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Detection of Tight Junction Barrier Function In Vivo by Biotin

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Abstract

Tight junctions (TJs) are the most apical component of the junctional complexes in mammalian epithelial cells and form selective paracellular barriers restricting the passage of solutes and ions across the epithelial sheets. Claudins, a TJ integral membrane protein family, play a critical role in regulating paracellular barrier permeability. In the in vitro cell culture system, transepithelial electrical resistance (TER) measurement and the flux of radioisotope or fluorescent labeled molecules with different sizes have been widely used to determine the TJ barrier function. In the in vivo system, the tracer molecule Sulfo-NHS-Biotin was initially used in *Xenopus* embryos system and subsequently was successfully applied to a number of animal tissues in situ and in different organisms under the experimental conditions to examine the functional integrity of TJs by several laboratories. In this chapter, we will describe the detailed procedures of applying biotin as a paracellular tracer molecule to different in vivo systems to assay TJ barrier function.

Keywords

Tight Junctions; Permeability barrier; Biotin tracer; Claudin-7; Epithelial cells

1. Introduction

Tight junction (TJ) is a gatekeeper of paracellular space between epithelial cells and prevents macromolecules and pathogens from entering the tissues while allowing the passive entry of ions and small molecules. Ample studies have reported that claudins, TJ integral membrane proteins, are essential for TJ barrier function (1–7). To study the TJ barrier function in vivo, Sulfo-NHS-Biotin has been commonly used as a tracer molecule. Biotins are small water-soluble molecules with their molecular weight ranging from 443 to 666 Da. Biotins are membrane impermeable reagents and allow efficient labeling of proteins and primary amine-containing macromolecules on the cell surface. Biotin reagents will not diffuse through the intercellular space if TJ is intact. However, if TJ structure/function is disrupted, the Biotin molecule will penetrate into the intercellular space. Sulfo-NHS-Biotin was first used in the early *Xenopus* embryo system by Chen et al. in 1997 to examine TJ integrity and barrier function after microinjecting various occludin deletion constructs into the 8-cell stage of blastomere (8). Subsequently, Furuse et al. (1) successfully applied it to the claudin-1-deficient mice to detect the epidermal barrier function. Nitta et al. (9) used this surface biotinylation technique to study blood brain barrier in claudin-5-deficient mice. TJ permeability assay using Sulfo-NHS-Biotin was also performed in pathogen-infected intestinal epithelia (10). More recently, Jeong et al. demonstrated that the blood brain barrier of adult zebrafish is functionally and molecularly similar to that of higher vertebrates by using Sulfo-NHS-Biotin and other molecular tracers and markers (11). Thus, it is clear that Biotin can be successfully applied to many different in vivo systems to examine TJ

permeability barrier. In this chapter, we will describe the step-by-step protocol of applying Sulfo-NHS-Biotin to the *Xenopus* embryo system and to our recently developed claudin-7 knockout mouse to detect TJ barrier function in vivo.

