2007

Whole-mount techniques to evaluate subepithelial cellular populations in the adult mouse intestine

Keely G. McDonald
Washington University School of Medicine in St. Louis

Rodney D. Newberry
Washington University School of Medicine in St. Louis

Whole-mount techniques to evaluate subepithelial cellular populations in the adult mouse intestine

Keely G. McDonald and Rodney D. Newberry
Washington University School of Medicine, St. Louis, MO, USA

BioTechniques 43:50-56 (July 2007)
doi 10.2144/000112514

Traditional immunohistochemistry on thinly cut frozen or paraffin-embedded sections has proven to be an invaluable technique for the analysis of cell-specific protein expression. While this technique performs well for specific point identification of cells within tissues, the feasibility of examining the entirety of large tissues or examining tissues in three dimensions is limited by the sectioning of the sample. Whole mounts of entire tissues combined with immunohistochemical techniques overcome some of these limitations. This approach is particularly useful in organs with a large surface area and regional diversity, such as the gastro intestinal tract. Despite these advantages, the use of whole mounts to evaluate aspects of the adult gastrointestinal tract have been largely limited to the study of submucosal neurons (1–3) or to the study of the overlying epithelium (4–7); studies of the diverse cellular populations within the mucosa have been hampered by technical issues. Techniques to study the neurons in the small bowel rely on removing the mucosal layer; therefore this approach is not useful to studying any cellular components of the mucosal layer. Our initial attempts to stain subepithelial cellular populations without removal of the epithelium were universally unsuccessful, suggesting that antibodies cannot effectively penetrate the serosa or the intact epithelium and associated mucous layer. To circumvent these limitations, we developed a technique that allows removal of the epithelial barrier while maintaining the underlying cellular components with their three-dimensional (3-D) structure. Combining this technique with standard immunohistochemistry has facilitated the rapid identification of specific cellular components, as well as the 3-D and regional organization of these cellular components within the intestinal lamina propria (8–10). The initial technique we described (8) allowed for only limited evaluation of tissues by low-power light microscopy. The protocol outlined here represents a significant advance, as it is amenable to examination by low-power dissecting microscopes and high-power light or fluorescent microscopes. In addition, the detection strategies are not limited to chromogenic substrates, but can use fluorescent detection alone or in combination with chromogenic detection. This significantly expands the utility and versatility of this technique and allows for the study of the macroscopic regional distribution and microscopic organization of multiple cellular populations within the entire intestine in one study.

Here, we describe this procedure in detail (see Protocol 1 for a brief overview of the procedure). To minimize the degradation of the intestine, it is crucial that all solutions be cold (4°C) or warmed to 37°C in the steps before the fixation process, and that all steps are performed quickly up to the point of fixation of the intestine. Practice may be required to perform these techniques in a timely manner. To make the plates to mount the intestine, dissecting wax (Carolina Biological Supply, Burlington, NC, USA) is melted by placing it in a beaker in a

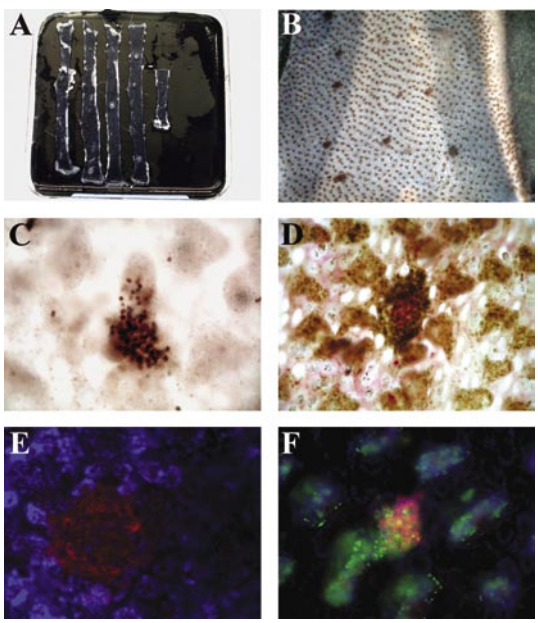

Figure 1. Examples of the intestinal whole-mount protocol and the flexibility of detection strategies. (A) View of the entire murine small intestine whole mount after removing the epithelium and after fixation. (B) Detection of B220, an isoform of CD45 expressed on the surface of B cells, with the enhanced 3,3′-diaminobenzidine tetrahydrochloride (DAB) metal chromogen under low-power microscopy (20×) using a dissecting microscope allows for rapid detection of clusters of positively staining B cells throughout the intestine. (C) A higher power (400×) examination of the proximal portion of the same intestine in panel B reveals the architecture of individual villi is preserved and the organization of the positively staining cells in orientation to villi structure. (D) Distal intestine stained with anti-B220 (brown) using DAB-enhanced metal substrate and anti-immunoglobulin M (IgM; red) using Fast Red substrate (Pierce) as a second chromogen, examined at 400×. Differences in the number of B220-positive cells between panel C, proximal intestine, and panel D, distal intestine, are reflective of regional differences in the distribution of B cells throughout the intestine and illustrate the utility of this approach. (E) CD11c (the integrin α-X chain, a widely used cell surface marker for dendritic cells) detected with DAB metal, appears as dark spots under fluorescent microscopy. Sections were co-stained with fluorescently detected anti-B220 (red) and nuclei fluorescently labeled in blue, as detected by Hoechst dye (Sigma-Aldrich, St. Louis, MO, USA) incorporation, and examined at 400×. (F) Fluorescent staining with anti-CD11c (green) and anti-CD90 (red), with nuclei in blue as detected by Hoechst dye incorporation, examined at 400×.

