

Whole-Mount Immunofluorescence Imaging of Zebrafish Eye Structures

Robust, automated confocal imaging using the
Agilent BioTek Cytation C10 confocal imaging reader

Author

Rebecca Mongeon, PhD
Agilent Technologies, Inc.

Abstract

Whole-mount fluorescence immunohistochemistry is a primary method of microscopy-based phenotypic analysis in the zebrafish model system. In this study, fluorescence immunohistochemistry and staining were combined to examine eye structures in embryonic zebrafish. For comparison purposes, the samples were studied in both widefield and confocal imaging modalities using the Agilent BioTek Cytation C10 confocal imaging reader. Results are presented that show the benefits of using the confocal optics of the Cytation C10 for processing fluorescence signals from deeper within relatively thick samples. Clear, detailed, high-quality confocal images of whole-mount zebrafish embryos labeled using fluorescence immunohistochemistry are shown.

Introduction

Zebrafish have long played an important role in eye developmental biology studies owing to their similarity to human ocular development.¹ With an ever-expanding tool kit of genetic manipulation techniques, zebrafish have proven a salient model system for research into human ophthalmological disorders, including late-onset disorders like glaucoma and cataracts.² Light microscopy techniques are vital for these research studies, where fluorescence immunohistochemistry is an essential tool for eye and retinal phenotypic analysis.

At the embryo stage, zebrafish are amenable to whole-mount immunohistochemistry methods due to their small size and optical transparency. However, traditional widefield microscopy methods are limited when imaging at depths beyond several microns into thick samples. Confocal imaging can overcome some of these limitations and provide a way to examine fluorescence signals deeper in zebrafish embryos. But, given the significant cost and specialized nature of most confocal microscopes, researchers often rely on tissue sectioning and reconstruction methods to produce high-quality images.³ The Agilent BioTek Cytation C10 confocal imager removes these barriers, allowing more laboratories access to robust, automated confocal imaging.

In this study, the confocal modality of the Cytation C10 instrument was used to examine the embryonic zebrafish eye through fluorescent whole-mount immunohistochemistry. To highlight the benefit of adding confocal capabilities to an automated imaging system for zebrafish research, the same structures were imaged using the traditional widefield modality on the Cytation C10.

Experimental

Chemicals

Unless otherwise noted, all chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA).

Sample preparation

General maintenance of zebrafish followed established methods.⁴ Wild-type male and female *Danio rerio* were maintained at 28 °C on a 14- to 10-hour light/dark cycle. Adults were crossed and eggs promptly collected in EM3 media.⁵ To reduce pigmentation, embryos were treated with 0.003% N-phenylthiourea in EM3 starting at 24 hours postfertilization.

Immunohistochemistry

Embryos were prepared for immunohistochemistry and staining using the following procedure. At three days postfertilization (pec-fin stage), embryos were manually dechorionated, anesthetized, and fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4 °C overnight. Following incubation, fixed embryos were removed from PFA and rinsed in PBS with 1% polysorbate 20 (PBST). Embryos were permeabilized in consecutive steps as follows: fixed embryos were rinsed in ultrapure laboratory water (water), submerged in prechilled acetone for 8 minutes at –20 °C, rinsed with water, followed by a final rinse in PBST. Embryos were then incubated at room temperature (RT) for one hour in permeabilization buffer (PBST with 3% Triton X-100 and 1% dimethyl sulfoxide (DMSO)), followed by incubation at RT for one hour in blocking buffer (PBST with 3% bovine serum albumin (BSA), 1% Triton X-100, and 1% DMSO). Permeabilized and blocked embryos were incubated overnight at 4 °C in a primary antibody and stain mixture of 1:200 DAPI (Invitrogen D1306), 1:100 phalloidin-Alexa488 (Invitrogen A12379), and 1:100 anti-acetylated tubulin (Sigma SAB5600134) in blocking buffer. Following incubation, embryos were rinsed extensively and incubated at RT in PBST for 3 hours. Embryos were then incubated overnight at 4 °C with a secondary antibody (anti-rabbit Alexa647, Cell Signaling 4414S), followed by extensive rinsing with PBST. Embryos were transferred to mounting media (80% glycerol, 20 mM TRIS pH 8.0, and 0.05% propyl gallate) on glass slides and imaged through a #1.5 glass coverslip.

Instrumentation

The Cytation C10 confocal imaging reader and Agilent BioTek Gen5 microplate reader and imager software were used to acquire all images, including brightfield, widefield, and confocal images. The Cytation C10 instrument was equipped with a 40 µm pinhole spinning disk, Hammamatsu Orca sCMOS camera, and emission/excitation filter cubes for both confocal and widefield modes. The imaging filter cubes used included: widefield: DAPI 1225100, GFP 1225101, CY5 1225105; and confocal: DAPI 1945103, GFP 1945104, CY5 1945102.

