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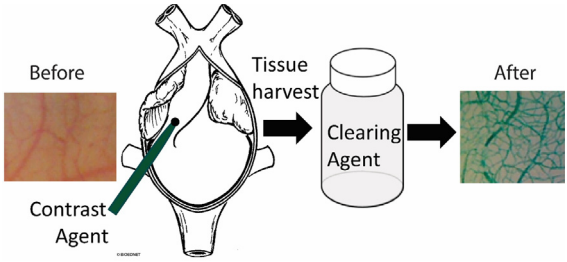
Method Article

Comparison of tissue processing methods for microvascular visualization in axolotls

Rodrigo Montoro, Renee Dickie*

Department of Biological Sciences, Towson University, 8000 York Road, Towson, MD 21252, USA

GRAPHICAL ABSTRACT



ABSTRACT

The vascular system, the pipeline for oxygen and nutrient delivery to tissues, is essential for vertebrate development, growth, injury repair, and regeneration. With their capacity to regenerate entire appendages throughout their lifespan, axolotls are an unparalleled model for vertebrate regeneration, but they lack many of the molecular tools that facilitate vascular imaging in other animal models. The determination of vascular metrics requires high quality image data for the discrimination of vessels from background tissue. Quantification of the vasculature using perfused, cleared specimens is well-established in mammalian systems, but has not been widely employed in amphibians. The objective of this study was to optimize tissue preparation methods for the visualization of the microvascular network in axolotls, providing a basis for the quantification of regenerative angiogenesis. To accomplish this aim, we performed intracardiac perfusion of pigment-based contrast agents and evaluated aqueous and non-aqueous clearing techniques. The methods were verified by comparing the quality of the vascular images and the observable vascular density across treatment groups. Simple and inexpensive, these tissue processing techniques will be of use in studies assessing vascular growth and remodeling within the context of regeneration. Advantages of this method include:

- Higher contrast of the vasculature within the 3D context of the surrounding tissue
- Enhanced detection of microvasculature facilitating vascular quantification
- Compatibility with other labeling techniques

* Corresponding author.
E-mail address: rdickie@towson.edu (R. Dickie).

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Method details

Rationale

Characterization of the vasculature is essential to understanding the complex role that blood vessels play during regeneration [1,2]. Well defined vessels, distinct from background tissue, are necessary for the quantification of vascular image data. In other regenerating vertebrate models systems, such as zebrafish, transgenic lines with fluorescently labelled vascular endothelial cells greatly facilitate vessel analysis [3]. Techniques for vascular visualization in salamanders, however, are still in their infancy.

In mammalian models, procedures for ink microangiography and optical clearing to visualize the vasculature in both healthy and tumor tissues [4–15] are well refined. The technique is sensitive; perfusion-based methods can result in a greater number of labelled vessels than immunohistochemical labeling [7]. While quantification of perfused vessels has been widely employed to examine mammalian vasculature and angiogenesis (e.g., [15–20]), we are not aware of any literature applying these techniques to allow quantification of urodele angiogenesis. Here, we use an alternative contrast agent, green pigment based ink, and compare aqueous and nonaqueous optical clearing methods, towards the goal of optimizing vascular image quality and facilitating quantification of the regenerative vasculature. The technique was assessed by comparing the clarity of the vascular network and the detectable vascular density across the treatment groups. The whole mount procedures described below provide a flexible system that preserves the three dimensional architecture of the vasculature and allows for more accurate estimation of the areal density of the intact and regenerating microvessels.

Methods

Using the methods of [21,22] as a starting point, we describe modified and expanded procedures for the perfusion of the vascular tree of axolotls, followed by tissue clearing techniques to reduce light scattering during microscopy.

Animal maintenance

Salamander maintenance followed standard procedures [23,24], outlined here briefly. Juvenile axolotls (*Ambystoma mexicanum*, albino strain) were purchased from the *Ambystoma* Genetic Stock Center (AGSC, Lexington, KY) and allowed to acclimate for at least a month prior to any experiments. The animals were housed individually in plastic tanks within an 18–20 °C room with natural light. Tank solution consisted 40% Holtfreter's salts [24] in dechlorinated water, manually changed on alternate days. The animals were fed every other day with anchovy-based food pellets (Rangen, Buhl, ID). All animal procedures were in accordance with an approved Institutional Animal Care and Use protocol and all local, state, and federal guidelines.

Tail amputations

To assess regenerated vasculature, the tail tips of adult (~12 cm snout vent length) axolotls were amputated and then allowed to regrow. Animals were first anesthetized until they were no longer