Benchmark

Injection of Evans blue dye to fluorescently label and image intact vasculature

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ABSTRACT

Blood vessels perform critical functions in both health and disease. Understanding how vessels form, pattern and respond to damage is essential. However, labeling and imaging the vasculature to ascertain these properties can be difficult and time-consuming. Here, the authors present a novel methodology for rapidly and efficiently labeling whole vascular networks *in vivo* by exploiting the fluorescent properties of Evans blue. By combining the labeling with fluorescence microscopy, this method enables visualization of whole tissue vasculature for a fraction of the time and cost compared with traditional methods.

METHOD SUMMARY

Evans blue is injected into the circulatory system of the experimental model. Following euthanasia, the tissue is collected, fixed and subsequently cleared (if necessary). Various fluorescent imaging modalities can be utilized to visualize the labeled vasculature.

KEYWORDS:

Evans blue (EB) ● fluorescence ● imaging ● vasculature

There are roughly 60,000 miles of blood vessels in the adult human body. This extensive vascular system is vital to animal physiology by providing a conduit for blood and immune cells, supplying nutrients, removing metabolic waste and supporting repair after injury. Additionally, recent studies have uncovered vascular heterogeneity between and within organs, thereby supporting tissue-specific physiologic functions, including the secretion of distinct 'angiocrine' factors [1–4]. As such, extensive research is devoted to understanding how vascular networks form during development and remodel after damage and how they are affected by genetic defects and disease.

Blood vessels traverse the body and infiltrate tissues, forming extensive networks. Additionally, the vessels that make up these networks vary in size, from the large aorta to the smallest capillaries. Therefore, visualizing vascular networks as a whole can provide important insights into form, function and any perturbations that occur because of genetic defects, injury or disease. Mice and zebrafish are examples of two vertebrate models commonly utilized to study vascular networks. Methods to visualize vascular networks include the injection of fluorescently labeled lectins, micro-computed tomography and *in vivo* photoacoustic imaging [5–7]. Additionally, transparent zebrafish embryos with transgenic fluorescent reporters enable the visualization of whole networks. However, the analysis of later stages and studies in mice are encumbered by complex methodology, expensive equipment and reagents, access to reporter lines and time-consuming protocols. Immunolabeling of vasculature within whole organs requires optimal tissue permeabilization, quality antibodies at high concentrations and sufficient antibody penetration. Deficiencies in any of these can produce subpar results such as partial staining [8]. Additionally, protocols for immunolabeling and clearing whole adult organs or entire embryos can take anywhere from 2 to 4 weeks [9]. Considerable troubleshooting is often necessary when testing new tissue samples or reagents. Current methodology therefore can present significant hurdles to visualizing vascular networks.

