 kDa 3–7 CS	• Two cleavage fragments generated by MMP2: 130 kDa (N-term) and 150 kDa (C-term) • N-term cleavage product, ~90 kDa (unknown protease)	650.24 (Millipore MAB5212)	Full-length and C-term [33]	r
		1F6 (DSHB)	Full-length and N-term [33]	r
		AF5800 (RD)	Full-length and C-term	r, m
Phosphacan 180 kDa 3–4 CS	• Soluble isoform of the phosphatase receptor RPTPζ • Multiple glycovariants with different O-linked glycans and CS chains	3F8 (DSHB)	O-linked mannosyl glycan [32]	r, h
		H300 (SCBT SC-25432)	N-terminal core protein	r, m, h
		122.2 (Millipore MAB5210)	Core protein	r

(continued)

Table 2 (continued)

Antigen	Major isoforms and fragments	Suggested antibodies	Epitope	Species[a]
Brevican 150 kDa 1–3 CS	• Splice variant bound to cell membrane by GPI • Multiple glycovariants in glioma compared to normal brain • ADAMTS-cleavage products: 50 kDa (N-term) and 90 kDa (C-term)	2/Brev (BD 610894) B6 (noncommercial)[b] B50 (noncommercial)[b]	Full-length and N-term fragment AA 506–529 of rat brevican [20] AA 389–395 of rat brevican [21]	m r, h r, m, h
Chondroitin sulfate ΔDi-4S	Chondroitin-4-sulfate (CS-A)	BE123 (Millipore MAB2030)	CS-A stubs detected after treatment with chondroitinase	r, m, h
Chondroitin sulfate ΔDi-6S	Chondroitin-6-sulfate (CS-C)	MK302 (Millipore MAB2035)	CS-C stubs detected after treatment with chondroitinase	r, m, h
Chondroitin sulfate	n/a	CS-56 (Sigma C8035)	CS-A and CS-C in intact CSPGs (no chondroitinase treatment)	r, m, h
Hyaluronic acid	HA of low (2,000–5,000 Da) and high (>1,000 kDa) MW	biotinylated HABP (Millipore 385911)	(GlcA-β(1,4)-GlcNAc)$_4$, abolished by hyaluronidase treatment[c]	r, m, h

Approximate MW calculated by denaturing SDS-PAGE
CS estimated number of chondroitin sulfate chains/protein, *SCBT* Santa-Cruz Biotechnology (Santa Cruz, CA), *BD* Becton-Dickinson Biosciences (San Jose, CA), *DSHB* Developmental Studies Hybridoma Bank (Iowa City, IA), *RD* R&D Systems (Minneapolis, MN), *GHAP* glial hyaluronate binding protein, *ADAMTS* a disintegrin and metalloprotease with thrombospondin motifs, *MMP* matrix metalloprotease
[a]Species where the antibodies have tested positive in western blot or immunochemistry (*r*, rat; *m*, mouse; *h*, human)
[b]Generated by Russell Matthews, Dept. Neuroscience and Physiology, SUNY Upstate Medical University
[c]Tetramer of (glucuronic acid)-β(1,4)-(*N*-acetylglucosamine), identified by D. Katta, *Proceedings of the 2003 Intl. Soc. of Hyaluronan Sciences Meeting* (unpublished)

2.5 General Stock Solutions and Materials for Immunochemistry

1. *Stock 10× PBS* (Dulbecco's phosphate-buffered saline, Harlow and Lane formulation [23]): 100 mM phosphate, 1.5 M NaCl, pH 7.4. Dissolve 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4 (anhydrous), and 2.4 g KH_2PO_4 (anhydrous) in 900 mL water. Complete volume to 1 L and store at room temperature. The pH of this buffer should be 6.75–6.95, and adequate pH adjustment may be needed for solutions using 1× PBS.
2. *4% w/v paraformaldehyde (PFA)*: PFA should be prepared in a chemical fume hood (*see* **Note 15**). Weigh 40 g of PFA

powder (Sigma-Aldrich 158127) and place in a 1,000 mL Erlenmeyer flask with a stir bar. Heat 600 mL of water in a microwave for 3–4 min, and add it slowly to the Erlenmeyer flask. Place the flask on a hot plate inside a chemical fume hood and heat it while stirring, until the water reaches 60 °C (do not heat over 70 °C). Add 3 mL of 5 N NaOH with a glass pipette to fully solubilize the PFA. Allow the solution to stir for 5 min until clear, and add 100 mL stock 10× PBS and 300 mL water. Let the solution cool to room temperature and adjust the pH to 7.2. Complete the final volume to 1 L, filter, and aliquot into 50 mL conical tubes. Store aliquots at −20 °C and use within 1 month.

3. *30 % w/v Sucrose solution*: To 400 mL water, add 150 g sucrose and 50 mL stock 10× PBS. Warm the solution to completely dissolve the sucrose, and complete the volume to 500 mL with water. Do not adjust the pH. Filter the solution and store in aliquots at 4 °C.

4. *Optimal cutting temperature (O.C.T.®) compound*. There are several commercial formulations available, based on glycerol and a mixture of low-freezing point resins. We recommend Sakura's Tissue-Tek O.C.T. Compound (Cat # 4583) supplied by VWR (Cat # 25608-930).

5. *Hydrophobic pen*: A hydrophobic pen (commonly known as "PAP pen") is a felt marker that dispenses a hydrophobic mixture to keep aqueous solutions (e.g., antibody solutions) confined to the tissue section. Hydrophobic pens are available from multiple commercial suppliers (*see* **Note 16**).

6. *Antibodies*: Primary and secondary antibodies for immunochemistry are usually available from commercial sources. Antibodies against CSPGs and carbohydrates have been identified in Table 2. Specific antibodies for laminins and their integrin receptors can be found in references [6, 15]. Aliquot and store antibodies according to the manufacturers' instructions. Avoid multiple freeze-thawing of the aliquots.

