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Rhodopsin–EGFP knock-ins for imaging quantal gene alterations

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Abstract

We have developed an imaging approach to monitor changes in gene structure in photoreceptors. We review here, the strategy and recent progress. Knock-in mice bearing a human rhodopsin–EGFP fusion gene potentially allow detection of a single molecular event: correction of a single copy of a gene within an entire retina. These mice can also be used for imaging rhodopsin distribution, membrane structure, and trafficking in normal mice or in disease states, using confocal or multiphoton fluorescence imaging techniques. They represent tools for studying molecular triggers of photoreceptor development, for following stem cell populations, and for evaluating retinal transplantation experiments.

Keywords: Photoreceptors; Gene knock-ins; Fluorescence imaging; Confocal microscopy; Retinal degeneration; Gene therapy

1. Detecting single molecular events in the retina

One approach to autosomal dominant neurodegenerative genetic diseases, such as autosomal dominant retinitis pigmentosa (ADRP), is to target the defective gene itself in the terminally differentiated neurons in which its expression causes disease. This is a daunting task, and in the early stages of research into methods for accomplishing it, success is likely to be rare, and the processes highly inefficient. Therefore, highly sensitive methods are needed to detect a single molecular event—for example, a single nucleotide change in one copy of a specific gene—within an entire retina. Sufficiently sensitive methods could also be used to study the natural mechanisms of mutagenesis and DNA repair in terminally differentiated neurons in vivo, about which we know relatively little (Nouspikel & Hanawalt, 2002).

Sensitivity at this level depends on a high degree of signal amplification. One of the most highly amplified events in any cell type is a change in gene structure. A single base pair change in a gene that is highly expressed can give rise to thousands of altered mRNA molecules, each of which can be translated into many molecules of altered proteins. In photoreceptor cells, the most highly expressed gene is rhodopsin. Assuming equal transcription from each copy of the rhodopsin gene, a single allele can give rise to a steady-state population of about 30 million rhodopsin molecules per rod (assuming 550–650 pmol rhodopsin per 6.4 million rods in wildtype mice with two alleles; Lyubarsky, Daniele, & Pugh, 2004). For this reason alone, rhodopsin is a good first choice for a gene to monitor at the single-cell level. Additional signal amplification can be provided by the detection technique itself. The most highly amplified optical detection technique available is fluorescence; a single molecule can emit on the order of 10^9 photons/s when maximally excited. The duration of such a signal depends on the photobleaching quantum yield. GFP has a photodestruction quantum yield of about 2.5×10^{-6} (Kubitscheck, Kuckmann, Kues, & Peters, 2000) so as many as 4×10^5 photons can be emitted by the average fluorophore before it is extinguished, and on the order of 1% of these detected. By linking EGFP to rhodopsin, the signal can be amplified enormously: a

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single altered rhodopsin gene can yield $\sim 10^{11}$ detected photons. In this way, the problem of finding a microscopic needle in a haystack is converted to the problem of finding a searchlight on a dark night.

2. Imaging strategy using knock-in mice

The basic strategy is as follows: a mouse strain is engineered to have one rhodopsin allele replaced by the genomic sequence derived from the human rhodopsin gene, with two or three types of alteration. First, the sequence encoding enhanced green fluorescent protein, EGFP, is inserted in frame between the codon for the last amino acid of human rhodopsin and the stop codon. Second, a mutation, ranging from a point mutation to a large insertion, is incorporated into the transcription unit. For the optimum signal-to-background ratio, this mutation will be one that completely eliminates translation of the carboxy-terminal region of human rhodopsin, including the fused EGFP sequences. Mutations that are not complete nulls, however, such as the P23H mutation found in many cases of ADRP, are also of interest for certain applications. Third, in some instances, the target sites for the Cre site-specific recombinase—loxH or loxP and lox511—are inserted to flank the coding region of the knocked-in gene. Originally, these sites were included to facilitate direct segmental replacement for repetitive knock-in of rhodopsin alleles in ES cells by co-transfection of an expression vector for Cre recombinase and a DNA segment flanked by the same sites. An additional benefit was found when these alleles were tested in vivo; the lox sites lead to a reduction in expression of the adjacent human rhodopsin–EGFP allele to about 20% of the level in the absence of lox sites (Chan, Bradley, Wensel, & Wilson, 2004). This property makes it possible to choose a level of expression appropriate for each experiment. If the protein product is deleterious (for example, the P23H mutant) expressing lower levels may promote cell survival.

