Generation of a genetically encoded marker of rod photoreceptor outer segment growth and renewal

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\textbf{Summary}

Vertebrate photoreceptors are specialized light sensing neurons. The photoreceptor outer segment is a highly modified cilium where photons of light are transduced into a chemical and electrical signal. The outer segment has the typical ciliary axoneme but, in addition, it has a large number of densely packed, stacked, intramembranous discs. The molecular and cellular mechanisms that contribute to vertebrate photoreceptor outer segment morphogenesis are still largely unknown. Unlike typical cilia, the outer segment is continuously regenerated or renewed throughout the life of the animal through the combined process of distal outer segment shedding and proximal outer segment growth. The process of outer segment renewal was discovered over forty years ago, but we still lack an understanding of how photoreceptors renew their outer segments and few, if any, molecular mechanisms that regulate outer segment growth or shedding have been described. Our lack of progress in understanding how photoreceptors renew their outer segments has been hampered by the difficulty in measuring rates of renewal. We have created a new method that uses heat-shock induction of a fluorescent protein that can be used to rapidly measure outer segment growth rates. We describe this method, the stable transgenic line we created, and the growth rates observed in larval and adult rod photoreceptors using this new method. This new method will allow us to begin to define the genetic and molecular mechanisms that regulate outer segment renewal, a crucial aspect of photoreceptor function and, possibly, viability.

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\textbf{Introduction}

Photoreceptors are morphologically specialized cells that have four functional and morphologically distinct compartments: two basal compartments; the synaptic region and the cell body, and two apical compartments; the inner segment and the outer segment. The outer limiting membrane is a specialized adherens junction that separates apical and basal compartments. The rod outer segment is a highly modified cilium that contains the phototransduction machinery and discrete intramembranous discs embedded with photon-capturing Rhodopsin. The inner segment is a specialized compartment containing organelles and is where most proteins and membranes are synthesized. The molecular and cellular mechanisms that regulate rod morphogenesis are poorly understood.

Photoreceptors have the exceptional and remarkable ability to shed and renew a part of themselves – the outer segment. The most distal tips of cone and rod outer segments are shed in discrete packets containing many discs, these packets are then phagocytosed by the neighboring retinal pigmented epithelium and renewal occurs at the base of the outer segment by the addition of new discs (Young, 1967; Young and Droz, 1968; Young and Bok, 1969; Young, 1971). Consequently, the oldest discs are at the tip of outer segments and the youngest are at the base. To maintain constant outer segment length, growth rates and shedding rates must match. The purpose of shedding and renewal is unclear but it seems likely to be an evolutionary solution to the inability to directly recycle old disk membrane and resident membrane proteins given the architecture of the outer segment, the disks, and the narrow connecting cilium. Very little is known about the cellular and molecular mechanisms that control outer segment shedding – what determines how much outer segment is shed and what is the composition of the machinery that sheds the tips. Equally obscure is how photoreceptors renew their outer segments – what determines how much outer segment is made each day, and what is the composition of the machinery that adds the new material.

Our progress towards understanding how vertebrate photoreceptors renew their outer segments has been hampered by at least three challenges. One, the renewal process seems to occur only in the intact retina where the relationship between photoreceptors and neighboring cells is maintained. Thus, studying the renewal process is challenging. Two, although photoreceptors in some arthropod species shed the tips of their microvillar sensory compartment (Williams and Blest, 1980; Stowe, 1980; Williams, 1982), there are no reports that photoreceptors in \textit{Drosophila melanogaster} shed, and thus, a comparative genetic approach using this species to identify conserved mechanisms of shedding is precluded. Three, the classical method of measuring rod outer segment renewal that uses injection of radioactive amino acids into free-living animals and measuring the displacement over time of radioactive proteins (mainly Rhodopsin) by autoradiography is tedious, has radioactivity containment issues, and experiments take a long time (i.e. up to 3 month exposure times). As a consequence,
experiments using this method have been used rarely in recent years. We have developed a powerful new tool to rapidly measure rates of outer segment renewal in rod photoreceptors that will allow us to begin to identify the molecular and cellular mechanisms that control outer segment renewal.