7. *HA-binding protein (HABP)*: Biotinylated HABP (EMD Millipore, cat #385911) is an HA-binding protein derived from bovine cartilage that has high affinity for high-MW HA and lesser affinity for the CSPG aggrecan [24] (*see* Table 2). It can be detected with fluorochrome-conjugated streptavidin (Invitrogen).

8. *Blocking solution for antibodies*: In a conical tube, mix 1 mL stock 10× PBS, 8 mL water, and 1 mL normal goat serum. Mix well and add 10 μL pure Triton X-100 (Sigma-Aldrich) or 50 μL of the 20 % Triton X-100 solution described in Sect. 2.1, step 6.

9. *Blocking solution for HABP*: Dilute 10 mL fetal bovine serum in 500 mL of water, filter through 0.45 μm-pore filter, and store at 4 °C until ready for use.

10. *PBS-T solution*: Mix 50 mL stock 10× PBS with 450 mL water, and add 50 μL pure Triton X-100 (or 250 μL of 20 % Triton-X100 solution) for a final concentration of 0.1 % v/v detergent solution.
11. *Mounting medium*: The selection of mounting medium after immunochemistry depends on the chosen method used to detect immunoreactivity (e.g., peroxidase, fluorescence, etc.). For epitopes detected by immunofluorescence, we recommend an aqueous mounting medium without glycerol to provide low background fluorescence, for example, *FluorSave Reagent* (cat #345789, EMD Millipore, Billerica MA). Many commercial alternatives are equally available.

3 Methods

The first method described here is a subcellular fractionation that yields one final fraction with soluble ECM proteins and several organelle-enriched pellets with insoluble or membrane-associated ECM molecules (mostly CSPGs). Membrane-bound CSPGs can be resuspended, deglycosylated, and analyzed by SDS-PAGE but must be extracted from the membranes for other biochemical tests such as immunoprecipitation. Partial solubilization can be achieved with detergent-containing buffers, but complete extraction usually requires guanidine hydrochloride to solubilize aggregated CSPGs. The second method described here is an immunostaining protocol that can be applied to both tissue samples as well as tumorspheres. Although the preparation of these two types of samples is similar, tumorspheres are typically too small to be seen by the naked eye, and their fixation and embedding steps are more challenging compared to tissue specimens. Special care (and patience) is recommended to manipulate tumorspheres as well as regular maintenance of these cellular derivatives using consistent culture conditions before they are ready to be embedded and sectioned.

3.1 Subcellular Fractionation for Biochemical Studies

1. Dissect and weight the tumor or normal tissue (*see* **Note 17**). Add 10 volumes of ice-cold homogenization buffer (e.g., 1 mL for every 0.1 g tissue), and thoroughly homogenize the tissue for 1 min (*see* **Note 18**). Avoid the formation of foam during homogenization. Reserve 1/10 of this total homogenate to measure protein concentration and to prepare for SDS-PAGE. Subsequent steps 3–7 are summarized in Fig. 1.
2. Weight the centrifuge tubes before adding the homogenized tissue; this will help estimate the weight of the remaining pellets after each centrifugation step.
3. Centrifuge the tissue homogenate at $900 \times g \times 10$ min. *Very carefully* collect the supernatant with a disposable plastic transfer pipette, and transfer it to a new centrifuge tube (*see* **Note 19**).

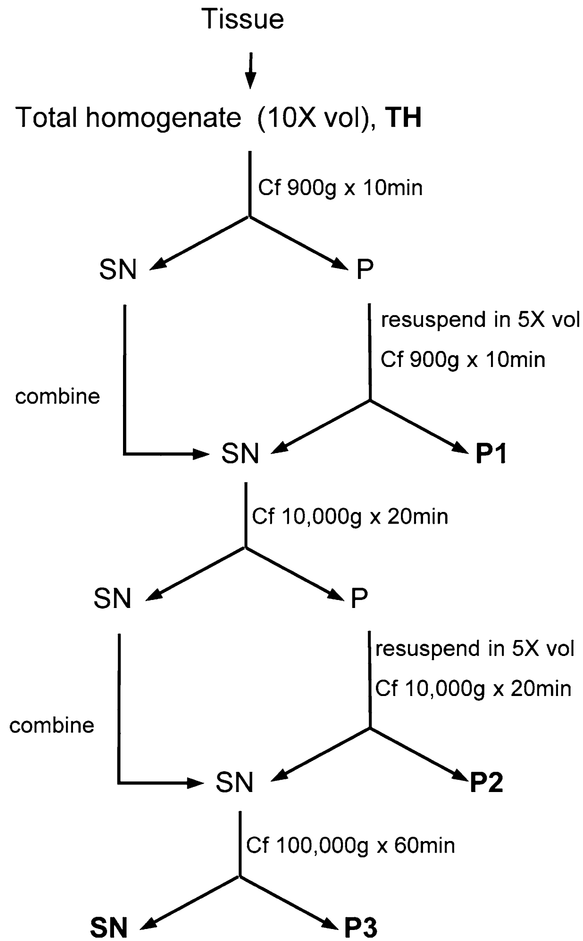

Fig. 1 Subcellular fractionation. Summarized procedure to fractionate tissue homogenates by differential centrifugation

4. Resuspend the pellet in five volumes of tissue homogenization buffer. Centrifuge the resuspended pellet again, and add the new supernatant to the supernatant collected in the previous step. The remaining pellet (P1) is the fraction enriched in cell nuclei and insoluble ECM. Reserve the pellet P1 on ice.
5. Centrifuge the collected supernatants at $10{,}000 \times g \times 20$ min. Collect the supernatant with a disposable transfer pipette, and transfer it to a new centrifuge tube.
6. Resuspend the pellet in five volumes of homogenization buffer, and repeat the centrifugation as in step 4, combining the supernatants. The second remaining pellet (P2) is the fraction enriched in heavy mitochondria, myelin, and microsomes.
7. Centrifuge the collected supernatants at $100{,}000 \times g \times 60$ min. Decant the supernatant into a new centrifuge tube. Rinse

the pellet with homogenization buffer without disrupting it. The final pellet (P3) is the fraction enriched in light endoplasmic reticulum and plasma membrane. The final supernatant (SN) is enriched in soluble cytosolic proteins and soluble ECM proteins.

