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Examination of Galectin Localization Using Confocal Microscopy

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Abstract

Confocal microscopy provides a unique modality to examine the expression and localization of biomolecules in a variety of settings. Using this technique, an image is acquired from the focal plane of the objective using focused laser light making it possible to work within the resolution limit of the optical system. In addition, by acquiring multiple images from a variety of focal planes, stacked series of images can provide clear spatial localization of a probed structure or protein. We describe herein the immunofluorescence methods for galectin staining in frozen sections of tissue for galectin localization using confocal microscopy.

Keywords

Confocal microscopy; Galectin; soft tissue; antibody; immunofluorescence

1. Introduction

Galectins are ancient lectins that bind to specific carbohydrate chains containing β -galactosides through their carbohydrate recognition domains (CRD) [1,2]. These proteins are found in both the intracellular (cytoplasm and nucleus) as well as the extracellular (cell surface and serum) compartments [3]. Depending on expression, secretion and local concentration, these lectins can mediate cell-cell, cell-matrix or cell-pathogen interactions, displaying various functions in physiological and pathological processes [4–8]. Although 15 members of the galectin family have been described [1], galectin-1 appears to be the most ubiquitously expressed member of the family. Mammalian galectin-1 is found in skeletal and striated muscle, liver, lung, brain, kidney, spleen, and intestine; in arterial walls, and in

¹⁷Centrifuging the diluted solutions of antibodies at maximum speed (~20,000 $\times$ g) for 30 min at 4°C before using them can help to avoid undesired background during the immunofluorescence procedure.

¹⁸It is important to use the minimal concentration of primary antibody to achieve a good staining. As a result, it is recommended to perform some test staining with multiple concentrations. If the antibody is a purchased antibody, begin with the minimum concentration indicated on the product datasheet. Usually, we see good staining by using 10 μ g/mL of purified antibody.

cytosol and nucleus of cultured human endothelial cells [9,10]. Additionally, galectin-1, along with galectin-3 is expressed in cells of myeloid and lymphoid lineage and appears to be involved in regulation of immune cell development and inflammatory processes [11]. Alternatively, some other galectins, show more selective expression patterns. For example, galectin-7 and -12 are localized to epithelial and adipose tissue, respectively [12–14], while galectin-4 resides primarily within tissue of the alimentary tract [15]. Thus, some galectins can be preferentially localized to selective tissues while others are more widely expressed.

Fluorescence microscopy offers a sensitive technique for observation of biomolecules in cells and tissues. This basic approach involves targeting the desired molecule with a probe that can either be fluorescently labeled itself or recognized by a secondary fluorescent probe. However, when using traditional fluorescence microscopy, out-of-focus light remains in the light path blurring the image of the sample and causing loss of resolution [16]. Laser Scanning Confocal Microscopy (LSCM) overcomes such technical limitations, providing the ability to collect light solely from the objective's focal plane. This eliminates blurring due to out-of-focus light to reveal finer detail than conventional systems could provide [16]. Furthermore, as the emitted light comes from a spatially determined location from the sample, it is possible to determine the discrete position of structures and protein expression within a cell or tissue. If the sample is moved thorough the focal plane a stack of images, referred to as a Z-stack, a three-dimensional localization and reconstruction of structures, can be obtained [16]. The use of this technique to spatially locate and analyze the expression of galectin family members can contribute greatly to knowledge of relationships between galectins and their ligands as well as normal expression patterns within an organism.

As confocal microscopy offers an exceptionally specific, high resolution method for protein localization, several studies have recently utilized this method for the localization and functional study of galectin family members [17–19]. LCSM can provide unprecedented levels of detail regarding the location and function of galectin family members (see Figures 1 and 2), but careful technique is important to achieve accurate information. Therefore, this chapter will outline methods for preparing and imaging tissue sections and cellular samples to analyze galectin localization and function by confocal microscopy.