Imaging procedure and processing

All 20x magnification images were acquired as a z-series at 2 μm intervals, for both widefield and confocal modalities. All 40x magnification images were acquired as a z-series at 1 μm intervals, for both widefield and confocal modalities. Images were processed using Gen5 software to flatten the background with a rolling-ball algorithm using a 30 μm diameter in all fluorescence channels. Z-projections were summated using the Gen5 software "Maximum" method. Maximum z-projection images were then deconvolved using default Gen5 calculated point spread function parameters. Optical depth estimations accounting for sample refractive index mismatch were determined using an axial distortion correction factor.⁶

Results and discussion

For a general examination of cranial structures using fluorescence imaging, a combination of nuclear staining (DAPI), cytoskeletal staining (phalloidin), and cytoskeletal immunohistochemistry (anti-acetylated tubulin) were used. Mounting a three-day postfertilization embryo in the dorsolateral position (Figure 1A) allows for an overview image of the entire head in a single-field-of-view at 20x magnification on the Cytation C10 (Figure 1B).

The brightfield image shown in Figure 1B (left panel) was taken at a single focal plane at an optical depth of $\sim 240\ \mu\text{m}$ from the dorsal surface of the head. At this position, the eyes are visible in cross-section and the retinal layering can be seen. A z-series of fluorescence images were collected automatically using both the widefield and confocal modules of the Cytation C10. The projections of the captured z-stack (Figure 1B, middle and right panels) represent an $\sim 60\ \mu\text{m}$ thick section. Although the z-stack optical imaging depth is $\sim 240\ \mu\text{m}$ from the dorsal surface of the head, due to the head geometry, much of the signal derives from light traveling through far less tissue.

The confocal images (Figure 1B, top row) demonstrate a significant reduction in the out-of-focus background light compared with widefield mode (Figure 1B, bottom row). This reduction in background is appreciated when comparing the processed image z-stacks obtained in confocal and widefield mode (Figure 1B, right). Structures such as the eye lens fibers (Figure 1B, arrowhead) are clearly visible in the confocal images, but cannot be distinguished from background in the widefield images.

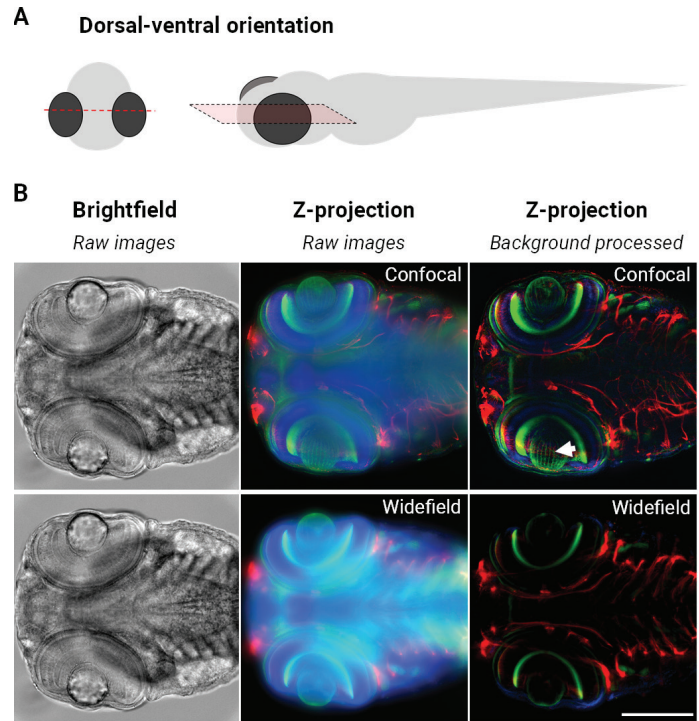


Figure 1. Dorsal-ventral whole-mount images of three-day old zebrafish embryo head in confocal and widefield modalities of the Agilent BioTek Cytation C10 confocal imaging reader. (A) Diagram of the imaging plane (dashed line) and embryo orientation. (B) At 20x magnification, the zebrafish head is captured in a z-series for both widefield and confocal modalities. Brightfield images taken at a single plane are shown alongside z-projection images ($\sim 60\ \mu\text{m}$ thick section), before ("Raw"), and after background reduction processing in the Agilent BioTek Gen5 software. Blue channel corresponds to DAPI nuclear staining, green channel corresponds to phalloidin staining of actin, and red channel corresponds to acetyl- α -tubulin immunostaining of neuron axons. Arrowhead indicates lens fibers visible in the confocal image. Scale bar indicates 200 μm .