Methods and Materials

Animals

Tg(xop:EGFP);Tg(hsp70:HA-mCherry)"alb" fish lines were maintained and staged according to Westerfield (1995). All experiments involving animals were performed with approval by and in accordance with the University of Massachusetts-Amherst Institutional Animal Care and Use Committee (IACUC). The Tg(xop:EGFP) line was provided by James Fadool (2003). We induced mCherry expression in larvae and adult fish using 45–60 minute incubation in 39°C water, after which the fish were returned to 28°C fish water.

Molecular Biology

The mCherry construct was generated by placing the N’terminal signal peptide (SP) sequence from zebrafish Crb2b (MRGLIVKVICLIVGLLV; SignalP 3.0 Server) upstream of the influenza hemagglutinin (HA) tag (YPYDVPDYA) followed by the transmembrane domain sequence from zebrafish Crb2a (AVPLACGILLVAILGFML) in frame with the coding region of mCherry (Shaner et al., 2004) followed by a poly-adenylation sequence at the 3’-end. This construct was cloned behind the zebrafish promoter for the heat shock protein 70 gene (hsp70; Halloran et al., 2000). The final hsp70:HA-mCherryTM construct was cloned into the pTol vector (Kawakami et al., 2000; Kawakami, 2004).

Transgenesis

The Tg(hsp70:HA-mCherryTM) line was used in all transgenic experiments using the pTol system (Kawakami et al., 2000; Kawakami, 2004). We co-injected 40 ng/mL of pTol-transgene construct plasmid with 40 ng/mL transposase mRNA into one-cell stage Tg(xop:EGFP);"alb" embryos. Injected embryos were grown to adulthood and out-crossed with the Tg(xop:EGFP);"alb" fish to produce offspring. We used PCR to identify transgenic offspring. PCR on fin DNA was performed to identify transgenic F1s and subsequent generations. F1 carriers were out-crossed with Tg(xop:EGFP);"alb" line to produce F2s. Tg(hsp70:HA-mCherryTM) were genotyped with the following primers; HSP Forward: AGAGACCGCAGAGAA, mCherry Reverse: ATGATGGCCATGTTATCCTCCTCG.

Immunocytochemistry and Microscopy

Larvae and adults were fixed in 4% paraformaldehyde for 1–2 hours. Cryostat sections (25–30 µm) were rehydrated with 0.1% Tween in PBS (PBS-Tw) for 15 min, incubated in 10% goat serum in PBS-Tw, rinsed briefly in PBS-Tw, and incubated overnight at 4°C in primary antibody (monoclonal anti-HA IgG1, 1:1,000 (Covance); rabbit anti-GFP, 1:200 (Invitrogen), and anti-Rhodopsin monoclonal Rb-5 (IgG2a), 1:50 (Rohlich et al., 1989)). Sections were washed, incubated with the appropriate secondary antibodies (FITC-conjugated goat anti-rabbit (Invitrogen) 1:200; rhodamine red-conjugated goat anti-mouse IgG1 (Jackson Laboratory), 1:100; Cy-5-conjugated goat anti-mouse IgG2a, 1:100 (Jackson Laboratory)), and samples mounted in Prolong Gold anti-fade reagent (Invitrogen). Samples were analyzed with a Zeiss LSM 510 Meta Confocal System. In larvae, we primarily analyzed the retinas in "alb" individuals to ensure that the entire outer segment was visible and not obscured by the RPE. Confocal images are a single scan (averaged 4 times) at about 1 µm optical thickness, or z projections with step increases of 0.37 µm. Measurements were acquired using Velocity 3D imaging software (Improvision PerkinElmer Company). The numbers of cells measured provided in the results were taken from an individual retina at each time point.

Results

A new method to measure rates of rod outer segment growth

The growth of rod outer segments was measured originally by injection of radioactive amino acids and subsequent autoradiography to measure the displacement of the band of radioactive proteins (predominantly rhodopsin) over time (Fig. 1A). Following the seminal studies using this radioactive method that revealed the phenomenon of outer segment renewal (Young, 1967; Young and Bok, 1969; Bok and Young, 1972; LaVail, 1973), the process of outer segment renewal has been largely unstudied and very little is known about the cellular or molecular mechanisms that regulate outer segment renewal. We became interested in the question of the molecular control of outer segment size and renewal because of our work studying the role of the Crumbs complex in photoreceptor morphogenesis and outer segment size (Hsu et al., 2006; Hsu and Jensen, 2010) and