2. Materials

2.1. Biotin Detection in *Xenopus* Embryo

1. Preparation for 25× MMR (Marc's Modified Ringer's) solution: For a total of 250 ml solution, add 36.525 g NaCl (2.5 M), 0.93 g KCl (50 mM), 0.75 g MgSO₄ (25 mM), 1.84 g CaCl₂ (50 mM), 1.25 ml of 0.5 M EDTA (2.5 mM), and 7.447 g HEPES (125 mM). The working solution is 0.1× MMR. To make the working solution, add 2 ml of 25× MMR to 498 ml dH₂O, and adjust pH to 7.8 using 1N NaOH.
2. 6-well and 60-mm petri dishes, forceps, plastic and glass transfer pipettes (see Note 1).
3. A refrigerated immersion cooler (Harvard Apparatus, Catalog # 724900).
4. To prepare 1 mg/ml EZ-Link Sulfo-NHS-Biotin (Pierce Chemical Co., Molecular weight: 443.43; Catalog # 21217), take Sulfo-NHS-Biotin out from −20°C freezer and let it sit at room temperature for 15 min before opening the cap. Dissolve Biotin in 0.1× MMR + 10 mM HEPES to make 1 mg/ml solution and cool it at 10°C.
5. Preparation for 5% paraformaldehyde fixation solution: dissolve 1.5 g paraformaldehyde in 15 ml H₂O, bring up to 19.5 ml, and then add 10.5 ml of 0.2 M sodium cacodylate.
6. Preparation for blocking buffer: make 1% fish skin gelatin (Sigma, Catalog # G7765) and 1% BSA (Sigma, Catalog # A8022) in PBS.
7. RITC-avidin (Pierce Chemical Co.) was diluted 1:500 in blocking buffer containing 1% fish skin gelatin and 1% BSA in PBS.
8. Tissue-Tek O.C.T. compound (VWR International, Catalog # 25608-930).
9. 2-Methyl butane (Fisher Scientific, Catalog # O3551) and liquid nitrogen.
10. Superfrost Plus slides (Fisher Scientific, Catalog # 12-550-15).
11. Cryostat Microtome (Microm HM 505, Richard-Allan Scientific, Kalamazoo, MI).
12. Zeiss Axioskop fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY).

2.2. Biotin Detection in Claudin-7 Knockout Mice

2.2.1. Biotin Injection

1. One-week old claudin-7 knockout (KO) mice recently generated in this laboratory were used for the biotin permeability assay.
2. Low pressure syringe pump (Harvard Apparatus, Catalog # 55-2219).
3. 1-ml syringe with 25G needle (see Note 2) connected to a polyethylene tubing (PE 50, VWR, Catalog # 427411).

¹For an easy transfer of the embryos from one plate to another, use the glass pipette with its end bent into a 90° angle and polish its tip on the flame of a Bunsen Burner.

²It is important to use the blunt end needle. The tip of the needle was cut by a Rotary Tool device (DREMEL, Racine, Wisconsin).

4. Stainless steel injection needle (30G, Small Parts, Catalog # HTX-30R) connected to a polyethylene tubing (PE 10, VWR, Catalog # 427401). The other end of this tubing is inserted into the end wall of above PE 50 tubing connected with the 25G needle. The connecting site of two tubings is sealed by parafilm.
5. Ketamine (18 mg/ml) and Xylazine (2 mg/ml) mixture was made from the stock solution of 100 mg/ml of Ketamine and 100 mg/ml of Xylazine and diluted with physiological saline (0.9% NaCl).
6. EZ-Link Sulfo-NHS-LC-Biotin was obtained from Pierce Chemical Co (Molecular weight: 556.59; Catalog # 21335) and stored in -20°C freezer with desiccant. Dissolve 15 mg Sulfo-NHS-Biotin in 2.97 ml of PBS (pH 7.5) and 0.03 ml of 100 mM CaCl_2 . This gives a final Biotin concentration of 5 mg/ml in PBS containing 1 mM CaCl_2 (see Note 3).

2.2.2. Biotin Detection

1. Tissue-Tek O.C.T. compound (VWR International, Catalog # 25608-930) for tissue embedding.
2. Disposable base mold (VWR International, Catalog # 60872-488) as tissue embedding container.
3. 2-Methyl butane (Fisher Scientific, Catalog # O3551) for freezing the tissue.
4. Superfrost Plus slides (Fisher Scientific, Catalog # 12-550-15) for mounting tissue sections.
5. A Cryostat Microtome (Microm HM 505, Richard-Allan Scientific, Kalamazoo, MI) for cutting frozen sections.
6. Ethanol and acetone (obtained from Fisher Scientific) for fixing the tissue sections on the slides.
7. Phosphate Buffered Saline (PBS): Prepare 10 $\times$ stock with 1.38 M NaCl (80 g/L), 26.67 mM KCl (2 g/L), 80.6 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (21.6 g/L), 14.71 mM KH_2PO_4 (2 g/L), and adjust pH to 7.4 using 1N NaOH if necessary. Make up to 1 L with dH_2O and autoclave it before stored at room temperature. Prepare working solution by 1:10 dilution of 10 $\times$ stock with dH_2O .
8. Blocking solution: 5% BSA in 1 $\times$ PBS.
9. TEXAS RED-conjugated Streptavidin (CALBIOCHEM, Catalog # 189738).
10. The ProLong Antifade reagents (Molecular Probes, Inc., Catalog # P7481).
11. A Zeiss Axiovert S100 microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with Metamorph Imaging Software (Molecular Devices, Downingtown, PA).

