Fluorescent Photoreceptors of Transgenic *Xenopus laevis* Imaged In Vivo by Two Microscopy Techniques

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**METHODS.** Albino and pigmented transgenic *Xenopus laevis* that express enhanced green fluorescent protein (GFP) in the major ("red") rods were generated. The distribution of GFP throughout the retina and within the rods was evaluated by confocal microscopy of frozen sections and immunoelectron microscopy. In vivo images of photoreceptors were obtained using conventional fluorescence microscopes to image through the lens of the eye or a laser scanning confocal microscope to image through the hypopigmented iris of albino eyes.

**RESULTS.** Confocal and immunoelectron microscopy of tissue sections showed that GFP was predominantly localized to the inner segments of the major rods; a smaller amount was in the outer segments. In a number of animals, not all the major rods expressed GFP. It was possible to identify these animals by obtaining fluorescence images of the retinas of intact, living tadpoles with conventional fluorescence microscopes, using the lens of the tadpole as part of the optical path. Confocal images of living animals could be used to visualize the distribution of GFP within the photoreceptors.

**CONCLUSIONS.** The ability to observe individual photoreceptors noninvasively allows in vivo longitudinal microscopic analysis of photoreceptor development in transgenic *Xenopus* tadpoles.


Amphibian retinas have long been a subject of study for anatomists, cell biologists, biochemists, and electrophysiologists. Procedures recently described by Kroll and Amaya for the rapid production of transgenic *Xenopus laevis* present new opportunities to test conclusions derived from in vitro studies of amphibian retinas. Our long-term goal is to use transgenic *Xenopus* to study protein targeting and retinal degeneration.

Transgenic frogs have many advantages as retinal research subjects. In particular, their rod photoreceptors have large (7 μm diameter) outer segments with a high rate of membrane turnover, making these cells valuable for the study of membrane biosynthesis and polarized transport of proteins. Unlike mice and rats, frogs have abundant cones that can be distinguished by simple microscopic techniques. Rapid development of the eyes in these animals permits studies of the retina within a week of fertilization. Unlike transgenic mice, the embryos develop outside the mother, and therefore all stages of development are easily monitored. Furthermore, several advantages are inherent in Kroll and Amaya’s technique itself. Large numbers of animals can be generated in a single day. Because the technique involves transgenic sperm nuclei, the entire animal is theoretically transgenic, making breeding potentially unnecessary.

Because many tadpoles are generated per experiment using this technique, we needed to develop rapid procedures for identifying transgenics and for studying transgenic effects. Using a construct consisting of a *Xenopus* opsin promoter fragment linked to an enhanced green fluorescent protein (GFP) cDNA, GFP was expressed in the rods of the *Xenopus* retina. Expression of the transgene in the retinas of living tadpoles and frogs was easily detected using a fluorescence-equipped dissecting microscope, a standard fluorescence microscope, or a laser scanning confocal microscope. Here we describe two microscopy techniques that allow rapid in vivo observation of fluorescent photoreceptors and permit evaluation of the uniformity of expression throughout the retina. Because these methods do not involve killing the animal, sequential data can be obtained in longitudinal studies of a single tadpole.

**METHODS**

**Construct**

The DNA construct used for transgenesis was based on the plasmid peGFP-C1 (Clontech, Palo Alto, CA). We excised the cytomegalovirus promoter from peGFP-C1 by digestion with the restriction enzymes *Ase*I and *Nhe*I and inserted a synthetic linker containing an *EcoRV* site and a *NotI* site. The sequences of the

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oligonucleotides used in the linker were 5'-CTAGCGATATCGCGGCCGCAT-3' and 5'-TAATGCGGCCGCGATATCG-3'. We then cloned the *Xenopus* opsin promoter,5 a 5.5-kb DNA fragment flanked by *Bam*HI sites, into the *Eco*RV site by filling in the overhanging *Bam*HI ends using the Klenow fragment of DNA polymerase 1. Correct orientation of the promoter was confirmed by *Bgl*II digestion. The final construct was named XOP-eGFP-C1. For transgenesis, the construct was linearized with *Not*I. Restriction enzymes were purchased from Life Technologies (Rockville, MD).

**Transgenesis**

Adult *Xenopus laevis* were obtained from Nasco (Ft. Atkinson, WI) or *Xenopus* Express (Homosassa, FL) and used to generate transgenic tadpoles by the procedure of Kroll and Amaya.1 Albino tadpoles were generated using albino gamete donors. We modified the original transgenesis procedure by eliminating the restriction enzyme (*Not*I) or reducing its concentration by 80% during incubation of the sperm nuclei with the linearized construct. This substantially increased the yield of late-stage tadpoles. We also reduced the egg extract concentration by 70% (Rob Grainger, University of Virginia, personal communication). Tadpoles were raised in 0.13 Gerhart's Ringers solution6 in clear plastic tanks. Each tank was equipped with bubbling air. Tadpoles were kept at 18°C, on a 12-hour light/dark cycle, and fed powdered Nasco frog brittle. Tadpoles were handled in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research and with NIH and AALAC guidelines.