Fig. 1. Methods to measure rates of rod outer segment growth. (A) The original method to measure outer segment growth used injection of radioactive amino acids, which are incorporated into newly synthesized proteins. The displacement of predominantly H-labeled Rhodopsin was measured over time. (B) A new method to measure outer segment growth using heat-shock induction to transiently express a red fluorescent protein that is incorporated into newly synthesized outer segment discs. The displacement of a stripe of red fluorescent protein can be followed over time. (C) A diagram of the construct used to generate a stable transgenic line to express heat-shock inducible red fluorescent protein. The hsp70 promoter was placed upstream of an expression construct where a signal peptide (SP) is fused to the hemagglutinin (HA) peptide tag followed by a transmembrane domain (TM) and mCherry fluorescent protein.
wanted to further explore the mechanisms of outer segment growth. We found the radioactive method unappealing because of the radioactive containment issues and the lengthy exposure times required. We wondered whether we could generate a genetically encoded inducible marker of outer segment growth and renewal that would simplify experiments. Could we transiently express a fluorescent protein that would incorporate into a subset of newly formed discs and then follow over time the displacement of the fluorescent proteins?

In particular, could we use heat-shock to transiently express a red fluorescent protein that incorporates into newly formed discs and then follow the distal displacement of the red fluorescent stripe over time (Fig. 1B)? We created a construct where a signal peptide is followed by a hemagglutinin (HA) peptide tag, a transmembrane domain and mCherry, all driven by the zebrafish hsp70 promoter (Fig. 1C). We epitope-tagged the protein construct to offer flexibility because our confocal microscope, like many others, is not currently optimized for mCherry excitation. Our previous work examining the structure and function of a Crumbs protein suggested that this simple construct would localize to outer segment discs without causing defects (Hsu et al., 2010).

We generated a stable transgenic line, Tg(hsp70:HA-mCherry\(\text{TM}\)), with the HA-tagged transmembrane bound mCherry construct using the pTol transgenesis method (Kawakami, 2004). We examined the photoreceptor layer at 68 hours post fertilization (hpf), not long after the first photoreceptor birthdays begin at around 48 hpf (L. Nowrocki, PhD thesis, University of Oregon, 1985; Larison and Bremiller, 1990). Following 45 minutes of heat-shock at 68 hpf, an examination of retinal sections labeled with anti-HA antibody shows that red fluorescence is largely membranous in Tg(hsp70:HA-mCherry\(\text{TM}\); alb\(\text{−}\)-/ alb\(\text{−}\)) larval retinal cells (Fig. 2A, B). Levels of red fluorescence in wild-type siblings subjected to heat-shock are similar to wild-type siblings that were not subjected to heat-shock (Fig. 2C, D, G, H). In the retina of Tg(hsp70:HA-mCherry\(\text{TM}\)) larva that were not subjected to heat-shock, some amacrine cells produce small amounts of HA-mCherry\(\text{TM}\) (Fig. 2E, F, arrows).