boiling water bath and then poured into a square (9 × 9 cm) Petri dish (Fisher Scientific, Pittsburgh, PA, USA) until approximately one-half full and allowed to cool. Multiple plates can be made ahead of time and used as needed. The intestine is removed intact and placed in cold phosphate-buffered saline (PBS) in a Petri dish, where it is flushed and opened. If the integrity of the Peyer's patches is important in the study, then the intestine should be opened along the mesenteric border to preserve these structures, which lie preferentially on the anti-mesenteric border. Once the intestine is opened, exposing the lumen, it is then transferred to the mounting plate containing cold PBS (Fisher Scientific). The intestines are mounted on dissecting wax in the square Petri dish lumen-side-up using 0.22-mm pins (Carolina Biological Supply). The intestine, pinned to the plate, is incubated with shaking (90 rpm) in warmed (37°C) Hank's balanced salt solution (HBSS; Fisher Scientific) containing 5 mM EDTA at 37°C for 12 min. We have found that this 12-min incubation time can vary between 10–15 min as needed to allow effective removal of the epithelial cells. After the 12-min incubation, the lumenal surface of the intestine is then washed vigorously with the HBSS containing 5 mM EDTA using a 30-cc syringe and 23-gauge needle to remove the epithelial cells. The intestine is rinsed once with cold PBS and then submerged in 10% formalin-buffered saline (Fisher Scientific) and placed at 4°C for at least 1 h; this can be extended to an overnight incubation if desired (Figure 1A). Following the fixation, the intestine is washed for 5 min in a solution of 1 M NaCl, 1 M Tris, pH 7.2, and 0.5% Triton® X-100 (TBST) three times. The addition of Triton X to the buffer at this point is necessary to facilitate the penetration of the antibody to the underlying cellular components. If detection using a horseradish peroxidase (HRP)-based substrate is anticipated, endogenous peroxidases can be quenched by treating the intestines with a solution of 1% H_2O_2 (Fisher Scientific) diluted in methanol at room temperature with shaking for 15 min. The intestine is subsequently washed three times for 5 min in TBST, followed by a blocking step in TBST containing 1% bovine serum albumin (BSA) (TBST-BB) for 30 min with shaking at room temperature. The protocol can be stopped at this point, and the tissue stored at 4°C in TBST-BB for extended periods of time. Further steps are largely determined by the detection method used to identify antibody staining and how the tissue will be analyzed. A standard protocol for staining these specimens is given below.

Primary antibodies diluted in TBST-BB are incubated with the intestine overnight at 4°C. On the following day, the intestine is washed as before in TBST, and the secondary antibody or conjugate, if required, is added in blocking buffer and placed on the tissue for at least 1 h with shaking at room temperature. After a 1-h incubation, the intestine is washed three times in TBST, and detection is performed using chromogenic or fluorescent substrates. Whole mounts can now be analyzed or further stained. If counterstaining is not desired, the chromogen-stained intestines can be put in distilled water, covered, and placed at 4°C for as long as 6 months. The protocol allows for significant flexibility in how the antibodies can be detected and the tissue analyzed. We have found detection using the chromogen 3,3′-diaminobenzidine tetrahydrochloride (DAB)-enhanced metal substrate (Pierce, Rockford, IL, USA) to be advantageous for identifying cellular staining at low power using light microscopy (Figure 1B). This allows for easy examination of the entire intestinal surface for the evaluation of the regional distribution of cellular components under low-power objectives (Figure 1B) and can easily be combined with examination under higher power objectives to evaluate the organization of cellular components (Figure 1C). The flexibility of the protocol allows for detection of additional antibody staining with a second chromogen (Figure 1D) or with high-power fluorescent microscopy with a second or third fluorescent reagent (Figure 1, E and F). We observed that TBST is no longer required after the initial staining and detection, and switching to PBS can improve the intensity of counterstaining. If a biotinylated reagent is used for the initial detection and counterstaining, available biotin and avidin are blocked using the Avidin/Biotin Blocking kit (Vector Laboratories, Burlingame, CA, USA). For convenience, staining can be performed on a smaller piece of tissue in a microcentrifuge tube; the tube containing the tissue can be shaken on a benchtop rotator during each incubation. If a nuclear counterstain is desired for fluorescent detection, the intestine can be treated for 10 min in bizbensamide (Fisher Scientific) and washed in PBS. To view the intestine using high-powered light or fluorescence microscopy with an upright microscope, the tissue can be mounted between two slides by applying a small bead of glue around the slide edges to keep the intestine secure and hydrated for viewing (Figure 1, C–F). If a combination of chromogenic and fluorescent detection strategies are used together, the chromogenically stained cells will appear as dark areas under the fluorescent filters, or alternatively they can easily be identified by switching to standard light microscopy (Figure 1E).