2. Materials

2.1 Preparation of glass slides

1. Gelatin from bovine source, Bloom 225 (Sigma, St Louis, MO)
2. ddH₂O
3. Chromium (III) potassium sulfate [chrome alum (Sigma, St Louis, MO)]
4. General-purpose filter paper (Sigma, St Louis, MO)
5. Glass slides (Knitel, Braunschweig- Germany)
6. Poly-L-lysine* 0.1% (w/v) in water (Sigma, St Louis, MO)

2.2 Processing of Tissue

1. Surgical material for tissue sampling (tweezers, scissors, etc.)
2. PBS 1X (137mM NaCl, 2.7mM KCl, 4.4mM Na₂HPO₄ and 1.4 KH₂PO₄ pH=7.3) and PBS 10X.
3. Paraformaldehyde* 4% in PBS (Sigma, St Louis, MO)
4. Sucrose* 30% in water (w/v) (Fisher, Hampton, NH)

3.3 2.3 Cryogenic technique

1. OCT Compund- Tissue-Tek (Sakura Finetek, Netherland)
2. [isopentane (Fisher, Hampton, NH) and liquid nitrogen] or [acetone (Fisher, Hampton, NH) and dry ice].
3. Polyethylene embedding cassettes (Fisher, Hampton, NH) or cryogenic vials (Corning Incorporated Life Sciences, Tewksbury, MA).

2.4 Preparation of sample sectioning and mounting

1. OCT Compund- Tissue-Tek (Sakura Finetek, Neetherland)
2. Pencil
3. Cryostat (Leica CM 1850, Wetzlar, Germany)
4. Gelatin coated glass slides (Section 2.1)

2.5 Tissue immunofluorescence

1. 0.25M glycine in PBS 1X
2. Hydrophobic barrier pen* (Vector Laboratories, Burlingame, CA)
3. Bovine Serum Albumine- Fraction V (BSA) (Sigma, St Louis, MO)
4. DAPI- 4,6-diamidino-2-phenylindole, dihydrochloride (Molecular Probes/Life Technologies, Grand Island, NY)
5. Triton X-100 (Fisher, Hampton, NH)
6. Tween™ 20 (Fisher, Hampton, NH)
7. Fc receptor Blocker- *ChromPure* whole molecule non-immune IgG from the same animal source as your secondary antibody (Jackson Immunoresearch, West Groove, PA)
8. Fluoromount G (Electron microscopy sciences, Hatfield, PA)
9. Primary antibody
10. Secondary antibody
11. PBS 1X (*see* Note 1)
12. Refrigerated micro-centrifuge

3. Methods

3.1 Preparation of glass slides

1. Clean glass slides thoroughly with ddH₂O and identify individual slides using a pencil. (*see* Note 2)
2. Prepare the gelatin solution by dissolving 2.5 g of gelatin into 500 mL of ddH₂O heated to 60°C to make a 0.5% solution (Do not exceed 60°C).
3. After gelatin is dissolved, add 0.25 g of Chromium (III) potassium sulfate (chrome alum) to make 0.05% chrom alum solution. (*see* Note 3)
4. Filter the solution using general-purpose filter paper.
5. Dip the slide once into the gelatin-coating solution for few seconds.
6. Place gelatinized glass slides at approximately an 80° angle to drain the excess solution onto a paper towel. Cover the slides with aluminum foil to keep off dust.
7. Allow slides to dry at room temperature for 2 days or overnight at 37°C. After drying, slides can be stored in the refrigerator (4–8°C).
8. Discard gelatin solution after use.
9. Alternatively, the glass slides maybe prepared by coating with 0.1% Poly-L-lysine. (*see* Note 4)

3.2 Processing of tissue

1. *Tissue sampling*: Excise the desired tissue and wash the biopsy briefly in PBS. If freezing tissue on the same day as tissue excision then proceed directly to section 3.3 Cryogenic technique. Otherwise proceed to step 2 (*see* Note 5).
2. *Tissue fixation*: Place excised tissue into a tissue cassette and fix in fresh 4% paraformaldehyde in PBS for 4 hours at room temperature. (*see* Notes 6, 7 and 8)

¹Alternatively, you may use TBS (10mM Tris and 100mM NaCl pH= 8.0).