3. Methods

Both occludin and claudins are tetraspan membrane proteins localized at TJs of epithelial cells. Expression of truncated occludin mutants has been reported to cause malfunction of TJ functions in *Xenopus* embryos and cultured MDCK II cells (8, 12). Claudin-7 is a member of the claudin family and has been reported to be involved in modulating paracellular Cl^- permeability in cultures (6, 13–15). Our recently generated claudin-7 KO mice model showed that these mice displayed salt wasting, chronic dehydration, and distinct growth

³The Biotin solution needs to be freshly prepared for each use. Do not prepare stock solution for storage.

retardation phenotypes (16). Most of these pups died around 8–9 days after birth. Biotin, as a tracer molecule, has been used in both the above *Xenopus* embryo system and our claudin-7 KO mouse to detect TJ barrier function. We observed the biotin leakage through the paracellular space of *Xenopus* embryos injected with occludin mutant constructs as well as the renal tubular epithelial cells of 1-week old claudin-7 KO pups. The biotin leakage in kidney tubular epithelial cells may not be directly caused by claudin-7 deletion since it occurs in some of the KO pups later in their lives (such as 7/8 days), but not at a younger age.

3.1. Tight Junction Permeability Assay in *Xenopus* Embryos Using Surface Biotinylation Method

1. At the 8-cell stage, the embryos were injected with full length or C-terminally truncated occludin RNA constructs. After RNA injection, the embryos were incubated at room temperature for 6 h.
2. Turn on the refrigerated immersion cooler and keep the temperature of water in a container at 10°C.
3. Add 1 mg/ml Biotin solution to a six-well dish and cool it at 10°C.
4. The embryos injected with WT and mutant occludin RNA constructs were placed into a 60-mm petri dish containing pre-cooled 0.1× MMR solution and kept at 10°C for 5 min (see Note 4).
5. The above embryos were carefully transferred into the six-well petri dish containing pre-cooled 1 mg/ml NHS-LC-Biotin in 0.1× MMR solution using the L-shaped glass pipette and then were kept at 10°C for 12 min (see Note 5).
6. The embryos were washed twice with 0.1× MMR solution at 10°C and fixed in 5% paraformaldehyde overnight at 4°C. The fixed embryos were rinsed three times with PBS.
7. Cut the aluminum foil into about 3 cm² and fold it into a funnel shape. Add the TISSUE-TEK O.T.C. compound into this funnel-shaped small container half full and embed two embryos into it, and then add O.T.C. compound to 80% full of the funnel-shaped container. The samples were immediately frozen in 2-methyl butane/dry ice and then in liquid nitrogen.
8. The frozen blocks were trimmed and cut into 14-μm thick sections on a Cryostat Microtome. The frozen sections were put onto the Superfrost Plus slides.
9. The sections were dried at room temperature for 30 min and incubated with the blocking buffer (1% fish skin gelatin and 1% BSA in PBS) overnight or for at least 5 h at room temperature.
10. The sections and the control sections from the embryo without Biotin labeling were incubated with RITC-avidin diluted 1:500 in blocking buffer for 1 h at room temperature.
11. All slides were washed three times with blocking buffer and two times with PBS, and then mounted with mounting medium.

