Published in final edited form as:

J Histotechnol. 2008 September ; 31(3): 129–134.

Time-Saving Benefits of Intravital Staining

Catherine MacGillivray, Jeremy Sylvan, Richard T. Lee, and Hayden Huang

Department of Medicine, Brigham and Women's Hospital, Cambridge, MA

Abstract

One of the challenges in labeling tissues for fluorescence microscopy is minimizing sample processing while maintaining or improving the information generated by the fluorescent label. Generally, tissues are extracted, fixed, and embedded in mounting media (such as paraffin), sectioned, and then postprocessed by removing the paraffin, blocking, labeling, and washing. Despite all of these steps, the consistency of labeling quality can vary as a result of several factors, including heterogeneity in labeling efficiency from slide to slide, the necessity of postprocessing to obtain information on sequential sections of tissue, interference from the mounting media, and loss of native three-dimensional structural information, especially in thicker sections. A method for embedding and processing tissues that have been labeled by intravital staining is described in this study. Intravital staining is the process in which live-cell dyes and other labels are injected into the bloodstream before fixation of the tissues. Tissues processed this way can be imaged upon sectioning without further staining and retain their native, three-dimensional information, thereby improving the information retained by the labels and speeding up sample processing.

Keywords

microscopy; paraffin; sectioning; three-dimensional imaging

Introduction

Laboratories that perform histological analyses can benefit from techniques that save time by prelabeling entire tissues before harvesting. Commonly used stains such as Sirius Red or Periodic acid-Schiff (PAS; Sigma 395B) can be used to measure cell size (1,2). However, processing the slides necessary for these measurements, starting from slide-mounted sections and ending with microscope-ready slides (which we define as labeled sections ready for imaging and analysis), could take from a half to a full day. On the other hand, intravital staining with the use of select labels would allow one to analyze the samples immediately after cutting them without requiring further staining. We hypothesize that, given a proper dye and protocol, intravital staining would save time by labeling an entire organism in one fast (<1 h) incubation. The procedure would eliminate the need to perform serial labeling postfixation on each slide containing a section of interest from the tissue. Additionally, multiple tissues would be simultaneously labeled, further decreasing the need for extensive postprocessing. These labels can be selected to provide information relevant to a given study.

This article describes the development of a protocol and selection of fluorescence labels that can be used for intra-vital staining. Because our interest was in measuring cell size, we chose a fluorescence-conjugated maleimide label to take the place of Sirius Red. By

injecting a solution containing the maleimide into a tail vein and sacrificing the animal after a 15- to 30-min incubation, we show that the slide is ready to be imaged immediately upon sectioning from the paraffin block, and data obtained this way can then be further analyzed (3). We also show that one can simultaneously label the cell nuclei and vasculature using this method to increase the amount of information obtained by this technique.

Methods

Triple Staining with Intravital Staining

Alexa Fluor 488 isolectin (Invitrogen, Carlsbad, CA) was purchased as a powder and dissolved to obtain 500 $\mu\text{g}/1\text{ mL}$ of phosphate-buffered saline (PBS), Texas Red maleimide (Invitrogen) was dissolved to obtain 25 mg/136 μL of dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO), and Hoechst 33342 was obtained as a solution of 10 mg/mL in water. Isolectin labels endothelial cells, and maleimide, typically being cell-impermeant, labels the extracellular matrix. Hoechst (a blue fluorescent dye) was used to label tissue nuclei. The wavelengths were chosen to permit three-color imaging; different arrangements, particularly for using fewer labels, can be used as well.

Mice were anesthetized with 0.3 mL of pentobarbital cocktail (stock consisted of 0.8 mL pentobarbital [BWH Pharmacy, Boston, MA] in 10 mL of saline). Level of arousal was determined by a footpad pinch. Mice tolerated injection when sedation was mild to moderate, as indicated by a slight startle response. All animal work was conducted in accordance with the Harvard Medical Area Standing Committee for Animals.