To circumvent many of these issues, the authors have developed a novel protocol that rapidly, simply and effectively labels intact vasculature for fluorescent imaging by any modality (Figure 1A). The authors have taken advantage of several unique properties of Evans blue (EB) to label vascular networks *in vivo*. EB (T-1824) is a 961-Da bis-azo dye developed by Herbert McLean Evans in 1914 and was initially employed to determine the proportion of water in blood plasma [10,11]. EB, like other azo dyes such as trypan blue, is non-cell permeable and can be utilized in cell viability assays [12]. More commonly, it has been used to detect vessel perfusion or damage to blood vessels by monitoring its leakage into the surrounding tissue, particularly the brain and skeletal muscle [13–15]. It has also been applied to genetic knockouts to detect abnormal vasculature development in organs such as the kidney [16]. Interestingly, EB binds tightly to serum albumin. A tetrasulfonic acid group on the dye and a reaction between this group and amino groups on albumin are responsible for the interaction [17]. When EB binds to albumin, it undergoes a conformational shift of a cis-trans isomerization, reducing rotational freedom and producing fluorescence in the red to far-red spectrum (Figure 1B) [18]. Excitation occurs at 620 nm and emission
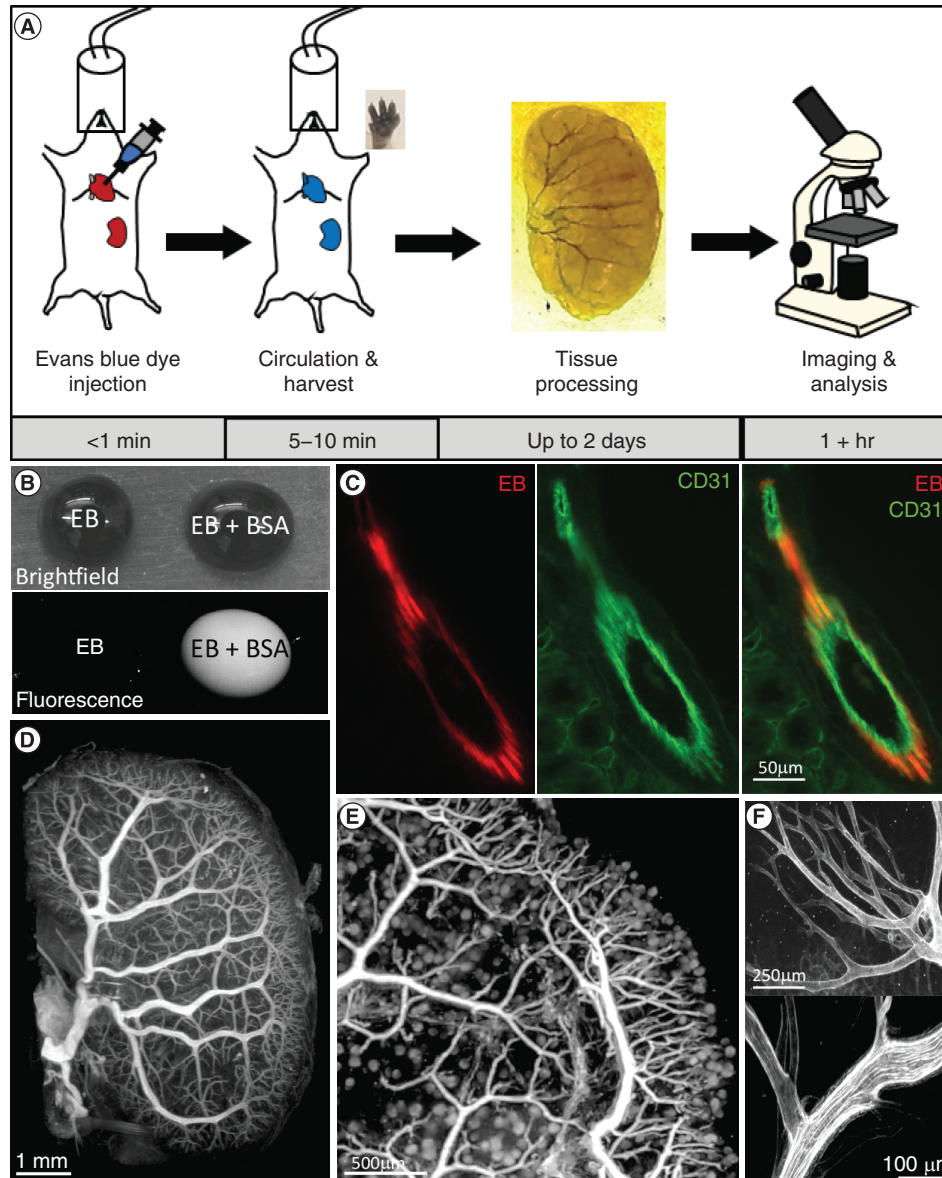
Figure 1. EB labels vasculature with high contrast. (A) Graphical method for labeling vasculature with EB. In brief, a solution of EB is injected intracardially under anesthesia and allowed to circulate. The harvested tissue is then processed for imaging with fluorescence microscopy. **(B)** EB fluoresces in the far-red spectrum only when bound to albumin, as shown with or without BSA under brightfield and fluorescence. **(C)** Colocalization of EB with an immunostain for the pan-vascular marker CD31 in an artery of a cryosectioned adult kidney. **(D)** Wholemount of an adult mouse kidney imaged by light sheet. **(E)** Zoomed view of mouse kidney vasculature imaged by light sheet, where glomeruli have been labeled in this instance. **(F)** Confocal images of labeled adult mouse kidney vasculature in the renal cortex.
BSA: Bovine serum albumin; EB: Evans blue.

