A method for fixing and paraffin embedding tissue to retain the natural fluorescence of reporter proteins

Akifumi Nakagawa, Kate Von Alt, Keith D. Lillemoe, Carlos Fernández-del Castillo, Andrew L. Warshaw, and Andrew S. Liss
Department of Surgery and the Andrew L Warshaw, MD, Institute for Pancreatic Cancer Research, Massachusetts General Hospital and Harvard Medical School, Boston, MA

BioTechniques 58:153-155 (September 2015) doi 10.2144/000114328
Keywords: FFPE; GFP; natural fluorescence

Supplementary material for this article is available at www.BioTechniques.com/article/114328.

Green fluorescent protein (GFP) and its derivatives are routinely employed as surrogate markers for gene expression and lineage tracing in genetically engineered mice. Tissues from these mice are commonly formalin fixed and paraffin embedded (FFPE) for histological studies. However, this results in inactivation of the natural fluorescence of these proteins, requiring their detection by immunological techniques. Here we present an ethanol fixation protocol that allows for the direct visualization of the natural fluorescence of reporter proteins while maintaining excellent tissue histology. We demonstrate the utility of this method for visualizing green and red fluorescent proteins in a wide range of murine tissues using both cytoplasmic and membrane-localized fluorescent reporter proteins. Tissues fixed by this method also allow for immunohistochemical studies, providing a single method to visualize the natural fluorescence of reporter proteins with subsequent detection of cellular proteins.

Fluorescent proteins such as green fluorescent protein (GFP) and red fluorescent protein (RFP) are valuable tools in engineered reporter mice (1). These proteins allow for identification of cells expressing a particular gene when inserted downstream of the endogenous promoter of the gene of interest. Fluorescent reporter proteins are frequently employed in lineage tracing studies where Cre recombinase activates their expression. An example of this is the mTmG mouse, which expresses a red fluorescent membrane-targeted tandem dimer Tomato (mT) prior to Cre-mediated excision and a membrane-targeted GFP (mG) after excision. Compared to experimental models with a single reporter protein, this mouse allows for visualization of both recombined (mG) and non-recombined (mT) cells (2).

Although the natural fluorescence of reporter proteins is widely visualized in both live and fixed cells in culture, their full potential in animal systems has not been realized. The preparation of standard formalin fixed and paraffin-embedded (FFPE) tissues, while providing optimal histology, results in the inactivation of the natural fluorescence of these proteins (3,4). Therefore, antibodies are frequently employed to detect reporter proteins by immunofluorescence or immunohistochemistry. Frozen sections from snap-frozen tissue are often employed for experiments where visualization of the natural fluorescence of the reporter protein is desired (5); however, frozen sections often have poor tissue morphology (6). Reduced formalin fixation time prior to paraffin embedding of the tissue has served as a compromise approach. However, not all tissue types can be adequately fixed by a short exposure to formalin, and high expression of fluorescent reporter proteins is required for best results (4,7).

Here we present a modified version of ethanol fixation and paraffin embedding of tissue that allows for clear visualization of the natural fluorescence of reporter proteins while maintaining excellent tissue morphology (8). mTmG reporter mice were bred with Pdx1-cre mice (both strains purchased from The Jackson Laboratory, Bar Harbor, ME) to allow for membrane-localized GFP expression in the pancreas. Reporter mice were sacrificed at 8–10 weeks of age and whole organs were harvested and fixed in pre-chilled 95% ethanol (Decon Laboratories, King of Prussia, PA) for 20–24 h at 4°C. Tissues were dehydrated in 4 changes of pre-chilled 100% ethanol for 1 h at 4°C, and the ethanol was subsequently cleared from the samples in 3 changes of pre-chilled xylenes (Fisher Scientific, Pittsburgh, PA) for 1 h, each at 4°C. Specimens were allowed to come to room temperature prior to being permeated with paraffin (Leica Biosystems, Buffalo Grove, IL) by 1 h immersions in 4 paraffin baths at 56°C. Blocks of paraffin embedded tissue were stored in the dark at 4°C until use. Sections of tissue 5 μm thick were cut on a Leitz 1512 microtome (Leica Biosystems), and mounted paraffin sections were dried for 30 min at 40°C.

Slides were deparaffinized and washed by 1 min of gentle agitation in 2 changes of pre-chilled xylenes, 3 changes of

METHOD SUMMARY

We describe a method to fix and paraffin embed tissues for visualization of the natural fluorescence of reporter proteins. This method preserves tissue histology and enables subsequent immunological detection of cellular proteins, providing a single source of tissue for diverse experimental goals.