8. For complete extraction of insoluble CSPGs and other aggregated ECM proteins (e.g., tenascins and collagens), go to the next section. Otherwise, resuspend P1, P2, and P3 in homogenization buffer (for every 1 mL of initial homogenate volume, use 0.2 mL to resuspend P1 and P2 and 0.1 mL for P3). Measure the protein concentration of the initial homogenate and the four isolated fractions (P1, P2, P3, SN) (*see* **Note 20**).

3.2 Extraction of Aggregated or Poorly Soluble ECM Molecules

1. Resuspend the desired pellets (P1–P3) in ice-cold proteoglycan extraction solution (use 1 mL for every 1 mL of initial tissue homogenate).
2. Stir or shake the samples at 4 °C for 2 h.
3. Centrifuge the extracts at 20,000 × *g* × 20 min. The supernatants will contain hard-to-solubilize ECM proteins, mostly CSPGs (*see* **Note 21**). Discard the pellets remaining after extraction (they contain insoluble tissue debris including DNA, myelin, and aggregated proteins).
4. For each volume of solubilized ECM proteins, add five volumes of ice-cold acid ethanol and let the extracts precipitate overnight at −20 °C.
5. Centrifuge the samples at 20,000 × *g* × 20 min. Carefully remove the supernatant and discard it.
6. Wash the pellet once in 1 volume of acid ethanol and centrifuge as in step 5.
7. Let the pellet air-dry for a few minutes to evaporate the ethanol.
8. Prepare a resuspension buffer suitable for the desired procedure (e.g., immunoprecipitation, deglycosylation, etc.), containing 0.5 % w/v CHAPS detergent.
9. Gently resuspend the pellet in one volume of CHAPS-containing buffer until it becomes solubilized. Solubilization may be improved at 37 °C.

3.3 Deglycosylation of Protein Samples Before SDS-PAGE

1. Prepare the samples to be deglycosylated as indicated in the last steps of methods Sect. 3.1 or 3.2. Adjust the protein concentration to 1.5–2.0 μg/mL using homogenization buffer.
2. To perform *non-denaturing deglycosylation*, combine 50 μL sample (75–100 μg total protein) with 50 μL of enzymatic deglycosylation cocktail. Incubate for 4 h at 37 °C (*see* **Note 22**) and continue with step 5.

3. To perform *denaturing deglycosylation* (*see* **Note 23**), add 6 μL 1 % w/v SDS and 4 μL 1.5 M 2-mercaptoethanol to 50 μL sample (75–100 μg total protein). Heat the sample for 5 min at 95 °C and let it cool on ice.
4. Combine the denatured sample from step 3 with 8 mL of Nonidet P-40 10 % w/v and 40 μL of enzymatic deglycosylation cocktail. Incubate for 4 h at 37 °C.
5. Stop deglycosylation by adding 50 μL of 3× reducing Laemmli buffer for every 100 μL sample.
6. Boil the samples for 5 min and cool on ice before loading the polyacrylamide gels. Continue with Sect. 3.5.

3.4 Deglycosylation of Blotted Proteins After SDS-PAGE

1. To perform *enzymatic deglycosylation* in blotting membranes (*see* **Note 24**), first separate the proteins by SDS-PAGE, and transfer them to PVDF or nitrocellulose membranes as described in Sect. 3.5. After transferring the proteins to the blotting membrane, place the membrane in an appropriate container, and cover with 1× deglycosylation buffer containing 0.05 % w/v Nonidet P-40 and 1 % w/v bovine serum albumin.
2. Add glycosidases to the membrane at the final concentrations listed in the enzymatic deglycosylation cocktail, and incubate the membrane for 4 h at 37 °C.
3. Wash the membrane with excess water three times (5 min each wash), and proceed with the western blotting steps described in Sect. 3.5.
4. To perform *nonenzymatic deglycosylation* in blotting membranes (*see* **Note 25**), proceed as in step 1 but transfer the proteins to PVDF (*do not use nitrocellulose*). Rinse the membranes with excess water for 5 min.
5. To remove sialic acid residues, incubate the membranes in 25 mM sulfuric acid for 1 h at 80 °C. Wash with excess water (three exchanges, 5 min each) and proceed with the western blotting.
6. To remove *O*-glycans (beta elimination), incubate the membranes in 55 mM NaOH for 16 h at 40 °C. Wash with excess water (three exchanges, 5 min each) and proceed with the western blotting.

3.5 SDS-PAGE and Western Blotting

1. Prepare gradient acrylamide gels adequate for the MW of the ECM proteins to be detected. Full-length CSPGs range in MW from 150 to 650 kDa and are therefore larger than most fibrillar monomeric proteins. A broad-range gradient (3.5–12 % acrylamide) will ensure detection of most CSPGs and other ECM proteins but will provide little resolution of isoforms and cleavage fragments. Using resolving gels with a single acrylamide

concentration will improve resolution for specific proteins (e.g., 5 % acrylamide for CSPG aggrecan and high-MW collagens; 6–6.5 % for phosphacan, neurocan, and tenascins; 7.5 % for brevican and laminins).