Although methods are well established for generating knock-in mouse strains, creating a new one is a time-consuming, tedious, and expensive prospect. In contrast, mini-genes directing rhodopsin or rhodopsin–EGFP expression can be readily introduced into the mouse genome in less time and with less expense using transgenic approaches (Campochiaro et al., 1996; Chen et al., 1997; Furukawa, Koike, Lippincott, Cepko, & Furukawa, 2002; Ichsan et al., 2005; Nie, Chen, Kumar, & Zack, 1996; Zhu et al., 2004). However, there are several reasons that make knock-ins preferable. First, although promoter constructs that direct expression to rod cells have been identified for the rhodopsin gene, these elements are clearly not the only ones regulating the timing and level of expression. Some transgenic lines expressing reporter genes driven by rhodopsin promoter constructs have displayed mosaic expression—that is, not all rods express at the same level—and others have displayed ectopic expression in cones. Knocking in the full human rhodopsin gene at the mouse rhodopsin locus ensures that all *cis* elements needed for proper regulation of transcription and splicing are present and that expression is uniform throughout the retina. Second, transgenic lines must be screened to find ones that express at an acceptable level, which for the rhodopsin gene is a critical consideration, since as little as a four- to sixfold overexpression, even of wildtype rhodopsin, can cause retinal degeneration (Olsson et al., 1992; Sung, Makino, Baylor, & Nathans, 1994). By contrast, either of two distinct levels of human rhodopsin–EGFP expression—80% or 16% of a normal mouse allele—can be chosen in advance by selecting the proper construct to be used for knock-in (Chan et al., 2004). Third, transgenic lines usually carry multiple copies of the transgene at the site of integration. The presence of multiple target genes complicates the analysis of experiments designed to modify the target, a complication that is absent for knock-in alleles. Finally, the ultimate goal is to develop approaches that might be applicable to manipulating genes with naturally occurring mutations. There is abundant evidence that the efficiency of processes such as recombination and mutation are influenced by the chromosomal context in which the gene of interest is found (Vasquez, Marburger, Intody, & Wilson, 2001), and so it is prudent to mimic the context in which the human rhodopsin gene is found as faithfully as is possible in a mouse model.

3. Initial versions of rhodopsin–EGFP knock-in mice

Mice bearing constructs of the kind described above—with mutations that block expression of rhodopsin–EGFP—have been generated (F. Chan, K. Sykoudis and A. Gross, unpublished results) but have not yet been studied extensively. However, analysis of previously described knock-in mouse strains (Chan et al., 2004) expressing wildtype human rhodopsin fused to EGFP, which were prepared essentially as controls, have proven very informative. As described below, they have allowed us to establish the feasibility of this approach and to define the effects of a modified C-terminus on rhodopsin function and phototransduction. In addition, they have provided valuable substrates for imaging various aspects of photoreceptor function in a range of applications.

In the case of termination or frameshift mutations, which completely block expression of the EGFP moiety, correction of the defective allele will give rise to bright green fluorescence in any cell undergoing the change. A single fluorescent cell, especially one that is only 1.4 μm wide, may be hard to detect in a tissue with

significant levels of autofluorescence. Therefore, it is necessary to know whether a single cell can be detected and how this can be done. It is also critical to make sure that expression of the rhodopsin–EGFP fusion does not, by itself, kill rod cells. If it did, then those cells undergoing gene repair would be subject to cell death, precluding their detection with high sensitivity.

To address these last two questions, we began by generating two strains of mice that express different levels of wildtype human rhodopsin–EGFP (Chan et al., 2004). One strain contains the *hrhoG* allele, which encodes human rhodopsin fused to EGFP through the linker peptide, APVAT. A single copy of the *hrhoG* allele expresses human rhodopsin–EGFP at 80% the level of a single mouse rhodopsin allele. The second strain contains the *hrhoG*(H) allele, which encodes the same human rhodopsin–EGFP, but is flanked on the 5′ side by a loxH site and on the 3′ side by a lox511 site (for details see (Chan et al., 2004)). A single copy of the *hrhoG*(H) allele expresses human rhodopsin–EGFP at 16% the level of a single mouse rhodopsin allele. These mice allowed us to examine what would happen in our gene correction scenario once the rhodopsin–EGFP fusion protein began to be expressed.