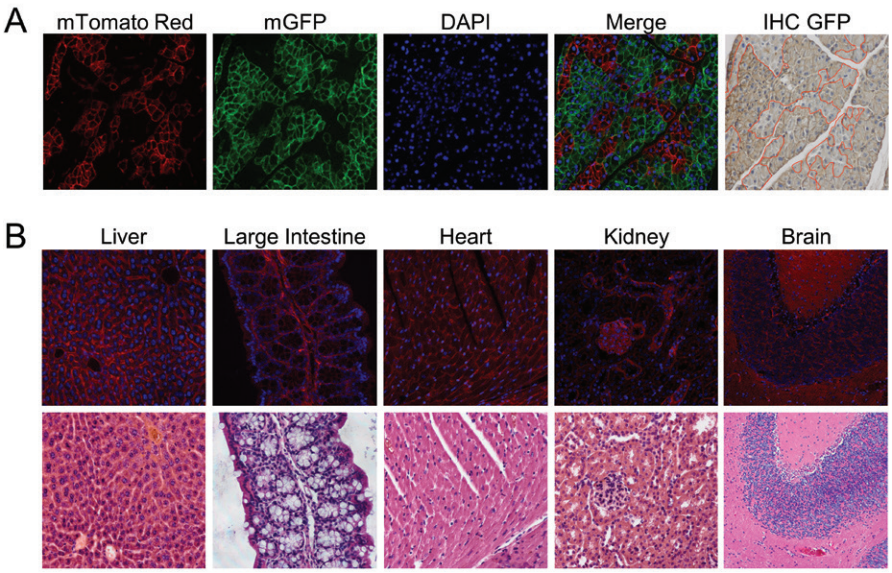

Figure 1. Natural fluorescence of membrane-localized reporter proteins. (A) Representative images from pancreas of Pdx1-cre;mTmG mice. Red and green fluorescence of membrane-localized reporter proteins as well as DAPI-stained nuclei (blue) are shown. Matched image from slide that was processed for GFP immunohistochemistry (IHC) is shown on the right. Areas of tissue that do not express GFP but do express mT are outlined in red. (B) Representative fluorescence images of mT and DAPI-stained nuclei in the liver, large intestine, heart, kidney, and brain of Pdx1-cre;mTmG mice. Matched hematoxylin and eosin (H&E)-stained images are shown in the bottom row.

pre-chilled 95% ethanol, and 3 changes of pre-chilled Tris-buffered saline (TBS). The cell nuclei were stained with DAPI (Sigma, St. Louis, MO) (1 μg/mL) for 5 min, followed by washing the slides with 2 changes of TBS and mounting with coverslips. DAPI and fluorescent proteins were visualized with a UV microscope. The typical mosaic pattern of expression of the Pdx1-cre construct can be readily observed by the natural fluorescence of the mT and mG proteins in the pancreas of Pdx1-cre;mTmG mice (Figure 1A). This method of fixation is applicable to a wide range of tissues, as tomato red fluorescence and tissue histology is preserved in liver, kidney, heart, brain, and small intestine (Figure 1B). The ethanol-based fixation method presented here also allows for traditional immunohistochemistry to be performed after visualization of fluorescence. The coverslips of slides visualized for fluorescence were removed, and the slides were washed in TBS, incubated in 0.3% H_20_2 (Fisher Scientific) for 20 min, blocked in TBS containing 0.1% BSA (Fisher Scientific) and 10% normal goat serum (Jackson ImmunoResearch Lab, West Grove, PA), and incubated with an antibody specific to GFP (Novus Biologicals Littleton, CO) overnight at 4°C. The following day, the slides were incubated with an appropriate biotinylated secondary antibody and developed with 3,3'-diaminobenzidine (DAB) (Invitrogen, Frederick, MD). Matched images of immunohistochemistry (IHC) and natural fluorescence revealed that membrane-localized GFP is readily detected by IHC in pancreatic specimens (Figure 1A, right panels).

A common problem with ethanol fixation of GFP-expressing cells in culture is that GFP is a soluble cytoplasmic protein and readily diffuses out of the cell (5). Employing Lgr5-EGFP-IRES-CreER mice (The Jackson Laboratory), which express enhanced GFP (EGFP) in the crypts of the small intestine, we observed that the standard ethanol fixation protocol described above suffers, at least to some extent, from a similar problem. EGFP fluorescence is readily observed in the crypts of the small intestine in freshly cut sections prior to deparaffinization (Figure 2A). However, after deparaffinization in xylenes, fluorescence is greatly diminished. It is worth noting that not all EGFP is lost, since its expression can still be detected by immunological methods. Therefore, it is likely that this method would allow for the detection of EGFP fluorescence in tissue where EGFP is more highly expressed. In order to visualize cytoplasmic EGFP fluorescence in tissue fixed using the ethanol-based fixation method described here, slides can be further fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 16–24 h at room temperature prior to deparaffinization. This additional fixation is sufficient to retain EGFP in the tissue during the deparaffinization and subsequent DAPI staining process (Figure 2B). The mechanism by which paraformaldehyde penetrates the paraffin to fix the tissue is unknown. However, the effectiveness of paraffin as a moisture barrier is proportional to its thickness and temperature (9). The use of thin (5 μm) sections combined with their immersion in paraformaldehyde immediately after fixing the tissue on the slides likely allows for sufficient penetration of the paraformaldehyde.