2. Load the samples (10–20 μg/lane) and MW standards in the gel.
3. Electrophorese the samples at 15 mA/gel until they have entered the resolving gel, and continue at 30 mA/gel until they reach the bottom of the gel.
4. Dismount the electrophoresis apparatus and prepare the gels and blotting membranes for transfer.
5. Transfer the samples at 225 mA (for 1 or 2 gels) for 3 h, to ensure complete transfer of the largest ECM proteins (fibronectin, versican, and non-deglycosylated CSPGs).
6. Dismount the transfer apparatus, and rinse the membranes briefly with water to remove debris and transfer buffer.
7. Stain the membranes briefly with amido black solution and quickly distain with excess water.
8. Prepare blocking buffer by dissolving a blocking protein in low-salt washing buffer (e.g., 5 % w/v nonfat milk or 3 % w/v bovine serum albumin). Block the membranes at room temperature for 1 h.
9. Incubate the membranes in primary antibodies (prepared in blocking buffer) overnight at 4 °C.
10. Wash the membranes exhaustively using three changes of low-salt and three changes of high-salt washing buffers (*see* **Note 26**). Each wash should be 5–10 min long.
11. Incubate the membranes in AP-conjugated secondary antibodies at room temperature for 1 h (*see* **Note 27**).
12. Wash the membranes exhaustively as in step 10.
13. Incubate the membranes in AP buffer at room temperature for 5 min.
14. Prepare the AP developing solution: 10 mL AP buffer + 66 μL NBT (50 mg/mL) + 33 μL BCIP (50 mg/mL). Mix well.
15. Add AP developing solution to the membranes, and wait until the bands develop in a purple color (2–20 min). Stop the reaction by rinsing the membranes with excess deionized water.
16. Let the membranes air-dry and image the blots (Fig. 2) using a scanner or a white light-illuminated gel imaging system.

3.6 Brain Tissue Preparation for ECM Immunochemistry

1. Experimental gliomas are usually obtained from mice injected intracranially with bulk tumor cells derived from human tumors or syngeneic sources. Once neurological symptoms are observed, euthanize the animals (*see* **Note 28**) and perfuse them transcardially with 10 mL 1× PBS followed by 10 mL 4 % PFA [25] (*see* **Note 29**).

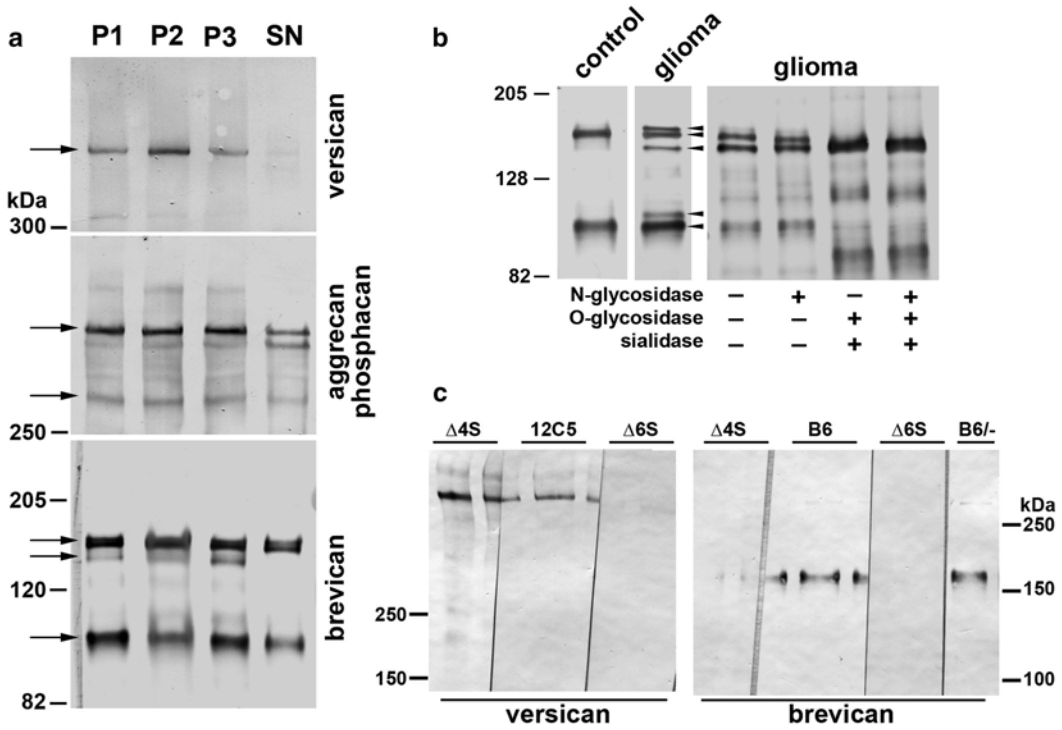

Fig. 2 Examples of CSPG variability in gliomas. (**a**) Subcellular fractions (P1-SN) of a grade III astrocytoma, treated with chondroitinase ABC and separated in a 3.5–12 % acrylamide gel. CSPGs were detected with the antibodies 12C5 (versican), Cat-315 (aggrecan and possibly phosphacan), and B6 (brevican full-length and C-terminal fragment). Notice how some CSPGs are largely insoluble (versican) or have specific membrane-bound isoforms (brevican). (**b**) Total homogenate of representative human control and glioblastoma samples, probed with anti-brevican antibody. Notice the different glycoforms in glioma (over-sialylated, hypo-glycosylated, etc., marked by *arrowheads*), which are absent in normal brain. Selective deglycosylation reveals the extent of brevican glycosylation [21]. (**c**) Soluble fraction of a human glioblastoma treated with chondroitinase and separated by SDS-PAGE under nonreducing (versican) or reducing (brevican) conditions. Membranes were cut along the middle of some lanes to line up the protein bands after blotting. "Stub" antibodies (Δ*4S* and Δ*6S*) reveal that versican is a constitutive CSPG that contains CS-A. In contrast, brevican is hardly, if at all, decorated with CS and is a facultative CSPG in glioma. In agreement, detection of brevican with (*B6*) or without (*B6*/–) chondroitinase treatment shows bands of indistinguishable MW

2. Carefully dissect the brains from the perfused animals, and place them in tubes or vials filled with 4 % PFA solution for postfixation overnight (*see* **Note 30**). Do not postfix brains with acetone (*see* **Note 31**), which will remove lipids and deform the brain tissue structure.
3. Remove the brains from the PFA solution, and place them in 50 mL conical tubes containing 30 % sucrose solution for cryoprotection and preparation for cryosectioning (*see* **Note 32**). Specimens should be kept in sucrose solution at 4 °C until they are ready for cryosectioning (*see* **Note 33**).