⁴It is critical to keep the embryos at 10°C because at this temperature, the blastomeres will stop the cell division, which is required for the experiments, while allowing for the normal development of these embryos after biotin labeling procedures.

⁵The 12-min labeling time was chosen since this was the maximum time at 10°C, which was 100% consonant with normal development of embryos to tadpole stages.

12. Sections were examined by epifluorescence using a Zeiss Axioskop microscope and photographed with TMAX-400 film (see an example in Fig. 1).

3.2. Biotin Injection Through the Cardiac Ventricle of Claudin-7 Wildtype and Knockout Mice

3.2.1. Injection Procedures

1. The vial containing Sulfo-NHS-Biotin powder was equilibrated to room temperature first before opening the cap. A small amount of Biotin was weighed at a time and directly dissolved in PBS (pH 7.5, Sulfo-NHS ester reacts with primary amines at pH 7.0–9.0) in the presence of 1 mM CaCl_2 to make 5 mg/ml Biotin solution.
2. One-week old claudin-7 wildtype (WT) and knockout (KO) pups were anesthetized by intraperitoneal injection with 0.05 ml/10 g of Ketamine (18 mg/ml) and Xylazine (2 mg/ml) mixture (see Note 6). After the pup was deeply anesthetized (see Note 7), thoracotomy was made to gain access to the thoracic cavity. All these procedures were approved by the East Carolina University (ECU) Animal Care and Use Committee and conducted in compliance with guidelines from the National Institute of Health and ECU on laboratory animal care and use.
3. The 1-ml syringe and its attached tubing were filled with 5 mg/ml of Sulfo-NHS-Biotin. The syringe was then connected to the low pressure pump and the injection speed was set at 100 $\mu\text{l}/\text{min}$. The 30G injection needle was inserted into the other end of the tubing.
4. The 30G injection needle with the blunt end was carefully inserted into the left ventricle of the heart and the biotin solution was perfused into the tissues through the blood stream (see Note 8). Five minutes after receiving 150 $\mu\text{l}/\text{g}$ (body weight) Biotin injection, the pup was sacrificed by decapitation and the kidneys were removed from the body.
5. The kidneys were immediately rinsed with PBS and placed into a base mold containing O.C.T. compound. This base mold was half immersed in 2-methyl butane on dry ice in a container until the O.C.T. compound was completely frozen (change the color into white) and then transferred to the liquid nitrogen.
6. The O.C.T. blocks were trimmed and sectioned on a cryostat. The 5 μm frozen sections were mounted on superfrost plus slides and moved to the next step (see Note 9) or stored at -80°C until needed.

3.2.2. Biotin Detection Using Fluorescent Light Microscopy

1. The frozen sections of mouse kidneys mounted on slides were thawed out at room temperature for 5 min and then fixed in 95% ethanol at 4°C for 20 min followed by 100% acetone at room temperature for 5 min.
2. After fixation, the sections were rinsed three times in PBS and blocked with 5% BSA in PBS (blocking buffer) for 50 min.

⁶The correct dose of the drug is critical since overdose of the drug will kill the pup.

⁷Whether the deep anesthesia is achieved or not can be tested by using a forceps to pinch the mouse tail. If there is no response after a sharp pinch, this means that a deep anesthesia is achieved.

⁸To test whether the perfusion system is working or not, the syringe and its attached tubing can first be filled with 1% Evans Blue diluted in 0.9% NaCl. Five minutes after injecting the Evans Blue into the left ventricle of the heart, the skin of the whole body of the pup will turn blue. This indicates that the perfusion system is working.

⁹To prevent the sections from coming off the slides, it is necessary to dry the slides at room temperature for about 30 min to make sure that the sections stick well to the slides.