at 680 nm [19]. Thus, injecting EB into circulation leads to its binding with albumin. Albumin associates with the endothelial glycocalyx and is also endocytosed, leading to fluorescent labeling of the endothelium [20]. This is supported by co-localization of EB fluorescence with endothelial-specific markers such as PECAM1 (CD31), as observed in an adult kidney artery (Figure 1C). Additionally, EB (unbound and bound) is visible with the naked eye, clearly labeling the vasculature a deep blue color and indicating successful administration has occurred. Labeling the vasculature with EB is extremely cost effective when compared with using antibodies or other vascular labeling strategies. For example, a 1-mg vial of fluorescently labeled tomato lectin, which can be similarly injected to label endothelium [5], is nearly equivalent in price to a 50-g bottle of EB powder. One vial of labeled lectin can be utilized for approximately ten mouse injections, whereas one bottle of EB can yield approximately 13,888 injections. Other methods, such as micro-computed tomography, can provide
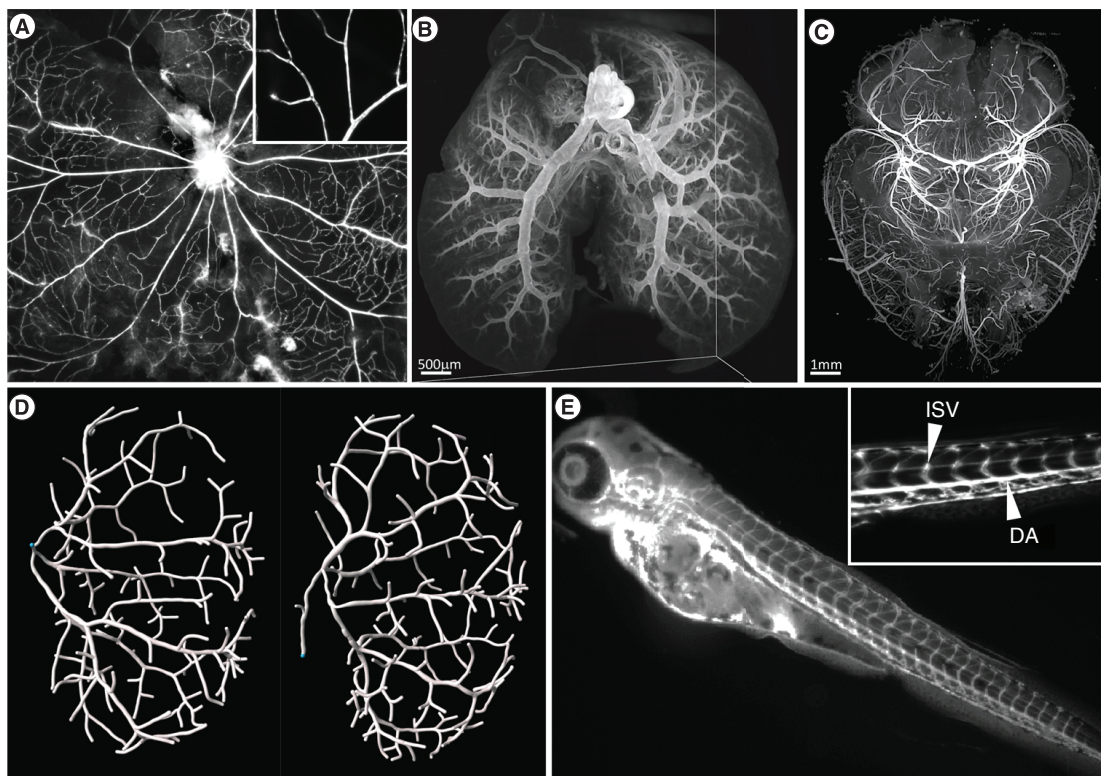
Figure 2. Examples of EB labeling in various models and tissues. (A) Unfixed mount of an adult mouse retina imaged 10 min after injection by widefield fluorescence. Inset shows higher magnification. **(B)** Wholemount of P6 mouse lungs, with heart left attached, imaged by light sheet. **(C)** Wholemount of an adult mouse brain imaged by light sheet. **(D)** Tracing of the two different vascular trees in the adult mouse kidney using Imaris software. The Filament Tracer module was utilized. **(E)** Zebrafish embryo 3 days post-fertilization imaged by widefield fluorescence. Labeled vessels such as ISVs and DA are observed (arrowheads, inset).
DA: Dorsal aorta; EB: Evans blue; ISV: Intersegmental vessel.

similar reconstructions of whole organ vasculature but require specialized x-ray imaging equipment and significant optimization of parameters [6].

