In Focus in HCB

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This issue features several articles presenting state-of-the-art methods and ongoing protocol advancements occurring in immunohistochemical techniques. We will briefly highlight two of those articles here. Lopez et al. (2016) provide a comprehensive review of methods and protocols for immunohistochemical studies of the human inner ear. Since the first decision often encountered in any prospective immunohistochemical study is choice of sample processing and potential embedding media, they compare and contrast immunostaining results obtained for inner ear tissue samples (as well as microdissected auditory and vestibular endorgans from human temporal bone) processed for subsequent frozen sections, paraffin sections and celloidin sections. Frozen sections and sections from formalin-fixed paraffin-embedded tissues are well-known methods in immunohistochemistry, but celloidin embedding may be in general less well known. Celloidin is a form of nitrocellulose polymer which has been used to great success for embedding human temporal bone samples, resulting in superb morphological detail with concomitantly less tissue shrinkage than seen with paraffin embedding; the negative aspect of celloidin embedding is the rather long tissue processing times involved. For immunohistochemistry, protocols have been developed for removal of celloidin from the section, with subsequent antigen retrieval protocols usually necessary. The authors illustrate the review with many examples of both fluorescence and chromogenic

immunostaining results obtained with all three tissue processing methods. Moreover, they provide a very useful section on advantages/disadvantages of the three types of tissue processing for immunohistochemistry on microdissected human auditory and vestibular endorgans, as well as whole-mount human cochlear and vestibular endorgans. The review concludes with an appendix providing full details on the methods and protocols highlighted therein, providing a most valuable resource for investigators currently involved with, or potentially contemplating immunohistochemical investigations of human inner ear samples.

Besides choosing the (hopefully) optimal tissue processing and embedding protocol, a further issue encountered in immunostaining techniques revolves around signal-to-noise ratios (SNR). This issue is particularly evident in immunofluorescence where background fluorescence (usually the result of tissue autofluorescent properties) degrades image contrast and resolution through a diminished SNR. Rosas-Arellano et al. (2016) describe modified signal enhancement protocols for both immunofluorescence and triple immunogold/transmission electron microscopy. They describe an antibody signal enhancer (ASE) solution, which is used in a blocking step, and as a diluent for the primary antibody, composed of a phosphate saline solution with added glycine, Triton X-100, Tween 20 and hydrogen peroxide. They compare the immunostaining results obtained using their enhancer solution to those obtained using a commercially available signal enhancing solution, by both qualitative and semiquantitative means. Importantly, they assessed SNR effects attributable to the individual ASE components themselves, demonstrating the synergistic effect produced by the combination present in the overall mixture. In addition, for a novel triple immunoelectron microscopy protocol, they describe using their ASE solution on ultrathin sections from brain samples

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embedded in the hydrophilic LR White resin. Since the authors detail all of the reagents and their concentrations in the ASE solution, other investigators will have the option of “fine-tuning” these components to optimize results for the individual antibodies and cellular/tissue samples used in their own investigations.

Quantitative analysis of light and electron microscopic immunolabeling has become an indispensable tool in basic and applied biomedical research. Equally important is the analysis of the probable colocalization of two or multiple different antigens. Most commonly, colocalization is investigated by immunofluorescence and defined as the overlap of different fluorescent dyes. Methods to analyze colocalization of immunolabeling, usually immunogold labeling, at the level of electron microscopy are less routine. By following the principle of the Manders’ colocalization coefficients used in immunofluorescence microscopy, Pastorek et al. (2016) introduce now the quantification of the colocalization level in pointed patterns for immunogold electron microscopy. For their approach, they redefined the light microscopic term colocalization to reflect the product of the spatial interactions at the single-particle (single molecule) level. This definition emphasizes more a physical and probability-based systematic spatial codistribution (co-occurrence) of the studied molecules that are detected by the use of different types of electron dense particles. As a rule, the quantitative image analysis is performed in three steps: the estimation of (1) the frequency distribution of particles; (2) the relative colocalization coefficient; and (3) the relative density of colocalization. For proof of principle, the level of spatial interaction between nuclear phosphatidylinositol 4,5-bis-phosphate and the nucleolus component fibrillarin was investigated as detected by double immunogold labeling and compared with the results of Manders’ colocalization coefficients for double confocal immunofluorescence and super-resolution structured illumination microscopy. The analysis of the double immunogold labeling revealed a higher association of fibrillarin with the nucleolus as compared to phosphatidylinositol 4,5-bis-phosphate. In addition, it was found that phosphatidylinositol 4,5-bis-phosphate has a higher tendency to colocalize with fibrillarin than vice versa. When compared to the results obtained with the Manders’ colocalization coefficients for double confocal immunofluorescence and super-resolution structured illumination microscopy, a similar tendency of the results was noticed suggesting the validity of the novel computational technique for quantifying the level of colocalization in pointed pattern in electron microscopic images. It is proposed that the technique can also be applied to other super-resolution microscopy imaging modes analyzing discrete pointed structures.