4. Images obtained with rhodopsin–EGFP

Fig. 1 illustrates the bright green fluorescence of the retinas of living mice bearing a single *hrhoG* allele (+/*hrhoG*). Mice of this genotype can easily be distinguished from wildtype mice by examining them under blue light illumination, using glasses designed to filter out the blue light, even in a background of normal white room light. Fig. 2 shows the images of rod outer segments that can be obtained by confocal scanning fluorescence microscopy of retinal whole-mounts from these animals. The samples display the brightest fluorescence when they are mounted and imaged without any fixing or staining. However, in Fig. 2 the retina was lightly fixed and stained for cone sheaths to allow visualization of cones, which are otherwise invisible in such images, and there is still very intense EGFP signal. Note that there is no EGFP signal in the cone cells—only the sheaths are stained by the peanut lectin, and the cone inner and outer segments appear as dark shadows framed by the sheaths. A *z*-stack of this kind (26 optical sections in the original file) can be collected with good signal-to-noise under sufficiently dim laser illumination to achieve only minimal photobleaching, and the entire three-dimensional extent of individual rod outer segments can be readily traced.

As an alternative imaging mode, confocal microscopy can be used to view unstained fixed cryosections (Figs. 3–5). Fig. 3 shows the healthy appearance and normal morphology of mice heterozygous for the *hrhoG* allele (+/*hrhoG*). These mice maintain healthy retinas throughout their lives, with only a slightly enhanced loss of photoreceptor nuclei, as compared to wildtype mice, both raised in standard animal facility lighting (Chan et al., 2004). Because nearly all the rhodopsin–EGFP is transported to the outer segment, the inner segment and cell body are difficult to see in individual optical sections under conditions in which the outer segment signal

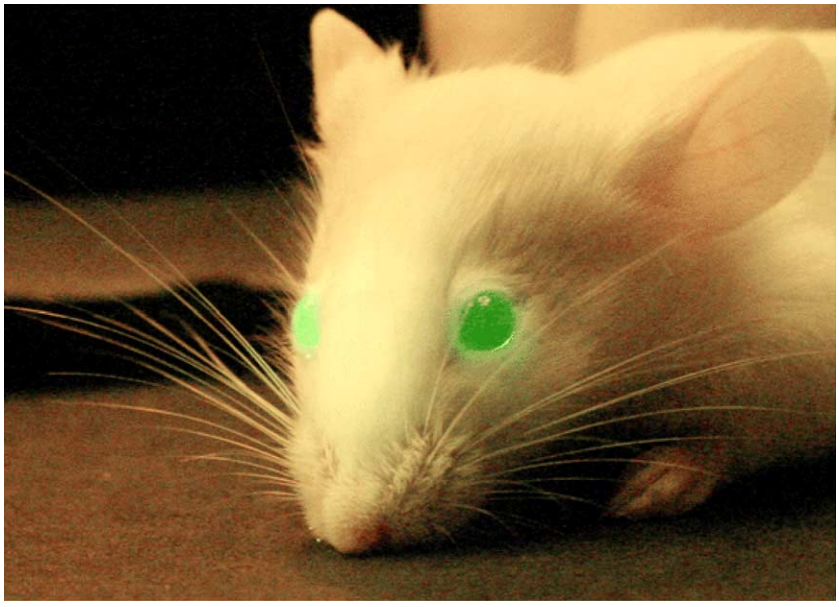

Fig. 1. Visual screen of *hRhoG* expression. Mice are routinely screened using a head-mounted photo-diode/emission-filter-goggle combination. For photography, the +/*hRhoG* mouse shown here was anesthetized and illuminated simultaneously with white light and diffuse 476-nm wavelength light from an argon-ion laser and photographed through a 515-nm long pass filter.