²When marking glass slides pencil should always be used. Markings made using pens are frequently erased during immunofluorescence techniques.

³Chrome Alum will positively charge the slides allowing them to attract negatively charged tissue sections.

⁴For coating glass slides with Poly-L-lysine, drop 10 µL of solution in the middle of a glass slide and distribute it evenly over the surface by the aid of another moving glass slide. Air-dry for 5 min at room temperature.

⁵As this chapter focuses on confocal analysis of galectin localization within solid tissue, unique considerations should be employed when examining galectin localization in suspension. Cells in suspension can be spread on a glass slide using routine blood smear techniques or overlaid on a poly-L-lysine coated coverslip[20,21]. When using this approach, evaluation of antibody engagement over a range of concentrations using flow cytometric examination may be beneficial to insure adequate saturation of the target antigen when seeking to ascertain potential changes under different conditions[22,23]. If fixation is required, this process typically follows adhesion of cells to the slide or coverslip followed by employing similar considerations as are utilized when staining a tissue section[24]. However, as cells may not adhere to the slide or cover slip as well as a tissue section, great care should be taken when incubating and washing the slide to not remove the cells.

⁶Several galectins, including galectin-1, galectin-2 and galectin-3 can undergo rapid oxidation following removal from a reducing environment[21,25,26]. Galectin oxidation results in significant conformational changes that may preclude antibody recognition depending on the antibody employed and the galectin being examined[21,27]. As a result, significant care should be taken to rapidly process tissue immediately after harvesting to reduce the potential impact of galectin oxidation on detection and localization of these proteins.

⁷To prepare 100 ml of 8% paraformaldehyde (2X) solution, add 100 µL 1.0M NaOH into a glass beaker containing 80 mL of water. Add 8.0 g of paraformaldehyde with agitation and heating to 60°C (be sure to keep a cap on or heat under a fume hood). Wait until complete solubilization (clear solution). Cool solution in ice until it reaches room temperature. Add 10 mL of PBS 10X and complete to 100mL with water. Mix and filter the resulting solution with a 0.2 µm pore size filter. Prepare a fresh 4% paraformaldehyde solution

3. *Post-fixation treatment:* To improve the cryopreservation of formaldehyde-fixed tissue, these materials should be incubated in sucrose solution (5–20%) and optimal cutting temperature (OCT) compound Tissue tek. (see Note 9)

3.4 Cryogenic technique

1. *Cryogenic technique-* Samples should be placed into a polyethylene embedding cassette (alternatively, cryogenic tubes can be used) filled with OCT. Be sure that the tissue biopsy is in the correct orientation for future sectioning (transversal, longitudinal or oblique). (see Notes 10 and 11)
2. Freeze the embedded sample using one of the two freezing techniques described below: (see Note 12)
 1. *Acetone plus dry ice:* Place dry ice into a single 250–500mL Becker and very slowly add acetone to cover the ice. Wait 5 minutes for acetone to chill and then, with the aid of tweezers, dip the cassette (or cryogenic tube) and wait at least 2 minutes to ensure sample has frozen completely. (see Note 13)
 2. *Liquid nitrogen plus isopentane:* Fill a polystyrene container (Nalgene, Rochester, NY) with liquid nitrogen to approximately $\frac{3}{4}$ of its total volume. Use tongs to lower a stainless steel or polypropylene container of isopentane into the liquid nitrogen. Begin chilling isopentane, at least 30 min before freezing samples. As isopentane nears freezing it will become opaque. With the aid of tweezers, dip the cassette (or cryogenic tube) into the chilled isopentane and wait at least 2 minutes to ensure sample has frozen completely.