Dynamics of hsp70:HA-mCherry\(\text{TM}\) in photoreceptors
An examination of single confocal z-sections of a 3 day postfertilization (dpf) retina at 5 hours post heat-shock (hpHS), shows that HA-mCherry\(\text{TM}\) is localized to the plasma membrane of Tg(hsp70:HA-mCherry\(\text{TM}\)) photoreceptors and in dense foci at the base of outer segments (Fig. 3A–C). A stripe of HA-mCherry\(\text{TM}\) can be seen at the base of GFP-expressing rods (arrows Fig. 4A, B). An examination of 4 dpf retina at 1 day post heat-shock (dpHS), shows that most of the plasma membrane HA-mCherry\(\text{TM}\) has disappeared from photoreceptor cell bodies and inner segments, while the stripe of HA-mCherry\(\text{TM}\) remains in the outer segment discs and has migrated distally (Fig. 3D–F). The rapid disappearance of HA-mCherry\(\text{TM}\) from the plasma membrane of photoreceptors suggests that it is rapidly endocytosed and degraded. In contrast, photoreceptor disc membrane is not retrieved and recycled, and so HA-mCherry\(\text{TM}\) is trapped in outer segment discs and serves as a measure of growth rates. Unlike rods, which have discrete discs stacked like slices of bread in a bread bag where proteins in one disc cannot diffuse into neighboring discs, cones have discs that are continuous like a ribbon and, thus, membrane proteins can diffuse through the entire continuous outer segment discs. In the single z-section of 4 dpf retina at 1 dpHS, HA-mCherry\(\text{TM}\) in cone outer segments is more continuous and broadly localized (arrowheads Fig. 3D–F), indicating that it has diffused widely.
Measurements of larval rod outer segment growth
Using heat-shock induction of HA-mCherry™ expression we measured rates of outer segment growth in larval rods. We heat-shocked 5 dpf Tg(hsp70:HA-mCherry™);Tg(Xop:EGFP);alb^-/- larvae and examined them at 1 dpHS and at 3 dpHS. Confocal z-projection of a 6 dpf larval photoreceptor layer at 1 dpHS and a 8 dpf larval photoreceptor layer at 3 dpHS shows HA-mCherry™ (red) in anti-Rhodopsin labeled outer segments (blue) in GFP-expressing rods (green), HA-mCherry™ can appear as a stripe, an oval, or a circle, depending on the orientation of the outer segment to the plane of the image (Fig. 4A, B). Using 3-dimensional Velocity software, we can measure in individual outer segments the distance from the base of the outer segment to the approximate middle part of the HA-mCherry™ stripe (arrows Fig. 4C, D). We measured in three dimensions the growth of outer segments of rods at 6 dpf 1 dpHS shown in Fig. 4A and found a mean growth distance of 1.35 μm/day (number of cells measured = 9, s.d. = 0.137 μm). We also measured in 3-dimensions the growth of outer segments of rods at 8 dpf, 3 dpHS shown in Fig. 4B and found a mean growth distance of 3.2 μm/3 days (number of cells measured = 13, s.d. = 0.42 μm) or 1.06 μm/day (s.d. = 0.14).

Measurements of adult rod outer segment growth
Using heat-shock induction of HA-mCherry™ expression we can also measure rates of outer segment growth in adult rods. Confocal z-projections of adult Tg(hsp70:HA-mCherry™);Tg(Xop:EGFP) photoreceptor layers at 1 dpHS (Fig. 5A), 4 dpHS (Fig. 5B), 7 dpHS (Fig. 5C), 9 dpHS (Fig. 5D), 15 dpHS (Fig. 5E) and 16 dpHS (Fig. 5F) shows HA-mCherry™ (red) in anti-Rhodopsin labeled outer segments (blue) in GFP-expressing rods (green). At 1 dpHS, HA-mCherry™ localizes near the base of the rod outer segments (Fig. 5A), at 15 dpHS HA-mCherry™ is near the tip of rod outer segments (Fig. 5E), and at 16 dpHS HA-mCherry™ has largely disappeared from rod outer segments (Fig. 5F). The disappearance of HA-mCherry™ from outer segments at 16 dpHS indicates that the rod outer segment is completely renewed in approximately 16 days in adult zebrafish. We measured in three dimensions the growth of outer segments of rods at 7 dpHS and found a mean growth distance of 11.09 μm/7 days (number of cells measured =14, s.d. = 0.861 μm) or 1.58 μm/day (s.d. = 0.123). These measurements indicate that the growth rate of rod outer segments in adults is similar to larval rates.

Discussion
Many of the molecular mechanisms underlying photoreceptor signal transduction, synaptic transmission, and ciliogenesis have been identified, but yet very little is known about how photoreceptors maintain their outer segments through the combined processes of outer segment growth and shedding and what molecular mechanisms underlie those processes. The
renewal of rod photoreceptor outer segments was described over forty years ago by following the fate of radioactively labeled proteins (largely Rhodopsin) in the outer segment over time in several different vertebrate species (Young, 1967; Young and Bok, 1969; Bok and Young, 1972; LaVail, 1973). The role of light in the process of renewal was studied and the rate of outer segment growth in *Xenopus laevis* rods was found to be the same in those maintained in constant light and those maintained to cyclic light, although growth was reduced significantly in those maintained in constant dark (Hollyfield and Rayborn, 1979). While outer segment growth was greatly reduced under constant dark conditions, this reduction could be due to a general reduction in protein synthesis, including reduced Rhodopsin levels (Hollyfield and Anderson, 1982). Light exposure, however, may play a role in outer segment growth, as experiments examining *Xenopus laevis* rods indicated that exposure to light accelerates outer segment growth during the first 8 hours of a 12:12 hour light-dark cycle and growth was undetectable during the last 8 hours of darkness (Besharse et al., 1977a, Besharse et al., 1977b). Light also plays a vital role in the shedding process; rods initiate outer segment shedding at the onset of light (Basinger et al., 1976).