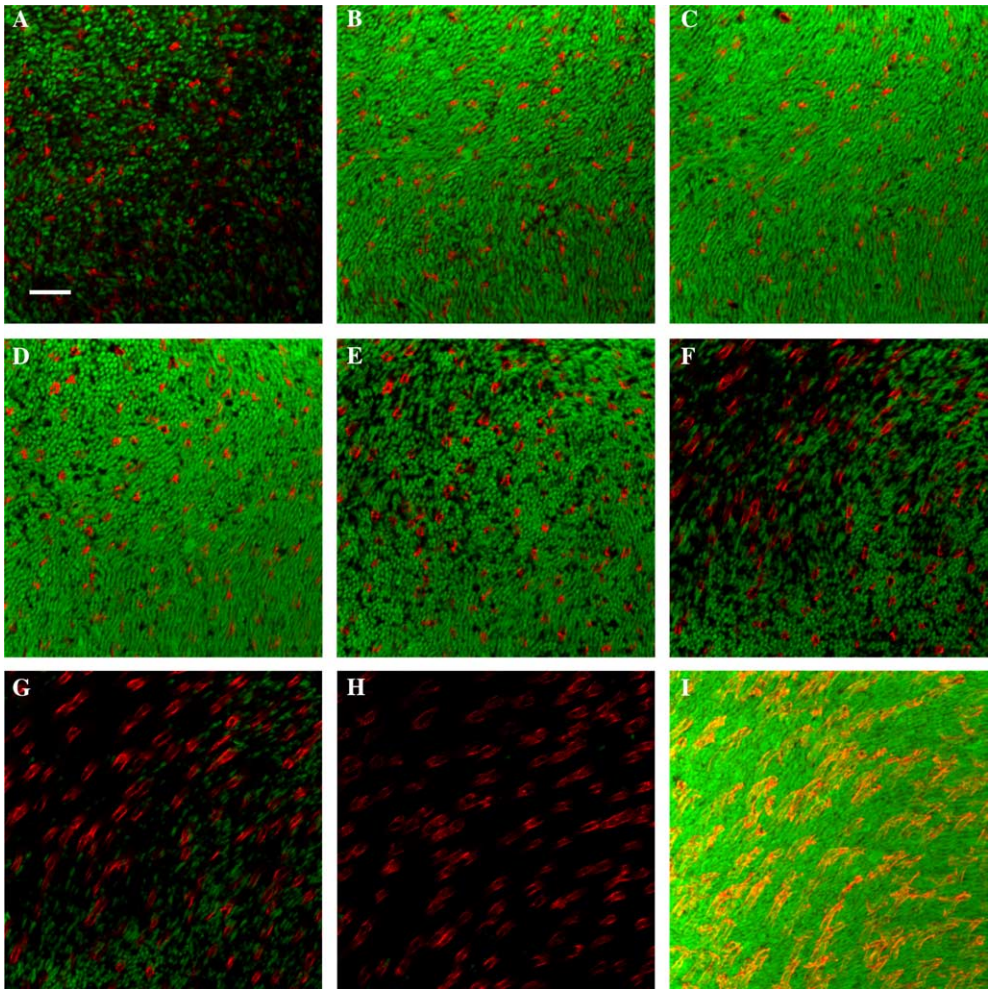

Fig. 2. Fluorescent confocal images of a retinal whole-mount from a 3-week-old +/*hrhoG* mouse. Rhodopsin–EGFP fluorescence and rhodamine-linked peanut agglutinin staining of cone sheaths are shown. (A–H) Twenty-six images were taken in 0.4 μm steps along the *z*-axis; every third one is shown. (I) Projection image from stack of pictures taken every 0.4 μm for 10 μm in *z*-axis. Scale bar: 10 μm.

is not saturated. However, the signal from traces of rhodopsin–EGFP in the plasma membrane is sufficient to be detected throughout the cell, as can be seen clearly in the projection through the *z*-stack of Fig. 3H. Outside the outer segment, the distribution of rhodopsin–EGFP is not uniform. In Fig. 3I, it can be seen that although the inner segment signal is weak, there is more rhodopsin–EGFP present in the inner segment than in the rest of the cell body extending to the outer plexiform layer.

These unstained sections are very useful for monitoring the progress of photoreceptor degeneration in mouse models. Examples are shown in Figs. 4 and 5. Mice lacking a wildtype rhodopsin gene and bearing two *hrhoG*(H) alleles (*hrhoG*(H)/*hrhoG*(H)) undergo a progressive retinal degeneration. Photoreceptors have abnormal morphology and they die over a period of 5–6 months after birth; only two rows of nuclei are left in the outer nuclear layer by 18 weeks. In *hrhoG*/*hrhoG* mice, the disruption of cell morphology is more severe (Fig. 5) and there is a more rapid decline in the number of photoreceptor nuclei, (Chan et al., 2004). Presumably, retinal degeneration occurs more slowly in *hrhoG*(H)/*hrhoG*(H) mice because they produce only 20% as much rhodopsin–EGFP as *hrhoG*/*hrhoG* mice. These results indicate that human rhodopsin–GFP, by itself, fails to provide an essential function necessary for proper rod cell formation and health. In heterozygotes, however, this missing function is provided by wildtype rhodopsin, allowing photoreceptors to achieve normal morphology and to maintain long-term survival.