The mouse tail was gently massaged to facilitate dilation of the tail vein. In some cases, a warm compress was used to assist in detection of the tail vein location. Fifty micro-liters of Hoechst solution was drawn into a 1-mL tuberculin syringe. The syringe needle was inserted close to the surface of, and perpendicular to, the upper surface of the tail, and the Hoechst solution was injected. After a 10-min incubation, 50 μL of the isolectin solution was injected in a similar fashion, but more proximal to the body compared with the first injection. The placement of this second injection site reduces leakage out of the first injection site. After a 5-min incubation, 20 μL of maleimide in DMSO, diluted 1:1 with 20 μL of saline, was injected into the lower surface of the tail to reduce leakage from the other injection sites. During the injections, the mouse was closely monitored for distress, which is especially critical during injection of DMSO-containing compounds.

After further incubation of 5 to 10 min, the animal was sacrificed and the tissues were harvested. The total incubation period for triple-labeling the tissues with intravital labels was approximately 30 min. The tissues of interest were fixed in 4% paraformaldehyde solution in PBS (Sigma) and incubated overnight. The samples were processed through alcohol, xylene, and embedded in paraffin (4). Specifically, samples were transferred from 4% paraformaldehyde to 70% alcohol for 1 h, incubated twice in 95% alcohol for 30 min per incubation, and incubated twice in 100% alcohol for 30 min per incubation. The tissues were then transferred to a fume hood and incubated four times in xylene for 30 min per incubation. After xylene, tissues were soaked in molten paraffin for three incubations at 45 min per incubation and finally embedded in paraffin blocks.

After embedding, the paraffin blocks were sectioned to 5- μm thickness and mounted on standard glass slides (Fisher Scientific, Hampton, NH). The sections were then imaged with the use of a fluorescence microscope (Olympus, Center Valley, PA) that included a 40 $\times$ NA 1.15 objective. Images were acquired with a Coolsnap HQ cooled CCD camera (Olympus). Sample images from the heart, intestine, and spleen are shown in Figure 1. These images are

brightness- and contrast-adjusted for clarity; such adjustments are necessary if postprocessing merging will be performed. Each tissue and each channel had different gains.

The images acquired from the intravital staining can then be used for quantitative or qualitative analysis. For example, relative localization of each of the components can be assayed by merging the images using artificial-color schemes (Figure 2). Such data are useful when one wishes to study the relative arrangement of the different labeled components.

One advantage to intravital staining is the relative uniformity of the labels across the tissue, without the need for further postprocessing. To demonstrate this, sections were acquired from tissues near the surface, at 100 or 200 μm deep, and at 1000 μm (1 mm) deep using the microtome as a guide for depth. Because the microtome is not an exact measure of section thickness, sections as far as 1 mm deep were taken to ensure the sections being examined were far removed from the original section. Although the tissue architecture may be different, the overall signal quality is remarkably consistent through the imaging depths (Figure 3).

To assess whether it is possible to make the process more efficient, we injected all three of the label injections simultaneously instead of serially. The results indicate that there is considerable loss of signal on the green (isolectin) label (Figure 4), suggesting that there is either interference from one label to another, or distortion in the readouts caused by the infusion of a large fluid volume. As the result of this finding, the protocol outlined in this study relies on serial injections. It should be noted, however, that the red and blue channels remained robust.

Immunofluorescence Staining of Intravital-Labeled Tissues

As powerful as the intravital staining is, one may wish to perform more specific immunofluorescence on the sections. Sections that are already double labeled with Hoechst and Texas Red conjugated maleimide can be further probed with standard immunofluorescence techniques, without significant loss of signal from the maleimide and or Hoechst stains. The tissues are prepared as described previously, with the isolectin stain omitted (i.e., the maleimide was injected 15 min after the Hoechst was injected with the use of different injection sites as described). After harvesting, fixation, and alcohol/xylene processing, the tissues were embedded in paraffin blocks.

Five-micrometer-thick slices were sectioned and mounted on standard glass slides. The sections were deparaffinized with ionized water as follows: two incubations of 5 min in Histoclear, two incubations of 2 min in 100% ETOH (ethanol), two incubations of 2 min in 95% ETOH, two incubations of 2 min in 70%, and then a 1 min incubation in water. The slides were then incubated in PBS with 20% horse serum for 20 min as a blocking step. Excess solution was tapped off the slides and the slides were then incubated in sarcomeric actin IgM (Sigma) for 1 h at a 1:100 dilution in PBS with 20% horse serum. The slides were then washed three times in PBS and incubated in secondary alexa 488 goat antimouse IgM (Invitrogen) in PBS with 20% horse serum for 30 min, washed three times, and covered with hydromount aqueous nonfluorescing mounting media (Fisher Scientific) and premium cover slip (Fisher Scientific). The slides were then imaged by fluorescence microscopy as before.