Combining the above technique to remove the epithelial barrier with basic immunohistochemical protocols has proven to be a rapid and cost-efficient method for evaluating the regional distribution and organization of cellular components within the intestine. Similar to the experience with techniques for the study of neuronal organization, our approach can be easily adapted to evaluate other hollow organs within the gastrointestinal tract.

ACKNOWLEDGMENTS

This work was supported in part by the Crohn's and Colitis Foundation of America, the Nicholas Costrini Award in IBD Research, and NIH grants no. DK064798 and AG028309.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

Protocol 1. Brief Overview of Small Intestine Whole-Mount Procedure

1.	Remove small intestine as a whole; place in cold phosphate-buffered saline (PBS).
2.	Flush intestine with cold PBS and open along anti-mesenteric border.
3.	Pin intestine lumen-side-up from proximal to distal ends on mounting plate.
4.	Incubate intestine in Hank's balanced salt solution (HBSS) + 5 mM EDTA for 12 min at 37°C shaking (90 rpm).
5.	Remove epithelial cells with needle and syringe.
6.	Wash in cold PBS once.
7.	Place in 10% formalin-buffered saline at 4°C for at least 1 h.
8.	Wash in 1 M NaCl, 1 M Tris, pH 7.2, and 0.5% Triton X-100 (TBST) three times.
9.	Incubate in 1% H_2O_2 in methanol for 15 min.
10.	Wash in TBST three times.
11.	Wash in TBST containing 1% bovine serum albumin (BSA) (TBST-BB) for 30 min.
12.	Incubate intestine in primary antibody overnight at 4°C.
13.	The following day; wash in TBST three times.
14.	Incubate in secondary antibody at room temperature for 1 h.
15.	Wash in TBST three times.
16.	Develop using a chromogen or fluorescent substrate.

REFERENCES

1. **Heuckeroth, R.O. and V. Pachnis.** 2006. Getting to the guts of enteric nervous system development. Development *133*:2287-2290.
2. **Vohra, B.P., K. Tsuji, M. Nagashimada, T. Uesaka, D. Wind, M. Fu, J. Armon, H. Enomoto, et al.** 2006. Differential gene expression and functional analysis implicate novel mechanisms in enteric nervous system precursor migration and neuritogenesis. Dev. Biol. *298*:259-271.
3. **Montedonico, S., T. Sri Paran, M. Pirker, U. Rolle, and P. Puri.** 2006. Developmental changes in submucosal nitrergic neurons in the porcine distal colon. J. Pediatr. Surg. *41*:1029-1035.
4. **Debard, N., F. Sierro, J. Browning, and J.P. Kraehenbuhl.** 2001. Effect of mature lymphocytes and lymphotoxin on the development of the follicle-associated epithelium and M cells in mouse Peyer's patches. Gastroenterology *120*:1173-1182.
5. **Li, Y.Q., S.A. Roberts, U. Paulus, M. Loeffler, and C.S. Potten.** 1994. The crypt cycle in mouse small intestinal epithelium. J. Cell Sci. *107*:3271-3279.
6. **Winton, D.J. and B.A. Ponder.** 1990. Stem-cell organization in mouse small intestine. Proc. Biol. Sci *241*:13-18.
7. **Delker, D.A., Q.S. Wang, A. Papanikolaou, H.E. Whiteley, and D.W. Rosenberg.** 1999. Quantitative assessment of azoxymethane-induced aberrant crypt foci in inbred mice. Exp. Mol. Pathol. *65*:141-149.
8. **Lorenz, R.G., D.D. Chaplin, K.G. McDonald, J.S. McDonough, and R.D. Newberry.** 2003. Isolated lymphoid follicle formation is inducible and dependent upon lymphotoxin-sufficient B lymphocytes, lymphotoxin beta receptor, and TNF receptor I function. J. Immunol. *170*:5475-5482.
9. **Lorenz, R.G. and R.D. Newberry.** 2004. Isolated lymphoid follicles can function as sites for induction of mucosal immune responses. Ann. N. Y. Acad. Sci. *1029*:44-57.
10. **McDonald, K.G., J.S. McDonough, and R.D. Newberry.** 2005. Adaptive immune responses are dispensable for isolated lymphoid follicle formation: antigen-naive, lymphotoxin-sufficient B lymphocytes drive the formation of mature isolated lymphoid follicles. J. Immunol. *174*:5720-5728.

Received 20 March 2007; accepted 24 March 2007.

Address correspondence to Rodney D. Newberry, Washington University School of Medicine, 660 South Euclid Ave., Campus Box 8124, St. Louis, MO 63110, USA. e-mail: rnewberry@im.wustl.edu