4. Remove the brains from the sucrose solution, and place them in tissue molds ("boats") of appropriate size filled with O.C.T. compound. Position the specimens in the desired orientation for cryosectioning, and place the boats on a freezing solution (*see* **Note 34**). Do not let the boats sink in the freezing solution as this will lead to uneven freezing and will complicate the cryosectioning process.
5. Once completely frozen, store the tissue blocks at −80 °C for a minimum of 24 h prior to sectioning.
6. Section brain tissue specimens using a cryostat to obtain 10 μm-thick sections. Arrange the sections on glass slides as desired. Cut sections can be stored at −80 °C indefinitely but should be tested to confirm that antigenicity is not lost due to effects of long storage.

3.7 Tumorsphere Preparation for ECM Immunochemistry

1. Glioma stemlike cells are obtained from dissociated human brain tumor tissue or from xenografts following standard procedures [26] and cultured in suspension to form tumorspheres (*see* **Note 35**). Let the cells grow several days in culture until they form well-defined spheres visible at low magnification.
2. Transfer the tumorspheres from their culture flasks to a conical tube, and let them settle at the bottom of the tube. *Do not centrifuge the tube as the spheres are extremely fragile and may break apart*. Carefully aspirate the culture medium, and add one volume of 4 % PFA or pure acetone (*see* **Note 31**) to fix the spheres for 10 min.
3. Carefully aspirate the fixative, and transfer the tumorspheres to a tissue mold with O.C.T. compound. Freeze the samples as indicated in Sect. 3.6, step 4, for tissue sections. Due to their low density, tumorspheres will not settle through the O.C.T. compound, and special care must be taken to arrange them on a single plane prior to freezing. The topside of the frozen block is the one that must be cut with the cryostat.
4. After freezing, store the blocks at −80 °C until sectioning. Section the block using a cryostat to obtain 10 μm-thick sections, and arrange them on glass slides as desired (*see* **Note 36**). Store sections at −80 °C as needed.

3.8 Immunochemical Staining of ECM

1. Remove slides containing tissue sections or tumorspheres from the freezer, and allow them to dry and warm up to room temperature. Once the samples are dry, mark a space around the samples of interest using a hydrophobic pen to keep the subsequent solutions confined around the samples.
2. If tissue or cells have been fixed with 4 % PFA, a permeabilization step is recommended for staining of intracellular antigens in addition to cell-surface or extracellular components.

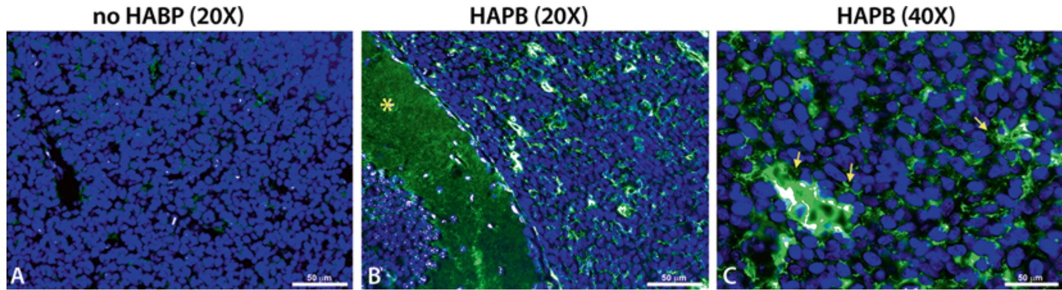

Fig. 3 Example of ECM staining in gliomas. (**a**–**b**) The figure shows tissue sections from an intracranial glioma derived from tumor stemlike cells, which have been stained with biotinylated HABP followed by Alexa 488-conjugated streptavidin. (**c**) Notice how at high magnification (40×) the intercellular deposited HA becomes visible in the interstitial spaces between tumor cells (*arrows*). The *asterisk* indicates a band of brain parenchyma enfolded by the growing tumor. Bars = 50 μm

To permeabilize the samples, add PBS-T on top of each sample, and incubate for 10 min at room temperature. Samples fixed with acetone usually do not require permeabilization.

3. Rinse the glass slides with excess 1× PBS, and incubate the samples with the appropriate blocking solution for antibodies or for HABP. Keep the samples in blocking solution for 30 min at room temperature, and wash the blocking solution with excess 1× PBS (two changes of PBS, 5 min incubation each).
4. Dilute the antibodies in PBS-T. Incubate the samples with the primary antibody or lectin at the recommended dilution, overnight at 4 °C. For biotinylated HABP (Fig. 3), the recommended starting dilution is 1/100.
5. Wash the primary antibodies using excess 1× PBS (two changes, 5 min each).
6. Incubate the samples with the appropriately labeled secondary antibody or streptavidin to detect the primary probe. For biotinylated HABP, we recommend Alexa 488-conjugated streptavidin (Invitrogen), diluted 1/500 in PBS-T. Incubate the samples for 2 h at room temperature. It is recommended to keep the samples protected from the light to prevent photobleaching of the fluorochromes.
7. Wash the secondary antibodies or streptavidin using excess 1× PBS (two changes, 5 min each). If desired, incubate the samples with a nuclear stain for 5 min to improve single-cell recognition. Typical nuclear staining agents include 4′,6-Diamidino-2-Phenylindole (DAPI, Invitrogen) and Hoechst 33258 (Invitrogen). Both can be purchased as pre-made solutions and are used at a final 1/1,000 dilution in 1× PBS.
8. Wash the glass slides with two additional changes of 1× PBS (5 min each), and remove excess liquid from the glass and samples.