5. Feasibility of detecting a single gene correction event

A mouse retina contains 6.4 million rods (Jeong & Ikeda, 1998), with a corresponding number of copies of each gene allele within their nuclei. Thus, if we can detect a single molecular event in one retina, screening through 20 eyes from 10 treated animals would allow detection of gene modifications as rare as one in 10^8.

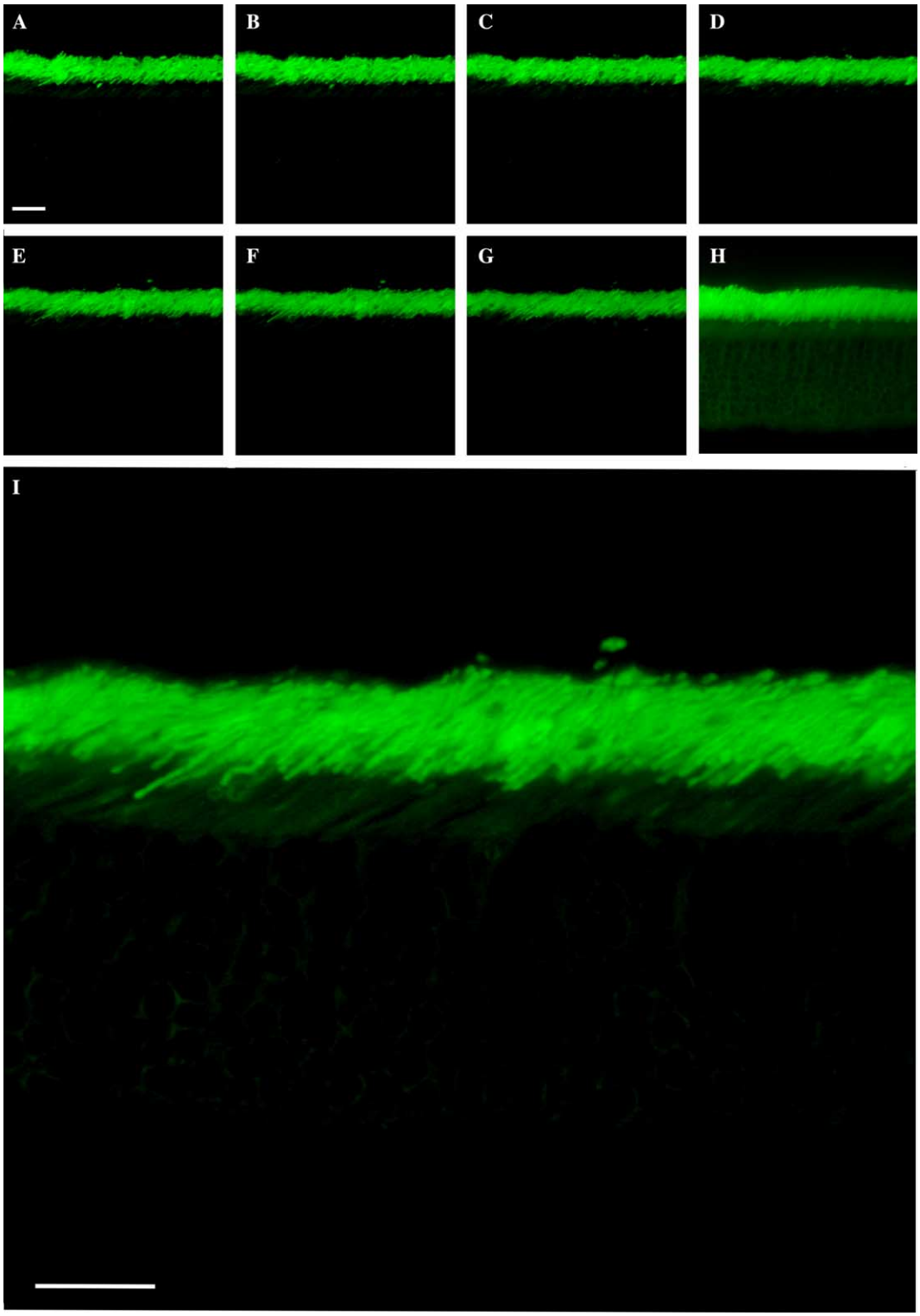

Fig. 3. Confocal scanning fluorescence images of retinal sections from +/*hrhoG* mouse at 9 weeks of age. (A–G) Images of cryosections every 1.8 μm along the *z*-axis through the slice. (H) Projection image compiled from 46 images taken every 0.225 μm in the *z*-axis. (I) Larger view of (F) showing resolution of individual cells. Scale bars: 20 μm.