In many mouse models of human retinal degeneration diseases where it has been examined, photoreceptor outer segments progressively shorten before photoreceptors die (Heckenlively et al., 1995; Chen et al., 1999; Hawes et al., 2000; Hong et al., 2000; Gao et al., 2002; Collin et al., 2005; Pang et al., 2005; Vasireddy et al., 2006). This observation raises the interesting question of...
whether there is a causal link between outer segment size and photoreceptor viability. We consider two possibilities. One, disease-associated mutations physically compromise photoreceptors and as they sicken they no longer are able to sustain the enormous metabolic load required to maintain their outer segments. Thus, as the cell sicken, the outer segment shortens secondarily and concomitantly, and finally the cell is so sick it undergoes apoptosis. Two, the cell undergoes apoptosis because its primary functional organ, the outer segment, is no longer functional. Thus, the loss of outer segment function induces photoreceptor apoptosis. If the latter possibility is true, then the question becomes could stimulating outer segment growth during photoreceptor degeneration disease prolong photoreceptor function and viability? In order to test this possibility, the underlying cellular and molecular mechanisms of outer segment growth must first be identified.

Given the fascinating and largely unaddressed question of how photoreceptors maintain their outer segments through the continuous processes of growth and shedding and the likely importance of these processes to retinal health, we developed a new method that allows us to rapidly measure growth rates of rod outer segments. Now that we easily measure rod outer segment growth, we can next ask the question what genes and molecular mechanisms contribute to outer segment growth. We can approach this question using two methods. One method to study gene function in outer segment growth uses mosaic analysis. This method uses the injection of a DNA construct where the Xenopus rod opsin promoter drives expression of a gain-of-function or loss-of-function candidate gene (Xop:gene X) in a subset of rods and then the rates of outer segment growth in these rods are compared to neighboring non-transgene-expressing rods (Fig. 6A). The other method is to generate stable transgenic lines, Tg(Xop:gene X), that express gain-of-function or loss-of-function candidate genes driven by Xenopus rod opsin promoter. The rate of rod outer segment growth in the transgenic line Tg(Xop:gene X);Tg(Xop:EGFP);Tg(hsp70:HA-mCherry) is compared to the rate of growth in the Tg(Xop:EGFP);Tg(hsp70:HA-mCherry) line. The advantage to the mosaic analysis is that it is rapid. The advantage to the stable transgenic analysis is that transgene copy number can be determined and growth rates in individual transgenic rods should be more consistent.

Given that we know virtually nothing about the cellular and molecular mechanisms of outer segment growth, what kind of genes and mechanisms might contribute to this process? We consider two different mechanisms that might control outer segment growth– cilia size control mechanisms and cell size control mechanisms. Although the ciliary axoneme appears to extend to the tip of cone outer segments, it does not in rods and as yet, there is no data supporting the renewal of the axoneme in rods (Roof et al., 1991; Eckmiller, 1996). There are, however, a number of molecular pathways identified that modulate ciliary and flagellar length that could be examined (see review by Ishikawa and Marshall, 2011). Photoreceptor outer segments are unlike typical cilia: they are longer and have a much greater volume, which is filled largely with disc membrane. Using data from LaVail (1973), we roughly calculate that a mouse rod makes about 230 μm² of membrane daily to replace that which was shed. The growth process clearly requires a great deal of membrane and protein synthesis and thus, pathways like the mTor pathway (for review see Zoncu et al. 2011) that are involved in cell size control may be regulators of outer segment growth. In the case of photoreceptor, increased cell growth could be invested in the outer segment. The generation of the Tg(hsp70:HA-mCherry) line should allow us to determine whether either of these two mechanisms– cilia size control or cell size control contribute to the growth of rod outer segments.

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