**Screening of Transgenic Tadpoles**

At developmental stage 40 to 42 (5 to 6 days old) animals expressing GFP were identified using a Leica MZ8 dissecting microscope (Leica Microsystems, Deerfield, IL), equipped with epifluorescence optics (Kramer Scientific, Elmsford, NY), including a GFP filter set. For routine observation, small tadpoles were captured in a large-bore Pasteur pipette, which could be rotated to easily view the eyes and retinas.

**Microscopy**

For in vivo confocal observation of photoreceptors, stage 46 tadpoles (11 days old) were anesthetized in 0.02% tricane (Sigma, St. Louis, MO) in 0.1× GRS and immobilized on coverslips using 0.8% low melting point agarose in the same solution. Eyes were imaged using a Zeiss 410 laser scanning confocal microscope (Carl Zeiss, Thornwood, NY). Alternatively, tadpoles were anesthetized in 0.02% tricane in 0.1× GRS and observed using a conventional fluorescence microscope (Zeiss Axioskop with FITC filter set). Photographs were taken using P3200 film (Kodak, Rochester, NY), push-processed to 12,500 ASA according to the manufacturer's instructions. High-speed film and rapid exposures were necessary to reduce blurring caused by movement in the
anesthetized animals due to the heartbeat. For clarity, images were digitized, and green color was added using Adobe Photoshop 3.0 (Adobe Systems, San Jose, CA).

For frozen sections, eyes were dissected from animals that had reached developmental stage 50 or higher (approximately 1-month-old) and fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.5). Fixed eyes were embedded in OCT tissue embedding medium (Tissue-Tek, Torrance, CA) and frozen in isopentane/dry ice, and 14-μm sections were cut using a cryostat. Sections were labeled with anti-rhodopsin monoclonal antibody mAbE,7 followed by a CY3-conjugated secondary antibody (Jackson, West Grove, PA). Labeling was carried out in phosphate-buffered saline in the presence of 0.1% Triton X-100 and 0.1% goat serum. Sections were observed in a Zeiss 410 laser scanning confocal microscope.

**Electron Microscopy**

Tadpoles were fixed in 4% paraformaldehyde buffered with 0.1 M sodium phosphate (pH 7.5) and embedded in LR Gold or LR White (Ted Pella, Redding, CA) according to the manufacturer’s instructions. Because pigment in the tadpole eye caused inefficient UV polymerization of LR Gold, the best results were obtained with heat polymerization of LR White. Thin sections were labeled with an anti-GFP polyclonal antibody (Clontech) and a secondary antibody conjugated to 10 nm colloidal gold particles (AP Biotech, Uppsala, Sweden).

**RESULTS**

We were able to generate 50 to 100 transgenic tadpoles in a single day from 6000 to 8000 injected eggs. Of the animals that survived to day 12 after fertilization (developmental stage 46), 30% to 40% were transgenic as determined by expression of green fluorescence in the eye. As previously reported, fluorescence was also observed within the pineal cells,4 but pinealocytes were not examined in detail in this study. Initial mortality was high relative to in vitro fertilized animals, presumably due to genomic damage during the procedure (typically 30% or fewer of transgenic tadpoles survived 1 month). A small (highly variable) percentage survived metamorphosis. Before modifying the original procedure, we were unable to raise any transgenic tadpoles to an age greater than 1 month.

Transgene expression was detected within 5 days of fertilization (stage 40), and sufficient tadpoles survived to later stages for analysis by frozen sections. Immunofluorescence analysis was carried out on frozen sections of transgenic tadpole eyes using mAbE, a monoclonal antibody that labels only the major (or “red”) rods.7 In nearly all transgenic tadpoles, expression of GFP within the eye was limited to the major rods (Fig. 1). Only 1% to 2% of the tadpoles that survived to day 12 displayed ectopic expression of GFP outside of the eye and pineal, and these embryos often developed abnormally and did not survive, again presumably due to genomic damage. Typically, transgene expression was nonmosaic (Fig. 1B). However, in a subset of transgenic animals, mosaic expression was evident, possibly because of late integration of the transgene after fertilization and cell division (Fig. 1C). In mosaic animals, the number of green fluorescent photoreceptors varied considerably (compare Fig. 1C and Fig. 3D).

Green fluorescence was found throughout the rods. However, fluorescence of the outer segment was lower than in the inner segment, cell body, nucleus, and synapse (Fig. 1D).
further localized GFP by immunoelectron microscopy using an anti-GFP polyclonal antibody, which indicated a similar distribution (Fig. 2). The density of labeling of the outer segment was consistently lower than that of the myoid (endoplasmic reticulum [ER]–containing) region. Labeling within the nucleus was similar to labeling in the myoid, although areas rich in heterochromatin were less labeled. The ciliary stalk was also intensely labeled (Fig. 2A). The difference in labeling between the myoid and outer segment likely reflects the proportionally smaller outer segment cytoplasmic volume. Label was greatly reduced within the lumens of the mitochondria, ER, and Golgi complex.