3. After removing the blocking buffer, the sections were incubated with TEXAS RED-conjugated Streptavidin (1:200 dilution in the blocking buffer) for 30 min and then washed three times each with the blocking buffer for 5 min and briefly rinsed with PBS twice before adding the mounting medium.
4. Kidney sections from claudin-7 WT and KO pups without biotin injection were also incubated with TEXAS RED-conjugated Streptavidin to serve as a negative control as well as to test for endogenous biotin reactivity.
5. One hour before the slides were ready, powdered Prolong anti-fade reagent (Component A) and Prolong mounting medium (Component B) were taken from -20°C freezer (see Note 10). Approximately 1 ml of Component B was added to one of the brown vials containing powdered Component A. The two components were mixed by very gently pipetting the mounting medium up and down. After the antifade reagent was no longer adhered to the sides of the vial, the vial was left at room temperature for about 30 min to eliminate the small air bubbles.
6. After the last rinse with PBS, the residual liquid on the slide was removed by a Kimwipe prior to coverslipping the sample. The antifade reagent/mounting medium mixture was applied to the sections with a 200- μl pipette. The sections were then covered by a coverslip. The slide was dried on a flat surface in the dark for at least 2–3 h before being viewed.
7. The sections were observed and photographed with an Axiovert fluorescent microscope. The images were taken using Metamorph Imaging Software as shown in Fig. 2.

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References

1. Furuse M, Hata M, Furuse K, Yoshida Y, Haratake A, Sugitani Y, et al. Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. *J Cell Biol.* 2002; 156:1099–1111. [PubMed: 11889141]
2. Colegio OR, Van Itallie CM, McCrea HJ, Rahner C, Anderson JM. Claudins create charge-selective channels in the paracellular pathway between epithelial cells. *Am J Physiol Cell Physiol.* 2002; 283:C142–147. [PubMed: 12055082]
3. Turksen K, Troy TC. Permeability barrier dysfunction in transgenic mice overexpressing claudin 6. *Development.* 2002; 129:1775–1784. [PubMed: 11923212]
4. Amasheh S, Meiri N, Gitter AH, Schoneberg T, Mankertz J, Schulzke JD, et al. Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. *J Cell Sci.* 2002; 115:4969–4976. [PubMed: 12432083]
5. Yu AS, Enck AH, Lencer WI, Schneeberger EE. Claudin-8 expression in Madin-Darby canine kidney cells augments the paracellular barrier to cation permeation. *J Biol Chem.* 2003; 278:17350–17359. [PubMed: 12615928]
6. Alexandre MD, Lu Q, Chen YH. Overexpression of claudin-7 decreases the paracellular Cl^- conductance and increases the paracellular Na^+ conductance in LLC-PK1 cells. *J Cell Sci.* 2005; 118:2683–2693. [PubMed: 15928046]

¹⁰If the Prolong mounting medium turns milky or is too viscous to manipulate, it should be placed in a 50°C water bath for 1 h before mixing it with Component A.