Fig. 6 shows an actual image of a single rod outer segment expressing human rhodopsin–EGFP from the *hrhoG*(H) knock-in allele, from a chimeric mouse generated from (+/*hrhoG*(H)) ES cells. The cell was imaged in a whole-mounted retina, and the surrounding background signal is due to autofluorescence from thousands of adjacent photoreceptor cells. This background has been computationally repeated in the figure to cover

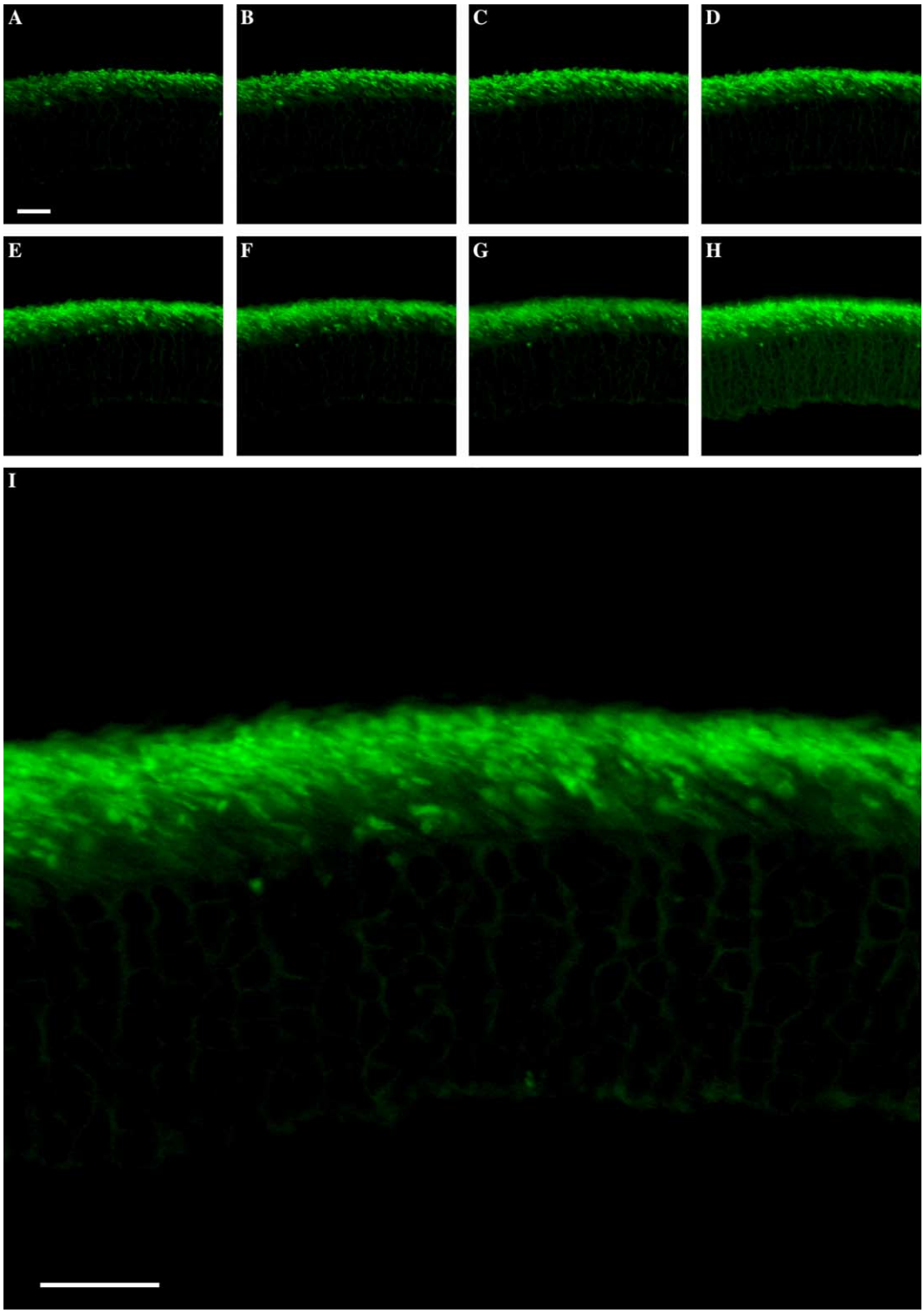

Fig. 4. Confocal fluorescence images of retinal sections from a 5-week-old *hrhoG*(H)/*hrhoG*(H) mouse. (A–G) Optical sections of cryosections spaced every 1.8 μm along the *z*-axis through the slice. (H) Projection image compiled from 46 images taken every 0.225 μm in the *z*-axis. (I) Larger view of single image. Scale bars: 20 μm.