3.4 Preparation of Sample sectioning and mounting

1. Carefully detach the frozen sample block from the cassette and identify it with a small piece of paper or other appropriate marking.
2. Handle the cryostat according to the manufacturer guidelines.

by diluting 8% paraformaldehyde in PBS (1X). The time of incubation may vary according to the kind and size of tissue (over four hours to several days). Search literature to determine the ideal time for tissue fixation.

⁸The desired concentration and content of paraformaldehyde solution may vary from depending on the chosen protein or structure that is being observed. (e.g. for microtubules visualization 4% paraformaldehyde plus 50mM EGTA and 50 μ M Taxol preserves cytoskeleton structure). Always consult literature for appropriate conditions.

⁹The post-fixation step in sucrose solution should not be performed if your tissue, was not fixed in formaldehyde, to avoid shrinking of non-fixed tissue. Following chemical fixation, tissue samples are sequentially immersed in solutions of sucrose (5, 10 and 20%) in PBS, pH 7.2. The transition from a concentration of sucrose solution to another should occur when the tissue samples under. Then transfer the tissue sample into a cassette (or cryogenic tube) filled with OCT. This procedure is indicated for solid tissues as brain and skeletal muscle.

¹⁰Marking facilitates identification and correct orientation of particular tissue pieces or surfaces during embedding and subsequent microscopical examination. Cassettes should be marked before embedding to establish orientation. Tissue blocks are simply marked after freezing by cutting a notch on the reverse side of the block face to be sectioned, or by trimming the block to a particular shape.

¹¹For some special tissues such as bones, wait at least 1h at 4°C in OCT for best infiltration, before freezing.

¹²It is important to freeze biological sample until it has been completely frozen. Freezing will begin on the outside move inward. The process will complete when tissue plus Tissue Tek becomes milky-white in appearance.

¹³Be careful when adding acetone directly to dry ice. It will “boil” fast, splashing acetone in all directions. Do not permit the acetone contact your sample directly.

3. Fasten the block into the block holder of the cryostat and adjust the tissue thickness for 5–10 μm .
4. Slice 5–10 μm repeatedly until a ribbon includes tissue forms (*see Note 14*).
5. Place a previously gelatin-coated slide into position so that the ribbon containing the desired tissue section settles onto the slide as it is sliced.
6. If your primary antibody is sensitive to paraformaldehyde fixation antigen retrieval may be required. Otherwise proceed directly to Section 3.5 (*see Note 15*)

3.5. Tissue immunofluorescence

1. If tissue was fixed in formaldehyde (Section 3.2.2), then block free formaldehyde residues by placing tissue in 0.25M glycine in PBS for 5 min at room temperature. Otherwise, proceed as follows:
2. Determine appropriate antibody conditions. (*see Notes 16–19*)
3. With a hydrophobic barrier pen, manually circumvent the tissue to restrain the antibody solution around the tissue during the incubation period.

All subsequent steps should be performed in a moisture chamber at room temperature.
4. Block tissue sections in 500 μL PBS containing 2% BSA and 0.5% (v/v) Triton X-100 plus Fc blocker (we used 50 $\mu\text{g/mL}$ donkey IgG) for 45 min.
5. Primary antibody should be prepared in PBS with 2% BSA.
6. Remove blocking buffer and add primary antibody at appropriate concentration to slide. Incubate for 1 hour.
7. Wash slides two times for 15 min in PBS plus 0.1% (v/v) Triton X-100 and then two times in PBS plus 0.05% (v/v) Tween 20.
8. Secondary antibody should be prepared in PBS with 2% BSA and 50 ng/mL DAPI. The optimal concentration of secondary antibody should be provided by the manufacturer.
9. Incubate slide with secondary antibody for 1 hour.

¹⁴Be sure to obtain additional sections for hematoxylin and eosin staining using commonly employed approaches to provide a histological reference for each slide[28].