The enzyme guanylyl cyclase C, which becomes activated by the peptides guanylin and uroguanylin, controls

the intestinal fluid homeostasis. Both guanylin and uroguanylin are locally produced and secreted by cells of the intestinal epithelium. Various studies have shown that depending on the studied species, guanylin and uroguanylin may be synthesized by different cell types. In human small intestine, guanylin transcripts as detected by in situ hybridization were observed in Paneth cells, whereas in rat, guanylin transcripts were detected in goblet cells and uroguanylin transcripts in enterochromaffin cells. In rat colon, guanylin was found in both goblet cells and columnar cells of the surface epithelium. In contrast, in guinea pigs, guanylin was restricted to enterochromaffin cells. Distinct but overlapping distribution patterns were reported for mouse intestine. Transcripts for uroguanylin were detected exclusively in the small intestinal villi, but transcripts for guanylin were revealed in both the villi and the crypts of the small intestine and in the surface epithelium of the colon. It is not certain whether these reported differences reflect species-specific differences or whether they are due to limitations of the conventional in situ hybridization technique. Therefore, Ikpa et al. (2016) have reinvestigated the expression pattern of guanylin (*Guca2a*), uroguanylin (*Guca2b*) and guanylyl cyclase C (*Gucy2c*) in mouse intestine by quantitative PCR and an improved in situ hybridization technique called RNAscope. In RNAscope, multiple (up to 20) independent pairs of oligonucleotide probes per transcript are used for hybridization to the target sequences for signal amplification. Therefore, RNAscope ensures selective amplification of target specific signals and as consequence a strongly improved signal-to-noise ratio. In agreement with the quantitative PCR analysis, *Guca2a* gradually increased from duodenum along the small intestine and peaked in colon, whereas *Guca2b* were low in duodenum and in colon and peaked in the middle to distal part of the small intestine. Transcripts for *Gucy2c* were expressed at low levels. The distribution pattern of *Guca2a*, *Guca2b* and *Gucy2c* compared well with that reported for rat intestine. The specificity of RNAscope for *Guca2a* was shown by the negative results in the intestine of *Guca2a* null mice. Furthermore, *Guca2a*, *Guca2b* and *Gucy2c* transcripts were undetectable in gastric and pancreatic epithelia. At the cellular level, *Guca2a* and *Guca2b* were detected mainly in Paneth cells of the crypts of Lieberkühn and in brush cells in the lower villus of duodenum, with the enterocytes of the villi being almost non-expressing. However, enterocytes of distal jejunum and ileum showed strong *Guca2a* and *Guca2b* expression. In colon, *Guca2a* but not *Guca2b* transcripts were found in the surface epithelium. *Guca2b* transcripts, however, were detected in cells at the base of colonic crypts probably corresponding to cKit-positive Paneth-like cells. The epithelium overlying lymphoid tissue in small intestine and in colon was strongly positive for both *Guca2a* and *Guca2b*. Expression of *Gucy2c*

was relatively uniform in the cells of the crypt and villus region of small intestine and the colonic enterocytes. Taken together, this study using an advanced in situ hybridization technique, RNAscope, revealed novel aspects of the cellular localization of guanylin, uroguanylin and guanylyl cyclase C in mouse intestine. This also suggests that guanylin and uroguanylin may play a role in chemoreception, stem cell proliferation and host defense.

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