Rotating the embryo 90 degrees, by mounting in the lateral orientation (Figure 2A), permits the zebrafish eye to be imaged in a single field-of-view on the Cytation C10 at 40x magnification. As with the whole head, the images of the eyes were taken in a z-series in both confocal (Figure 2B, top row) and widefield (Figure 2B, bottom row) modes on the same instrument. The z-projection shown in Figure 2B encompasses $\sim 20\ \mu\text{m}$ optical depth section starting at about 35 μm from the surface of the eye. To include the entirety of the anterior pole lens structure in these images, the green channel z-projection includes additional z-slices. These z-slices start at the eye surface, and therefore the green channel includes a larger total optical depth section ($\sim 55\ \mu\text{m}$).

For clarity, each imaging channel is shown individually, and then as a final composite image (Figure 2B). The nuclear DAPI staining (blue) identifies the retinal nuclear layers as indicated by the labels. The phalloidin staining (green) identifies the plexiform layers and lens fiber structures. Acetylated α -tubulin staining (red) identifies neuronal axons throughout the retina, highlighting the connections between the retinal cell layers.

The improvement in image quality provided by confocal mode compared to widefield mode is clear. The confocal images distinguish individual nuclei in the DAPI channel, lens fibers in the phalloidin channel, and a multitude of fine neuronal axon processes throughout the retina. This level of detail isn't visible in widefield mode.