To compare the efficacy of immunohistochemistry and to avoid issues from increased background, we acquired images on the green fluorescence channel (the channel for the secondary antibody) for the same exposure times and were not brightness- or contrast-adjusted. Results indicate that the immunofluorescence is quite strong in the labeled specimen compared with a similar section that was also double labeled by intravital staining

but not processed for immunofluorescence (Figure 5). Occasionally, the existing intra-vital labels faded somewhat with immunofluorescence processing. This did not occur on all specimens but is something to consider when imaging valuable tissues.

Discussion and Conclusions

The technique described in this article has significant benefits for multiple staining of tissues while reducing postprocessing required for certain tissue labels. Intravital staining can stain multiple organs and, in some samples, the sections have maintained their fluorescence for over a year. A single incubation of less than an hour before harvesting of the tissues can label the nuclei, endothelium, and matrix in different whole organs, with microscope-ready slides after mounting the sections, without the need for further processing. Further, tissues that have already been labeled with intravital techniques are suited for immunofluorescence staining. This technique can improve efficiency significantly, reducing the need for labeling multiple slides across a single organ, and across multiple different tissues. Because entire organs are being labeled simultaneously, there may be significantly less variability in stain quality from different sections taken from the same organ. We note, however, that the number of tested dyes that can be used for intravital staining is currently low (three presented in this article) compared with those available for immunohisto-chemistry or immunofluorescence labeling. It may be worth trying other labels, including conjugated antibodies, with intravital staining to assess whether specific surface receptors can be detected using this technique.

This technique also has scientific advantages because it supports efficient, three-dimensional (3D) imaging of tissues. Creating a 3D image using conventional histological techniques requires considerable time and effort. For example, if the total desired imaging thickness of an organ were 500 μm and a single section were 5 μm thick, then one would need to process and keep track of 100 individual slices. In contrast, using the intravital staining technique, one simply sections and images tissues. Further, one can stop sectioning intermittently (e.g., every 100 μm) to keep the number of active slides manageable, without requiring the repetition of staining protocols. Intravital staining not only speeds the 3D imaging process, but it also enhances the scientific value of the images. As a result of the tissues being labeled in situ, the labels better reflect the organization of the tissues' native local environments. After intra-vital staining, an entire tissue can be sectioned and mounted. Serial images of the same section can be readily identified for highly structured tissues, such as the heart. With the use of cross-registration techniques, it is possible to recreate a 3D representation of a tissue, and judicious selection of labels can be used to extract individual cell information (3,5). This has been done to examine cell volume, with the finding that cell volume changes can be quantitatively different from those measured using cross-sectional areas (5). Finally, with deep-tissue imaging techniques such as two-photon microscopy, one can image the tissues in 3D while maintaining the native architecture of the tissue (6). Intra-vital staining eliminates damage or distortion of tissues often caused by pressure pumps. Thus, intravital labeling provides an elegant alternative for the assessment of structure in whole tissues.

The use of paraformaldehyde as a fixative can lead to issues with background autofluorescence. This is evident when the sections are imaged with long exposures, particularly using blue emission filters. Thus, if only one or two labels are required, green and red would be the best combination despite the greater wavelength separation afforded by using red and blue, because it would minimize autofluorescence. We have attempted to use frozen sections to avoid using paraformaldehyde completely; however, the stain quality was not as good, with significant smearing on the red and green labels (maleimide and isolectin, data not shown). Although it may be possible to develop a protocol to use frozen embedding

with intravital staining, standard paraformaldehyde fixation appears to be suitable for most tissues and applications.

We conclude that intravital staining is a powerful addition to the toolbox for histological studies of tissue architecture. Compared with standard postprocessing (e.g., the use of Sirius Red), intravital staining yields results across multiple tissues in a much shorter period of time. Further, the combination of multiple, whole-organ labeling while maintaining viability for further immunohistochemistry and the possibilities for 3D studies make this technique well-suited for a broad range of applications.

Acknowledgments

The authors thank Scott Perkins for assistance in developing this technique, and Peter So for his expertise in two-photon imaging.