The authors have generated a convenient protocol using the injection of EB into mice to label and visualize vascular networks. Injection is routinely performed with EB mixed in a saline solution that, upon circulation, will bind to albumin, thereby fluorescently labeling the vasculature [14]. The authors' approach uses intracardial injection performed under isoflurane-induced anesthesia (Figure 1A). The chest cavity is opened to ensure accurate injection into the left ventricle. However, an experienced injectionist could perform intracardial administration without opening the chest cavity or deliver EB via tail vein. Tail vein injection does not require the use of anesthesia, has been utilized to inject EB in vascular leakage assays and would prevent any damage to the heart from intracardial injection [15]. However, this route of administration requires significant expertise and practice to master [21]. Following intracardial injection, EB is allowed to circulate for 5 min to ensure sufficient labeling. This is monitored by watching for the paws and other areas of thin hair coverage to turn blue. Once circulation is complete, the animal is appropriately euthanized. The authors found that perfusion with paraformaldehyde is not necessary if strictly labeling the vasculature with EB. However, if accompanying immunolabeling will be performed, perfusion can be done and will not affect the efficiency of EB labeling. The albumin–dye complex coats the endothelial wall and remains associated through all processing steps, holding up to perfusion, extensive washing, reagent changes and organic compounds with little to no loss. Following euthanasia, the tissue of interest is harvested, fixed and processed for imaging. The authors have performed extensive analyses on the adult mouse kidney, which allows for visualization of vascular networks by light sheet microscopy (Figure 1D & E) and high-resolution confocal microscopy (Figure 1F). Although the authors' injection strategy primarily labels large vessels like arteries in major organs when visualized in wholemount, labeling of smaller vessels and glomerular capillaries is occasionally seen (Figure 1E). Altering processing and imaging parameters as well as increasing resolution may help with visualizing such vessels. Labeling of smaller vessels may also be enhanced by the addition of glycerol to EB, although the authors have not independently verified this [16]. In tissues such as the retina, capillaries are perfused and can be immediately visualized following dissection, allowing rapid assessment of any phenotypes (Figure 2A). Thicker tissues and whole organs like the kidney require clearing, which is accomplished with modified iDISCO methods [9].

In short, tissue is dehydrated and cleared utilizing dichloromethane and dibenzyl ether, allowing for analysis of the vascular network in 24 h or less. The authors employ a LaVision UltraMicroscope II (LaVision BioTec, Bielefeld, Germany) for light sheet microscopy of whole organs. Imaris (Bitplane Inc, MA, USA) imaging software is used to reconstruct 3D images for visualization and can also trace vascular networks for quantitative analyses (Figure 1D, E & 2B−D & Supplementary Figure 1). The 3D networks the authors generated for the lungs closely match those previously obtained with micro-computed tomography, confirming the utility of the method (Figure 2B) [22]. Any fluorescent widefield or confocal microscope may be utilized for smaller tissues and/or greater resolution images. Additionally, EB holds up to tissue processing for cryo- or paraffin section. The fluorescence is stable, with no additional precautions required other than those necessary for traditional fluorescent labeling techniques. Photobleaching may be possible, although the authors have reimaged whole tissues several times at high laser power with little or no loss of fluorescence. When cleared tissues are properly stored in the dark, they can be reimaged months to years later. Finally, the authors tested the utility of EB in labeling the embryonic vasculature of zebrafish. Cardinal vein injection allows for near-instantaneous labeling of the vasculature and visualization of structures such as intersegmental vessels, highlighting its efficacy in other organisms (Figure 2E).

Conclusion

We have shown the utility of EB labeling to visualize vascular networks in 3D using light sheet microscopy or traditional fluorescence microscopy methods. We have demonstrated the application of this technique in various tissues and model systems. In addition to efficient labeling of the vasculature, we also show that it is relatively high throughput, requiring only a few days to go from administration to visualization, or in the case of thin tissue, such as the retina, only a few minutes to visualization. The cost savings and short time from injection to imaging are an advance over current methodology and enable a greater number of researchers to analyze whole vascular networks within their laboratory.

Future perspective

Imaging methodology is constantly evolving, with new techniques, protocols and instrumentation being generated at a rapid pace. However, combining current methodology can result in novel labeling and imaging protocols, such as we have presented here for the vasculature, enabling broader applications and ease of access to a growing number of researchers.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2020-0152

Ethical conduct of research

All animal studies were approved by the Office of Animal Care and Use at the University of North Carolina at Chapel Hill. Procedures were performed under approved institutional animal care and use committee protocols for mice (16-276.0, 19-183.0) and zebrafish (18-004.0).

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