7. Hou J, Paul DL, Goodenough DA. Paracellin-1 and the modulation of ion selectivity of tight junctions. *J Cell Sci.* 2005; 118:5109–5118. [PubMed: 16234325]
8. Chen Y, Merzdorf C, Paul DL, Goodenough DA. COOH terminus of occludin is required for tight junction barrier function in early *Xenopus* embryos. *J Cell Biol.* 1997; 138:891–899. [PubMed: 9265654]
9. Nitta T, Hata M, Gotoh S, Seo Y, Sasaki H, Hashimoto N, et al. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J Cell Biol.* 2003; 161:653–660. [PubMed: 12743111]
10. Guttman JA, Li Y, Wickham ME, Deng W, Vogl AW, Finlay BB. Attaching and effacing pathogen-induced tight junction disruption in vivo. *Cell Microbiol.* 2006; 8:634–645. [PubMed: 16548889]
11. Jeong JY, Kwon HB, Ahn JC, Kang D, Kwon SH, Park JA, et al. Functional and developmental analysis of the blood-brain barrier in zebrafish. *Brain Res Bull.* 2008; 75:619–628. [PubMed: 18355638]
12. Balda MS, Whitney JA, Flores C, Gonzalez S, Cereijido M, Matter K. Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. *J Cell Biol.* 1996; 134:1031–1049. [PubMed: 8769425]
13. Hou J, Gomes AS, Paul DL, Goodenough DA. Study of claudin function by RNA interference. *J Biol Chem.* 2006; 281:36117–36123. [PubMed: 17018523]
14. Alexandre MD, Jeansonne BG, Renegar RH, Tatum R, Chen YH. The first extracellular domain of claudin-7 affects paracellular Cl^- permeability. *Biochem Biophys Res Commun.* 2007; 357:87–91. [PubMed: 17400193]
15. Tatum R, Zhang Y, Lu Q, Kim K, Jeansonne BG, Chen YH. WNK4 phosphorylates ser(206) of claudin-7 and promotes paracellular Cl^- permeability. *FEBS Lett.* 2007; 581:3887–3891. [PubMed: 17651736]
16. Tatum R, Zhang Y, Salleng K, Lu Z, Lin JJ, Lu Q, et al. Renal salt wasting and chronic dehydration in claudin-7-deficient mice. *Am J Physiol Renal Physiol.* 298:F24–34. [PubMed: 19759267]

Fig. 1.

Expression of mutant occludin disrupted the barrier function of TJ in *Xenopus* embryos. mRNAs transcribed from full-length or mutant occludins were microinjected into the antero-dorsal blastomere of eight-cell embryos. Six hours after injection (2,000 cell blastula), the embryos were labeled by incubation in 1 mg/ml NHS-LC-Biotin for 12 min at 10°C, then washed and fixed. Frozen sections were stained with RITC-avidin. The staining of NHS-LC-Biotin appeared as a thick continuous line on the surface of blastomeres. The TJs in the embryos injected with full-length (**a**, 504 amino acids), or the least COOH-terminally truncated (**b**, 486 amino acids) occludin mRNAs were impermeable to the biotin tracer. In contrast, in the embryos injected with two COOH-terminally truncated occludins (**c**, 386 amino acids, and **d**, 336 amino acids), the TJs were leaky and therefore, the biotin tracer penetrated into the intercellular spaces. The *arrowhead* (**c**) reveals that the membranes of internal cells beneath those at the embryonic surface have also been biotinylated, indicating that the tracer penetrated beyond the first tier of cells. *Bar*, 10 μ m. (From Chen et al., J Cell Biol 1997; 138(4):891–899 (8), by copyright permission of the Rockefeller University Press).

Fig. 2.

Leakage of tight junctions in renal tubules of claudin-7 KO mice. Two 7/8-day old claudin-7 KO pups (**b** and **d**) and their two WT littermates (**a** and **c**) were anesthetized by intraperitoneal injection of Ketamine/Xylazine mixture. After deep anesthesia was achieved, Sulfo-NHS-LC-Biotin was perfused through the left ventricle of the heart. Five minutes after biotin perfusion, the pups were sacrificed and kidneys were removed from the body. Five-micrometer frozen sections were fixed in 95% ethanol at 4°C for 20 min and then fixed in 100% acetone at room temperature for 5 min. Sections were incubated with blocking buffer (5% BSA in PBS) for 50 min before incubating with TEXAS RED-conjugated Streptavidin for 30 min at room temperature. The distribution of injected biotin tracer was visualized by immunofluorescence microscopy. Images (**e**) and (**f**) were taken from WT and KO kidneys of the same litter without biotin injection to serve as a negative control for TEXAS RED-conjugated Streptavidin. All images were taken from the kidney cortex region and the *arrows* indicate the lumen of the renal tubule. The *arrowhead* in insert of image (**b**) points to the biotin labeling between tubular epithelial cells. (**g**) Glomerulus. *Bar*: 30 μ m.