Apply a droplet of mounting medium to the tissues, and carefully lay a glass cover slip on top (*see* **Note 37**).

9. Allow the mounting medium to harden for at least 1 h at room temperature prior to imaging as excessive movement may dislodge the cover slip and damage the tissue. A representative example of HABP staining is illustrated in Fig. 3.
10. Glioma stemlike cells can also be dissociated from the tumorspheres and cultured as dissociated adherent cells before fixation and staining, without the need for freezing and sectioning described in Sect. 3.7. For this, pre-coat culture vessels or glass cover slips with Geltrex® (Invitrogen, Cat #A1413301) to induce adherence of cells dissociated from tumorspheres (*see* **Note 38**).
11. After 24–48 h, wash the adhered glioma cells to remove their culture medium, and fix them with one volume of 4 % PFA or ice-cold acetone for 10 min at room temperature.
12. Wash the fixed cells with 1× PBS for 5 min, and block them with the appropriate blocking solution for 30 min at room temperature.
13. Follow the steps to incubate primary and secondary probes, as well as intermediary washes, as indicated in steps 3–7. Include a nuclear staining step if desired.
14. Wash the stained cells in their culture vessels and keep them in 1× PBS until imaging. Since mounting media is not added to the cultured cells, imaging should be done promptly to avoid loss of fluorescence.

4 Notes

1. We have tested the following protease inhibitors without any noticeable differences: Complete®, EDTA-free, protease inhibitor tablets (Roche Applied Science, Indianapolis, IN); HALT® protease inhibitor cocktail solution, EDTA-free (Pierce/Thermo Scientific, Rockford, IL); and Sigma protease inhibitor cocktail solution, EDTA-free (Sigma-Aldrich, St Louis, MO).
2. Although EDTA is a good choice as broad-spectrum metalloprotease inhibitor, the association of CSPGs to cell membranes is in part Ca^{++} dependent and therefore disrupted by Ca^{++} chelation. Omitting EDTA in the tissue homogenization solution usually has no major effects if the tissue is processed quickly and is maintained on ice at all times.
3. 1,10-phenantroline is a broad-spectrum metalloprotease inhibitor that chelates Zn^{++} from the active site of the enzymes. Prepare a 200 mM stock solution in dimethyl sulfoxide, and

store in aliquots at −20 °C for several months. Use at 1 mM final concentration.

4. Tissue homogenization should cause minimal subcellular disruption to avoid breaking intracellular organelles. Glass-teflon (Potter-Elvehjem) or glass-glass (Dounce) homogenizers are the best suited for this purpose and can process volumes ranging from 0.5 to 50 mL of homogenate. Aggressive tissue disruption (e.g., Turrax-type homogenizer or sonication) should be avoided. Alternatives such as small plastic homogenizer tips that fit in microcentrifuge tubes are appropriate for lysis of cultured cells but do not provide sufficient tissue homogenization.

5. Nonidet P-40 ((octylphenoxy)polyoxyethanol) is sold by Sigma as Igepal CA-630. It should not be confused with NP-40 ((nonylphenoxy)polyoxyethanol), which is also provided by Sigma (# 74385) and Pierce (# 85124). However, since their structure is minimally different, either detergent can use to maintain enzyme stability during deglycosylation.

6. Different manufacturers list different enzyme activities, calculated using different methods, which are difficult to compare. In this protocol we have chosen sialidase and *O*-glycosidase from Roche Applied Sciences, *N*-glycosidase and chondroitinase ABC from Sigma-Aldrich, and other enzymes from New England Biolabs, as previously described [20, 21]. Pilot tests must be conducted with enzymes from other manufacturers to establish equivalence of enzymatic activity.

7. If a glycosidase is not desired in the cocktail, replace it with an equal volume of water to maintain a total volume of 50 μL.

8. DTT is used to reduce samples that cannot be boiled (because some proteins may precipitate or aggregate, as it happens with some glycosyltransferases). Samples mixed with DTT-containing Laemmli buffer can be reduced by heating them at 37 °C for 5 min.

9. Acrylamide is a dangerous carcinogen and neurotoxin that forms long-lasting aerosols when weighed. Commercial pre-made solutions are recommended.

10. If results suggest poor transfer of CSPGs to the blotting membranes, add SDS to the Towbin buffer (0.1 % w/v final concentration). However, SDS may reduce binding of other proteins to the membranes and its effect must be tested on a case-by-case basis.

11. Unsupported nitrocellulose has excellent affinity for proteins and is easily wetted in Towbin buffer. Either side can be used to face the gel, which is an important advantage over supported nitrocellulose. However, it is more fragile than supported nitrocellulose or PVDF, and it may break during stripping or harsh chemical treatments.