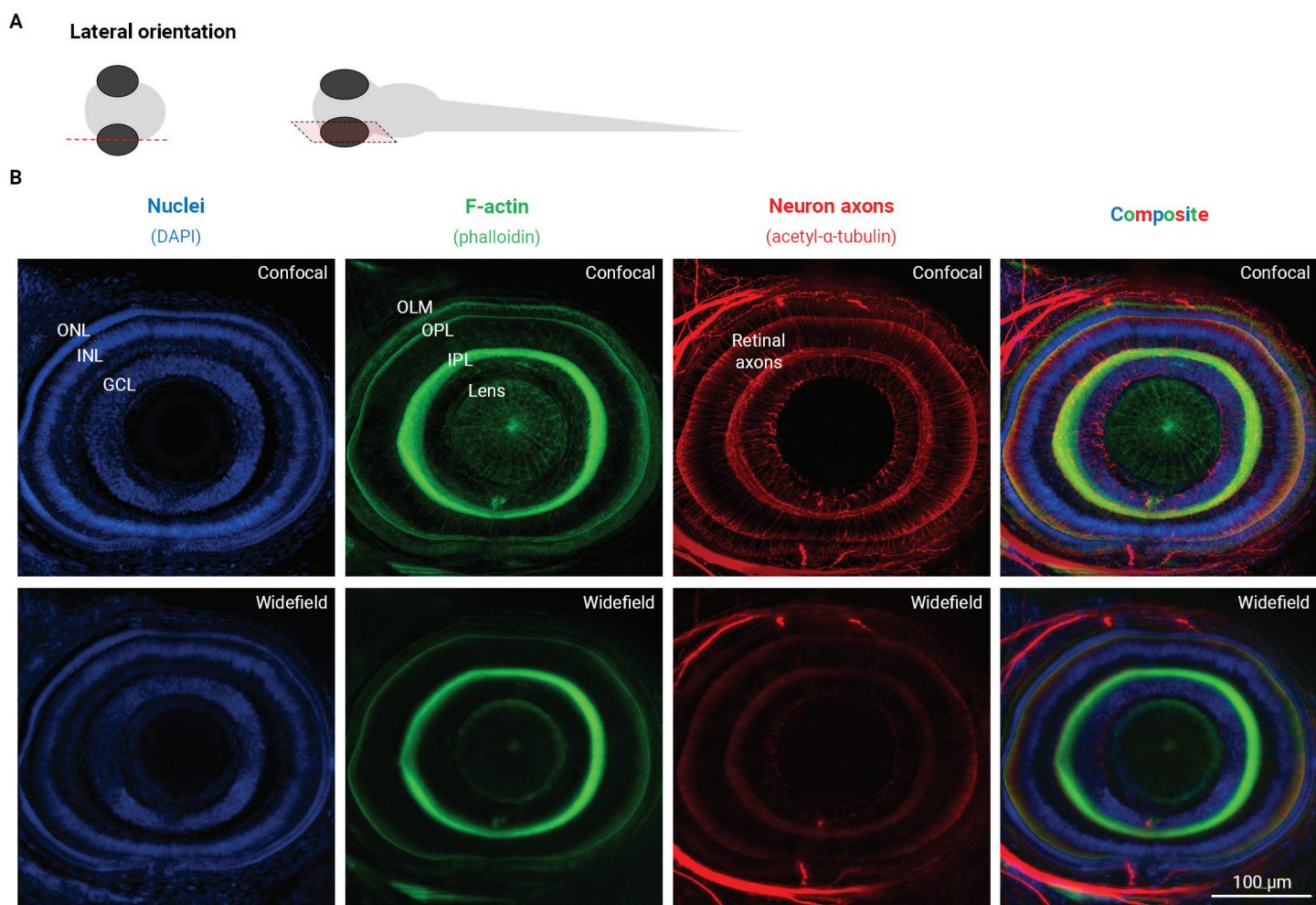


Figure 2. Lateral whole-mount images of the eye of a three-day-old zebrafish embryo in confocal and widefield modalities of the Agilent BioTek Cytation C10 confocal imaging reader. (A) Diagram of the imaging plane (dashed line) and embryo orientation. (B) At 40x magnification, the zebrafish eye is captured in a z-series projection image for both confocal and widefield imaging modalities as indicated. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; OLM, outer limiting membrane; OPL, outer plexiform layer; IPL, inner plexiform layer.

A digital zoom of the 40x images shown in Figure 2B highlights the lens structures that can only be observed in confocal imaging modality (Figure 3). Phalloidin stains the actin filaments in the cytoskeleton of lens fiber cells. As lens cells differentiate and mature, the cytoskeleton is excluded from the cytoplasm and associates closely with the plasma membrane at the periphery of the cell. In cross-section, lens fiber cells appear as elongated hexagons with two broad sides and several narrow sides in close juxtaposition that contribute to the intense phalloidin staining observed along the narrow lateral edges.⁷ In the single z-slice near the equator of the lens shown in Figure 3, phalloidin staining identifies the narrow and broad sides of lens cells in the outer cortex of the lens. In a z-projection of the entire lens anterior pole, the narrow edge staining appears as striations that highlight the fiber cell boundaries as they converge at the anterior suture. Lens epithelial cell labeling with phalloidin is also visible as an outer monolayer of hexagonal patterning overlaying the fiber cell striations.

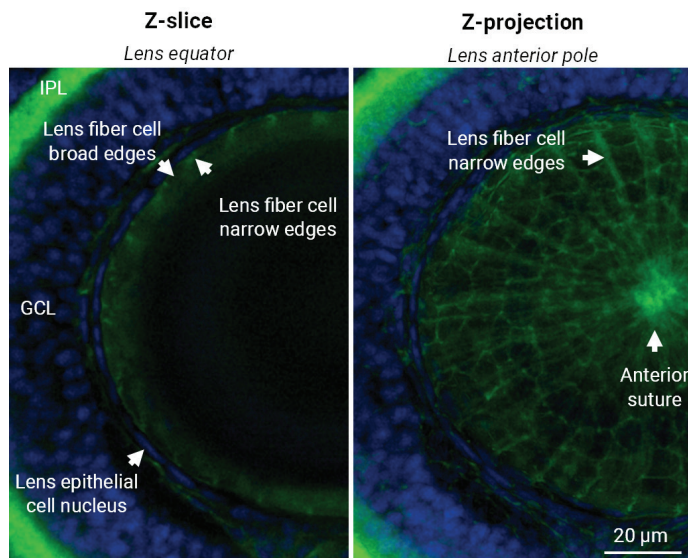


Figure 3. Lateral whole-mount images of the lens of a three-day-old zebrafish embryo obtained in confocal mode on the Agilent BioTek Cytation C10 confocal imaging reader. At 40x magnification and digitally zoomed, zebrafish lens structures are shown in a single optical plane (left) and a z-series projection (right). DAPI nuclear staining (blue) indicates cell nuclei, including the lens epithelial cell monolayer as indicated. Phalloidin staining of actin filaments (green) highlights lens fiber cell edges as indicated. Abbreviations: IPL, inner plexiform layer; GCL, ganglion cell layer.

Conclusion

The Agilent BioTek Cytation C10 confocal imaging reader adds confocal optics to the Cytation instrument line without compromising the multimodal features and ease-of-use expected from an automated Cytation imaging system. Confocal optics enable high-quality imaging of whole-mount zebrafish embryos labelled using fluorescence immunohistochemistry. Through comparisons to widefield imaging, this application note demonstrates that the optical sectioning capability of the Cytation C10 spinning disk confocal is essential to accurately capture fluorescently labelled cellular structures deep in zebrafish embryos, enabling detailed evaluation of retinal and lens structures central to zebrafish ophthalmological research.

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