Two different techniques were used to observe photoreceptors in living tadpoles. The first used standard Leica dissecting or Zeiss Axioscop microscopes equipped with fluorescence optics. Anesthetized animals were manipulated so that their eyes were turned toward the objective. By moving the objective toward the eye such that the exterior of the eye was out of focus, a magnified image of the photoreceptors came into view, using the tadpole’s own lens as an optical element (Fig. 3A). The image was generally of better quality in smaller animals, between stages 40 and 50. In older animals with larger eyes, individual photoreceptors were difficult to resolve, because the image diameter of individual cells decreased as the focal length of the maturing lens changed. Tadpoles with mosaic expression patterns could readily be distinguished from those expressing GFP uniformly because their retinas had ir-
regular patterns of fluorescent rods and contained fewer fluorescent cells (Fig. 3C). Frozen sections of these animals at later stages of development confirmed that GFP expression was nonuniform (Figs. 3B, 3D).

The second technique for in vivo observation of photoreceptors used an inverted Zeiss 410 laser scanning confocal microscope. We anesthetized and immobilized tadpoles so that one eye was oriented toward the objective. By optically sectioning through the eye we could observe individual rods (Fig. 4). Depending on the depth of sectioning, photoreceptors with different orientations were observed. In midplane axial images the contrast in GFP expression between inner and outer segments was readily discernible. The level of fluorescence was not uniform around the circumference of the eye. This was because small quantities of irregular pigmentation were present in the iris that blocked fluorescence. Irregularities in the depth of tissue, primarily in the region of the optic fissure, also affected the fluorescence level. By increasing the sensitivity of detection in the confocal microscope, fluorescent photoreceptors were seen throughout the circumference of the eye. However, this resulted in saturation and loss of detail in brighter areas.

We found this technique most useful for observing very small albino tadpoles (stage 46). In larger animals, the anterior segment of the eye becomes more opaque, and the distance of the photoreceptors from the coverslip limits optical sectioning. Images of photoreceptors could also be obtained through the lens of the eye but were not substantially different from those obtained with a conventional microscope (not shown).

CONCLUSIONS

We have expressed GFP under the control of the Xenopus opsin promoter in transgenic Xenopus laevis. The transgene is expressed in the major rods of the retina and was not detected in other retinal cells. Fluorescence and immunoelectron microscopy indicate that GFP is primarily localized to the inner segment, cell body, and synapse, with a lower concentration in the outer segment. This likely reflects differences in volume accessible to the soluble GFP protein, because the impermeable disks occupy a large portion of the outer segment volume. GFP accesses the outer segment compartment via the connecting cilium. Label was also greatly reduced within the interiors of the mitochondria, suggesting that they are not permeable to GFP. Although the intensity of labeling varied substantially between animals, and sometimes between cells of the same retina, outer segment labeling was always less intense than inner segment labeling. The presence of GFP did not adversely affect photoreceptor morphology or survival, at least not to a significant extent in animals up to 1 year of age.

We modified the original protocol of Kroll and Amaya1 for generating transgenic Xenopus laevis by eliminating or reducing the restriction enzyme originally used to enhance integration of the transgene construct into the chromosomal DNA. The modified procedure yielded greater numbers of late-stage transgenic animals. However, a significant number of animals showed mosaic transgene expression, most likely as a result of postfertilization integration. We see no reason why similar postfertilization integration could not also occur in the presence of restriction enzyme. We therefore caution that when using transgenic Xenopus laevis produced by these methods as an experimental model, the possibility that transgene copy number may not be constant throughout the animal should be considered.

The robust expression of GFP under control of the Xenopus opsin promoter allows us to image the rods of living tadpoles using two different techniques. First, we can obtain a magnified image of the fluorescent rods by recruiting the tadpole’s lens into the optical path. This image is useful for determining whether a tadpole’s retina exhibits mosaic transgene expression. This type of analysis will also be useful for monitoring retinal degeneration in these animals. One would expect a progression from a nonmosaic to a mosaic image if photoreceptors were gradually eliminated. Second, we can obtain images of photoreceptors by optically sectioning through the anterior of the eye of albino animals using a confocal microscope. Confocal images can discern between outer segment and inner segment labeling, because the photoreceptors can be observed in various orientations by sectioning at different depths.

We found that analysis of tadpole eyes by frozen sections before stage 50 was difficult because very few sections are obtained from one eye. The alternative of plastic embedding and thin sectioning is time-consuming and does not preserve fluorescence. In contrast, the techniques we have described for examining photoreceptors in live animals are rapid and are most effective on small tadpoles; researchers can further analyze the tadpoles by frozen sections or other techniques at a later date. Furthermore, because the tadpoles are not killed, longitudinal studies can be performed on individual eyes.

Transgenic frog technology is relatively new; however, amphibian retinas have long been a common experimental subject, and the simplicity of Kroll and Amaya’s technique,1 coupled with the large body of literature available on the Xenopus retina, suggest that this technology will be valuable to vision researchers. The ease with which transgenic Xenopus laevis photoreceptors can be screened and analyzed in vivo should enhance their value in such investigations.

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