an area corresponding to 1/35 of a mouse retina (0.5 mm^2). Thus, this image provides a realistic view of what a single molecular event in a null background would look like. Screening through thirty-five such fields on each of 20 retinas, to yield a total of 700 images like the one shown, would allow the desired detection sensitivity of 10^{-8}. Note that because the fluorescent rod expresses human rhodopsin–EGFP *and* wildtype mouse

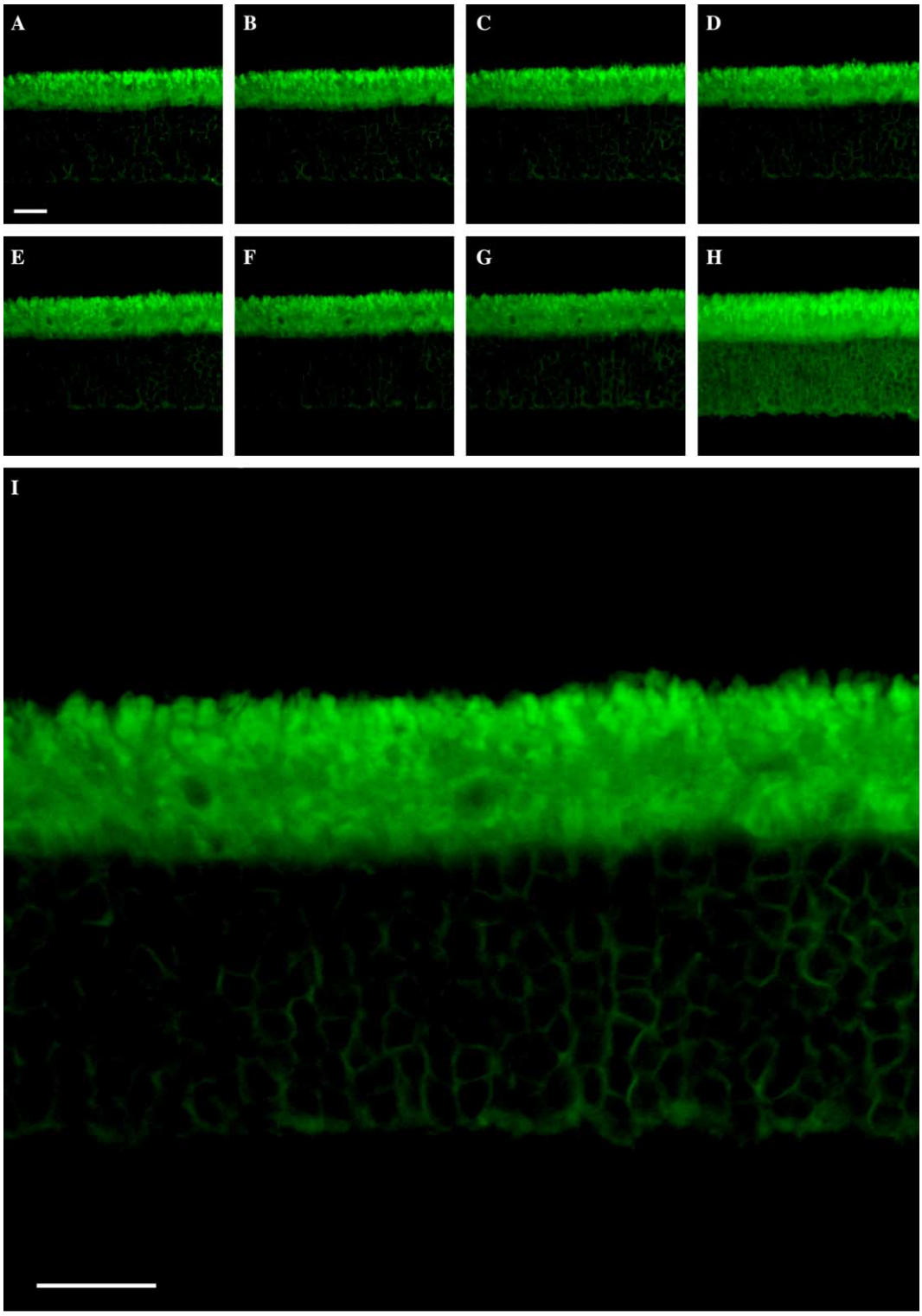

Fig. 5. Images of retinal sections from *hrhoG*/*hrhoG* mouse at 6 weeks of age. (A–G) Images of cryosections every 1.8 μm in along the *z*-axis through the slice. (H) Projection image compiled from 46 images taken every 0.225 μm in the *z*-axis. (I) Larger view of single image. Scale bars: 20 μm.

rhodopsin, it is expected to remain healthy and visible for a very long time.