The method of tissue fixation and paraffin embedding described here allows for the visualization of the natural fluorescence of reporter proteins and offers several advan-

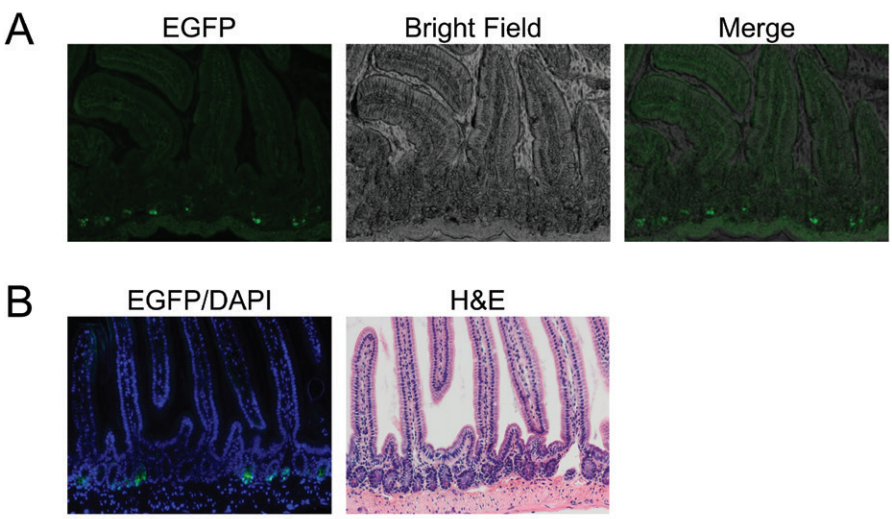

Figure 2. Natural fluorescence of cytoplasmic enhanced GFP (EGFP) reporter protein. (A) Representative EGFP fluorescence and matched bright field images from the small intestine of an Lgr5-EGFP-IRES-CreER mouse prior to deparaffinization. (B) Representative images of EGFP fluorescence with DAPI-stained nuclei and matched hematoxylin and eosin (H&E) staining of the small intestine.

tages over existing methods. While immunological detection of reporter proteins is commonly employed, these methods may result in either false negative or false positive expression (10). Furthermore, due to the generally poor tissue histology of frozen sections, the use of these samples is often restricted to fluorescence analysis and therefore requires additional experimental animals for studies. Since this ethanol fixation method maintains tissue histology and provides samples that are readily amenable to immunological detection of cellular proteins, tissue fixed by this method offers a single source of material for analysis. Moreover, the ability to detect fluorescence within freshly cut slides provides for the rapid identification of tissue sections with appropriate reporter gene expression.

Author contributions

A.N. and A.L. conceived the study. A.N., K.V., and A.L. designed and carried out the experiments. A.N., K.V., C.F., and A.L. analyzed the results. All authors contributed to the writing and editing of the manuscript.

Acknowledgments

We would like to thank Patricia Della Pelle for her advice and technical support. This work was supported in part by the Andrew L Warshaw, MD, Institute for Pancreatic Cancer Research and a grant from the NIH: P01CA117969. This paper is subject to the NIH Public Access Policy.

Competing interests

The authors declare no competing interests

References

1. **Fuchs, E. and V. Horsley.** 2011. Ferreting out stem cells from their niches. Nat. Cell Biol. *13*:513-518.
2. **Muzumdar, M.D., B. Tasic, K. Miyamichi, L. Li, and L. Luo.** 2007. A global double-fluorescent Cre reporter mouse. Genesis *45*:593-605.
3. **Jiang, X., Z. Kalajzic, P. Maye, A. Braut, J. Bellizzi, M. Mina, and D.W. Rowe.** 2005. Histological analysis of GFP expression in murine bone. J. Histochem. Cytochem. *53*:593-602.
4. **Swenson, E.S., J.G. Price, T. Brazelton, and D.S. Krause.** 2007. Limitations of green fluorescent protein as a cell lineage marker. Stem Cells *25*:2593-2600.
5. **Jockusch, H., S. Voigt, and D. Eberhard.** 2003. Localization of GFP in frozen sections from unfixed mouse tissues: immobilization of a highly soluble marker protein by formaldehyde vapor. J. Histochem. Cytochem. *51*:401-404.
6. **Onodera, Y., H. Shimizu, S. Yamashita, and T. Nishikawa.** 1992. Cryofixed, freeze-dried and paraffin-embedded skin enables successful immunohistochemical staining of skin basement membrane antigens. Histochemistry *98*:87-91.
7. **Williams, J.H., B.L. Mepham, and D.H. Wright.** 1997. Tissue preparation for immunocytochemistry. J. Clin. Pathol. *50*:422-428.
8. **Sainte-Marie, G.** 1962. A paraffin embedding technique for studies employing immunofluorecence. J. Histochem. Cytochem. *10*:250-256.
9. **Kline, G.M.** 1937. Permeability to moisture of synthetic resin finishes for aircraft. J. Res. Natl. Bur. Stand. *18*:235-249.
10. **True, L.D.** 2008. Quality control in molecular immunohistochemistry. Histochem. Cell Biol. *130*:473-480.

Received 26 May 2015; accepted 30 June 2015.

Address correspondence to Andrew S. Liss, Department of Surgery, Massachusetts General Hospital, Boston, MA. E-mail: aliss@mgh.harvard.edu