This work was supported by grant R21 EB4646 and grant HL081404 from National Institutes of Health.

References

1. Frantz S, Hu K, Widder J, Bayer B, Witzel CC, Schmidt I, et al. Peroxisome proliferator activated-receptor agonism and left ventricular remodeling in mice with chronic myocardial infarction. *Br J Pharmacol* 2004;141:9–14. [PubMed: 14662734]
2. Yoshioka J, Imahashi K, Gabel SA, Chutkow WA, Burds AA, Gannon J, et al. Targeted deletion of thioredoxin-interacting protein regulates cardiac dysfunction in response to pressure overload. *Circ Res* 2007;101:1328–1338. [PubMed: 17916779]
3. Cruz, FU.; Lee, RT.; Huang, H. *Current Protocols in Molecular Biology*. John Wiley & Sons; New York: 2006. Three-Dimensional Reconstruction of Tissues.
4. Carson, FL. *Histotechnology: A self-instructional text*. 2nd ed. American Society of Clinical Pathologists; Chicago: 1997.
5. Yoshioka J, Prince RN, Huang H, Perkins SB, Cruz FU, Mac-Gillivray C, et al. Cardiomyocyte hypertrophy and degradation of connexin43 through spatially restricted autocrine/paracrine heparin-binding EGF. *Proc Natl Acad Sci USA* 2005;102:10622–10627. [PubMed: 16020536]
6. Ragan T, Sylvan JD, Kim KH, Huang H, Bahlmann K, Lee RT, et al. High-resolution whole organ imaging using two-photon tissue cytometry. *J Biomed Opt* 2007;12:014015. [PubMed: 17343490]

Figure 1. Fluorescence microscope images of (A) heart, (B) intestines, and (C) spleen from a mouse, labeled with Hoechst (nuclear stain) at left, isolectin (endothelial stain) in the center, and maleimide (matrix stain) on right of each image strip. Images were acquired at 40 $\times$ magnification. These images were brightness- and contrast-enhanced for clarity. The scale bars in the upper left of (A) and (C) and lower left of (B) = 20 μ m.

Figure 2.

False-colored and merged images from Figure 1A showing the relative location of the different components. Note that the green label indicating the location of blood vessels appears at edges or corners of the matrix stain (red), whereas the blue label, indicating nuclei, typically appears in the blank spaces. This image was brightness/contrast enhanced to show color balances clearly. The scale bar in the upper left corner = 20 μm .

A

B

Figure 3A.

Images of a mouse heart taken at 5 (top), 200 (middle), and 1000 (bottom) μm , with channels blue, green and red (left to right), demonstrate the consistency of the label without any further post-processing. Scale bar at the lower left = 20 μm . Image sets were adjusted for brightness and contrast only after the set was assembled to preserve the relative brightness/contrast of each section.

Images of a mouse liver taken at 5 (top), 100 (middle) and 1000 (bottom) μm , with channels blue, green and red (left to right). Some focal bright spots can be detected in the red channel; the source of these is not known. Scale bar at the lower left = 20 μm . Image sets were adjusted for brightness and contrast only after the set was assembled to preserve the relative brightness/contrast of each section.

Figure 4. Simultaneous injections (right) exhibit significant signal degradation on the green (isolectin) label compared with that obtained by serial injection (left), in a mouse liver. The exposure of the simultaneous injection was increased to double that of the serial injection due to a lack of signal at the same exposure time. Scale bar at the lower left = 20 μm . Images were brightness- and contrast-adjusted as a pair (after assembling the figure).

Figure 5.

(A) Double-labeled section in mouse heart using intravital labels Hoechst (left) and maleimide (right). The center image is the green channel, which only depicts background fluorescence since no labels were used in this fluorescence emission range. (B) Triple labeling in mouse heart using both intravital staining (left, Hoechst; right, maleimide) and immunofluorescence (center, IgM against sarcomeric actin). The center images for (A) and (B) were taken at the same exposure and not brightness-adjusted after acquisition. Thus, the increase in green fluorescence signal is from the immunostaining protocol, which labels actin in the cells. In some specimens as shown here, the intravital staining diminishes, but does not disappear, after the immunofluorescence labeling protocol. Scale bar in the upper left of each image set = 20 μm .