Aside from their utility in studying gene repair and inactivation, the *hrhoG* and *hrhoG*(H) strains can be used to image rhodopsin expression and rod morphogenesis with exquisite sensitivity in a range of experimental paradigms. In ongoing unpublished studies, they have been used for monitoring the course of retinal degeneration (for example, in *rd*/*rd*; *hrhoG*/*hrhoG* mice (Chan et al., 2004); Figs. 4 and 5), and to follow the

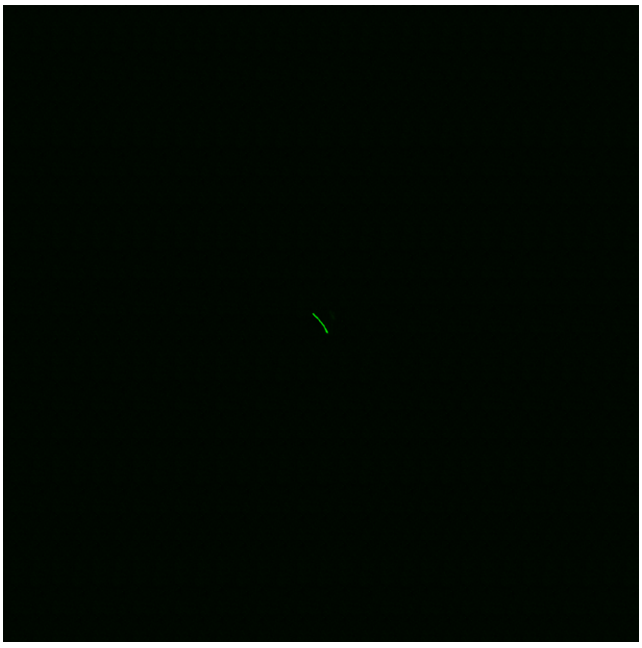

Fig. 6. Signal needed for quantal detection of gene correction. A single rod outer segment in a retinal whole-mount from a mouse chimeric for the +/*hrhoG*(H) genotype was imaged along with the background from the surrounding wildtype photoreceptors. The background was digitally copied and replicated to cover an area equal to 1/35 of a mouse retina (0.5 mm^2); the outer segment is about 24 μm long. Imaging conditions were as in Fig. 2.

success of retinal transplantation in living mice. A recent report (Otteson et al., 2005) suggests that they can be useful for studying regulation of rhodopsin gene expression, and they could also be used to screen for factors (gene products or chemicals) that induce differentiation of rods from precursor cells. The brightness of their fluorescence makes them excellent substrates for initial efforts at high-resolution in vivo fluorescence imaging in mice using two-photon or adaptive optics techniques (see other articles in this issue). Finally, these mice provide highly sensitive fluorescent substrates for experiments designed to knock down expression of rhodopsin alleles, using antisense oligonucleotides, ribozymes (Lewin et al., 1998), siRNAs, or triplex technology (Intody, Perkins, Wilson, & Wensel, 2000; Perkins, Wilson, Wensel, & Vasquez, 1998).

6. Methods

6.1. Fluorescence microscopy

All procedures were performed in accordance with approved animal use protocols. Retinal whole-mount was prepared as previously described (Chan et al., 2004) and imaged using a Zeiss LSM 510 laser scanning confocal microscope. For cryosections, eyes were collected from euthanized mice, fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.3) for 1 h at room temperature with gentle rotation, followed by 1 h in 30% sucrose in phosphate buffered saline, pH 7.3. The eyes were frozen on dry ice in 100% Tissue-Tek O.C.T. compound (Sakura Finetek USA, Torrance, CA) and sliced in 12 μm-thin sections with a Microm HM 500 microtome (Microm Instruments, Heidelberg, Germany). The slices were air dried, washed three times in PBS at room temperature for 30 min each. Images were captured after mounting with Gel/Mount (Biomeda, Foster City, CA) on an Olympus Fluoview 300 confocal scanning system interfaced to an IX-70 microscope.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.visres.2005.07.016.

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