12. Amido black is more sensitive than the Ponceau-S red staining and can be destained with 10 % v/v acetic acid or excess deionized water. It is highly recommended to detect efficient transfer of high-MW proteins. The solution can be refiltered and used a few times until it becomes diluted.
13. NBT and dimethylformamide are highly toxic substances with mutagenic risk. Any solutions containing dimethylformamide (NBT and BCIP) must be prepared in a chemical fume hood and manipulated on the bench using appropriate personal protective equipment. Commercially available mixtures of NBT/BCIP (in solution or tablets) may be used as a convenient and safe alternative.
14. Do not confuse BCIP p-toluidine with BCIP sodium salt, which is not soluble in dimethylformamide.
15. PFA is a flammable, corrosive, irritant, and suspected carcinogen. Care should be taken to avoid direct contact with PFA powder or the fumes it produces once dissolved. Appropriate protective equipment, including eye and breathing protection, should be worn at all times while handling PFA. Old PFA solutions become unstable and can explode at high temperatures.
16. Surrounding a tissue section with the hydrophobic lining provided by a PAP pen ensures that the antibody solution will not leak from the section and also reduces the total amount of antibody needed. This method allows multiple tissue sections on the same slide to be stained with different antibodies without cross contamination. If a PAP pen is unavailable or if multiple tissue sections on the same slide require the same antibody, then the sections can be covered with small pieces of Parafilm® after adding the antibody.
17. It is recommended to work always with freshly resected tissue. Subcellular structures will break during freezing, and their contents will spread over several subcellular fractions.
18. Keep the tissue and fractions on ice, and use ice-cold solutions throughout the procedure to minimize protein degradation.
19. Do not decant the supernatant. The first pellet is very loose and may dislodge from the tube.
20. If further fractionation is needed, the P2 (mitochondria-rich) fraction can be separated by centrifugation in a discontinuous sucrose gradient yielding layers of myelin, light microsomes, and isolated mitochondria [20].
21. Do not try to run guanidine-extracted proteins for SDS-PAGE because guanidine will precipitate with SDS. You must precipitate these proteins using acid ethanol and resuspend them in an adequate buffer or directly in 1× Laemmli buffer.
22. Incubation may be increased up to 8 h to ensure complete removal of O-linked glycans and chondroitin sulfate from

heavily glycosylated CSPGs such as aggrecan. After deglycosylation the samples must be frozen or kept at 4 °C until adding Laemmli buffer.

23. Denaturing deglycosylation is usually required to remove N-linked carbohydrates buried in the core protein. Denaturation is rarely required to remove O-linked glycans and is not required to remove chondroitin sulfate chains.
24. Deglycosylation in membranes is used to analyze expression of glycoepitopes, which may be unmasked by removal of carbohydrates, without changing the MW of the protein because it has already been transferred to the blotting membrane.
25. Nonenzymatic deglycosylation by oxidative elimination removes terminal sialic residues and O-linked glycans, without releasing N-linked glycans [27].
26. Most anti-CSPG antibodies require exhaustive washing (~60 min in total) to reduce nonspecific detection of glycoepitopes from other proteins.
27. Blots developed using enzymatic detection of alkaline phosphatase (e.g., NBT/BCIP or Fast Red) will show sharper bands than chemiluminescence-developed blots, facilitating identification of CSPG isoforms. However, results obtained with this method are not quantitative. Chemiluminescent detection can be used subsequently to quantify specific CSPG bands.
28. Euthanasia is achieved by intraperitoneal injection of an overdose of ketamine (500 mg/kg) plus xylazine (10 mg/kg), followed by immediate perfusion when the animal loses consciousness, reflexes, and muscle tone. Ketamine is a DEA class II controlled substance and requires special authorization to be used in research.
29. For an excellent visual guide to perfusion techniques and brain dissection, *see* [28]. Perfusion must be performed using a syringe, peristaltic pump, or a gravimetric device to slowly dispense the fixatives (usually 0.5 mL/min or less). Sterile techniques should be maintained throughout perfusion. To improve perfusion of the CNS, use a hemostat to clamp the abdominal aorta at the beginning of the perfusion procedure.
30. Formaldehyde will penetrate the tissue approximately 15–18 mm after 24 h at room temperature [29].
31. Pure acetone (pre-chilled at −20 °C and used ice cold) can be used to fix cells in suspension or monolayers. However, it should not be used for high-fat tissue (brain), and we do not recommend it for brain tissue sections.
32. Paraffin-embedded sections preserve the architecture of the cells better than frozen sections but also require harsh antigen-retrieval steps due to extensive cross-linking and denaturation

of the original epitopes. Many antibodies that work in frozen sections will not detect epitopes in paraffin-embedded tissue and require extensive additional validation.

33. Specimens will be ready for cryosectioning when the aqueous content of the tissue equilibrates with the sucrose solution. In practice, the brains will sink to the bottom of the tubes.
34. Freezing solutions include liquid nitrogen, isopentane/dry ice, and isopropanol/dry ice. A mixture of ethanol/dry ice is inadequate for freezing purposes.
35. The most widely used culture medium for glioma stemlike cells is *Neurobasal* (Invitrogen) supplemented with epidermal growth factor (20 ng/mL), basic fibroblast growth factor 2 (20 ng/mL), L-glutamine (1 mM), sodium pyruvate (1 mM), and the vitamin supplement B27 (Invitrogen) [15].
36. Sectioned tumorspheres will be usually invisible to the naked eye. A small, portable, bright-field microscope will be useful to detect them during sectioning.
37. Usual cover slips are 0.17 mm thick but thinner cover slips (0.10–0.13 mm) may be used for special applications. Do not drop the cover slip on top of the tissue, but gently let it fall from one border, in order to displace air between the tissue and the cover slip. Take special care to ensure that air bubbles are not trapped under the cover slip.
38. *Reduced growth factor Geltrex®* is a commercial basal lamina mixture composed of laminin, collagen type IV, entactin, and heparan sulfate proteoglycans. To make a coating solution, dilute 80 μL of stock Geltrex in 50 mL culture medium (usually Dulbecco's MEM containing 10 % fetal bovine serum and 1× penicillin/streptomycin). This yields a mixture with a final total protein concentration ~24 mg/mL that is used directly to cover the cultureware.