¹⁵Fixation should immobilize antigens while retaining cellular and subcellular structure and permeabilize samples to allow for access of antibodies to all cells and subcellular compartments. Crosslinking reagents, such as paraformaldehyde serve this purpose, but may reduce the antigenicity of some cell components as the crosslinking can obstruct antibody binding. As a result, antigen retrieval techniques may be required. We use an additional acetone fixation if antigen retrieval is needed. For acetone fixation, dip the slide into -20°C acetone for 10 min. Before proceeding with immunofluorescence, rehydrate sections with PBS (1X). Remember that acetone fixation will promote cell permeabilization.

¹⁶To confirm antibody specificity, perform immunoblotting with its specific purified protein and/or tissue/cell extract to verify if the protein recognized is the expected size. In addition, antibody specificity may be demonstrated by performing the immunofluorescence stain with primary antibody that has been previously neutralized with its specific purified protein.

¹⁹Negative controls such as absence of primary antibody or use of an isotype control should be included in the staining protocol in order to achieve interpretable results.

10. Wash slides four times for 15 min each in PBS plus 0.05% (v/v) Tween 20.
11. *Mounting slides:* Dip the slide briefly in Milli-Q water. With the aid of a soft paper towel, dry around the sample very carefully.
12. To mount the coverslip over the tissue section, gently add 20–30 μ L of Fluoromount, directly to the slide and carefully lay the coverslip over the tissue section taking care to avoid bubbles.
13. Dry gently around the coverslip and seal with colorless nail polish (with no formaldehyde in its formulation) to prevent drying and displacement of biological specimen.
14. Observe the prepared tissue slide using a conventional fluorescence microscope to determine whether fluorescence is detectable in the sample.
15. In the case of positive results, place them in a LCSM and proceed as indicated by manufacturer. (*see* Note 20)

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²⁰When using the confocal microscope, select the best parameters for your samples, such as a low laser power and a quick scan, to avoid photobleaching. The pinhole should be set to the optimal size for each objective. Care should be taken to avoid overlapping signals between fluorochromes by setting each channel excitation power and emission bandwidth correctly. The PMT (Photo Multiplier Tube) gain should be high enough to have a bright image without saturated pixels. If the signal is weak, increasing laser power at maximum may be useless, because you probably will create a false positive image.

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Figure 1.

Gal-1 exhibits diffuse cytosolic localization in various adult tissues. Frozen sections of porcine tissue were subjected to confocal analysis using anti-Gal-1 (α hGal-1) and secondary antibody (α hGal-1 + 2°) or secondary alone (2° alone) as indicated. (A–C) α hGal-1 followed by secondary stain of liver (A), brain (B) or stomach (C). (D–F) Staining with the secondary antibody alone for liver (D), brain (E) or stomach (F). This research was originally published in *Glycobiology*. Dias-Baruffi M, Stowell SR, Song SC, Arthur CM, Cho M, Rodrigues LC, Montes MA, Rossi MA, James JA, McEver RP, Cummings RD. Differential expression of immunomodulatory galectin-1 in peripheral leukocytes and adult tissues and its cytosolic organization in striated muscle. *Glycobiology*. 2010 May;20(5):507–20. Oxford University Press

Figure 2.

Gal-1 displays organized cytosolic localization in striated tissues. Frozen sections of cardiac or skeletal porcine muscle tissue were subjected to confocal analysis using anti-Gal-1 (α hGal-1) and secondary antibody (α hGal-1 + 2°) or secondary alone (2° alone) as indicated. (A–B) α hGal-1 followed by secondary stain of (A) cardiac muscle and (B) skeletal muscle. (C–D) Staining with the secondary antibody alone for (C) cardiac muscle and (D) skeletal muscle. This research was originally published in *Glycobiology*. Dias-Baruffi M, Stowell SR, Song SC, Arthur CM, Cho M, Rodrigues LC, Montes MA, Rossi MA, James JA, McEver RP, Cummings RD. Differential expression of immunomodulatory galectin-1 in peripheral leukocytes and adult tissues and its cytosolic organization in striated muscle. *Glycobiology*. 2010 May;20(5):507–20. Oxford University Press