References

1. Louis DN (2006) Molecular pathology of malignant gliomas. Annu Rev Pathol Mech Dis 1:97–117
2. Viapiano MS, Matthews RT (2006) From barriers to bridges: chondroitin sulfate proteoglycans in neuropathology. Trends Mol Med 12:488–496
3. Gritsenko PG, Ilina O, Friedl P (2012) Interstitial guidance of cancer invasion. J Pathol 226:185–199
4. Bellail AC, Hunter SB, Brat DJ et al (2004) Microregional extracellular matrix heterogeneity in brain modulates glioma cell invasion. Int J Biochem Cell Biol 36:1046–1069
5. Ruoslahti E (1996) Brain extracellular matrix. Glycobiology 6:489–492
6. Lathia JD, Li M, Hall PE et al (2012) Laminin alpha 2 enables glioblastoma stem cell growth. Ann Neurol 72:766–778
7. Gladson CL (1999) The extracellular matrix of gliomas: modulation of cell function. J Neuropathol Exp Neurol 58:1029–1040
8. Paulus W, Huettner C, Tonn JC (1994) Collagens, integrins and the mesenchymal drift in glioblastomas: a comparison of biopsy specimens, spheroid and early monolayer cultures. Int J Cancer 58:841–846
9. Mahesparan R, Read TA, Lund-Johansen M et al (2003) Expression of extracellular matrix components in a highly infiltrative in vivo glioma model. Acta Neuropathol 105:49–57

10. Viapiano MS, Lawler SE (2009) Glioma invasion: Mechanisms and Therapeutic Challenges. In: Van Meir E (ed) CNS cancer: models. Prognostic factors and targets. Humana, Jersey City, NJ, pp 1219–1252
11. Varga I, Hutoczki G, Petras M et al (2010) Expression of invasion-related extracellular matrix molecules in human glioblastoma versus intracerebral lung adenocarcinoma metastasis. Cent Eur Neurosurg 71:173–180
12. Hu B, Kong LL, Matthews RT et al (2008) The proteoglycan brevican binds to fibronectin after proteolytic cleavage and promotes glioma cell motility. J Biol Chem 283:24848–24859
13. Wu Y, Chen L, Zheng PS et al (2002) beta 1-Integrin-mediated glioma cell adhesion and free radical-induced apoptosis are regulated by binding to a C-terminal domain of PG-M/versican. J Biol Chem 277:12294–12301
14. Lorente G, Nelson A, Mueller S et al (2005) Functional comparison of long and short splice forms of RPTPbeta: implications for glioblastoma treatment. Neuro-Oncology 7:154–163
15. Lathia JD, Gallagher J, Heddleston JM et al (2010) Integrin alpha 6 regulates glioblastoma stem cells. Cell Stem Cell 6:421–432
16. Rauch U (2004) Extracellular matrix components associated with remodeling processes in brain. Cell Mol Life Sci 61:2031–2045
17. Matthews RT, Kelly GM, Zerillo CA et al (2002) Aggrecan glycoforms contribute to the molecular heterogeneity of perineuronal nets. J Neurosci 22:7536–7547
18. Rodriguez de Lores A, Alberici M, De Robertis E (1967) Ultrastructural and enzymic studies of cholinergic and non-cholinergic synaptic membranes isolated from brain cortex. J Neurochem 14:215–225
19. Jones DH, Matus AI (1974) Isolation of synaptic plasma membrane from brain by combined flotation-sedimentation density gradient centrifugation. Biochim Biophys Acta 356:276–287
20. Viapiano MS, Matthews RT, Hockfield S (2003) A novel membrane-associated glycovariant of BEHAB/brevican is up-regulated during rat brain development and in a rat model of invasive glioma. J Biol Chem 278:33239–33247
21. Viapiano MS, Bi WL, Piepmeier J et al (2005) Novel tumor-specific isoforms of BEHAB/brevican identified in human malignant gliomas. Cancer Res 65:6726–6733
22. Davis L, Kuehl M, Battey J (1994) Electrophoresis of proteins on sodium dodecyl sulfate polyacrylamide gels. In: Davis L (ed) Basic methods in molecular biology, 2nd edn. Appleton & Lange, Norwalk, CT, pp 661–668
23. Harlow E, Lane D (1999) Using antibodies: a laboratory manual. CSHL Press, Cold Spring Harbor, NY, pp 682–689
24. Binette F, Cravens J, Kahoussi B et al (1994) Link protein is ubiquitously expressed in non-cartilaginous tissues where it enhances and stabilizes the interaction of proteoglycans with hyaluronic acid. J Biol Chem 269: 19116–19122
25. Scouten CW, O'Connor R, Cunningham M (2006) Perfusion fixation of research animals. Microsc Today 14:26–33
26. Lee J, Kotliarova S, Kotliarov Y et al (2006) Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell 9:391–403
27. Duk M, Ugorski M, Lisowska E (1997) beta-Elimination of O-glycans from glycoproteins transferred to immobilon P membranes: method and some applications. Anal Biochem 253:98–102
28. Gage GJ, Kipke DR, Shain W (2012) Whole animal perfusion fixation for rodents. J Vis Exp 65:3564, doi:3510.3791/3564
29. Fox CH, Johnson FB, Whiting J et al (1985) Formaldehyde fixation. J Histochem Cytochem 33:845–853
30. Tarentino AL, Plummer TH Jr (1994) Enzymatic deglycosylation of asparagine-linked glycans: purification, properties, and specificity of oligosaccharide-cleaving enzymes from Flavobacterium meningosepticum. Methods Enzymol 230:44–57
31. Glasgow LR, Paulson JC, Hill RL (1977) Systematic purification of five glycosidases from Streptococcus (Diplococcus) pneumoniae. J Biol Chem 252:8615–8623
32. Dino MR, Harroch S, Hockfield S et al (2006) Monoclonal antibody Cat-315 detects a glycoform of receptor protein tyrosine phosphatase beta/phosphacan early in CNS development that localizes to extrasynaptic sites prior to synapse formation. Neuroscience 142:1055–1069
33. Massey JM, Amps J, Viapiano MS et al (2008) Increased chondroitin sulfate proteoglycan expression in denervated brainstem targets following spinal cord injury creates a barrier to axonal regeneration overcome by chondroitinase ABC and neurotrophin-3. Exp Neurol 209:426–445