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Tools and resources for analyzing gene expression changes in glaucomatous neurodegeneration

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Abstract

Evaluating gene expression changes presents one of the most powerful interrogative approaches to study the molecular, biochemical, and cellular pathways associated with glaucomatous disease pathology. Technologies to study gene expression profiles in glaucoma are wide ranging. Qualitative techniques provide the power of localizing expression changes to individual cells, but are not robust to evaluate differences in expression changes. Alternatively, quantitative changes provide a high level of stringency to quantify changes in gene expression. Additionally, advances in high throughput analysis and bioinformatics have dramatically improved the number of individual genes that can be evaluated in a single experiment, while dramatically reducing amounts of input tissue/starting material. Together, gene expression profiling and proteomics have yielded new insights on the roles of neuroinflammation, the complement cascade, and metabolic shutdown as important players in the pathology of the optic nerve head and retina in this disease.

Keywords

Glaucoma; Optic nerve head; Retina; Gene expression profiling; Transcriptome; RT-qPCR; Retinal ganglion cells; Microarray; Proteomics

1. Introduction

Understanding the pathological processes involved in glaucoma is paramount to developing therapeutic strategies. Animal models of the disease are critical to this research process, and provide a platform to evaluate the progression of cellular and molecular mechanisms that are affected. Central to this, assessment of what genes are turned on and off provides perhaps the most insightful information on how cells are affected in the disease. To that end, methodology for studying changes in gene expression must provide quantitative information on what genes are being differentially regulated, and qualitative information to identify what cell types are responsible for expressing those genes. Finally, since functional pathways

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usually require interactions of multiple genes, differential gene expression provides important insight into cellular processes that are critically involved.

1.1. The definition of gene expression

For the purposes of this review, it is relevant to define what is meant by “gene expression”. Technically, this phrase refers to a process by which a gene sequence is copied into a coding RNA (such as mRNA), which is then converted into a functional product inside the cell. Conversion can be splicing of a primary transcript of a non-protein coding RNA into a functional siRNA, or translation of an mRNA into a protein, as examples. Methodology to evaluate “gene expression” is generally limited to assessing a single intermediate or product of the gene expression process. These assessments may not necessarily reflect the end result of gene expression. For example, quantitative measurement of an mRNA does not reflect rates of translation, and protein turnover from cellular pools. Nevertheless, quantitative measurements can provide an informative snapshot into the molecular environment of a cell, especially if they reflect a significant change from one condition to another.

2. Cellular anatomy – what cell types are present to express something?

2.1. The optic nerve head and adjacent optic nerve

The initial site of ocular hypertension-induced damage in glaucoma is believed to be the optic nerve head (Nickells et al., 2012). Therefore, a great deal of investigation on gene expression changes has focused on this tissue. The structure of the optic nerve head differs among species, principally in the presence or absence of connective tissue beams providing support for the scleral opening through which axons pass as they exit the eye. Aside from this difference, however, the optic nerve heads of all species share similar cellular components.

Axons from the retinal ganglion cells (RGCs) pass through the optic nerve head in bundles. Surrounding these bundles are supporting glial cells, which are comprised of astrocytes that express glial fibrillary acid protein (GFAP) and lamina cribrosa (LC) cells, which are thought to have astrocyte-like supporting functions, but lack GFAP expression (Hernandez et al., 1988). In rodents, this structure is completely cellular, with columns of glia fitted between the axon bundles. Adjacent to these, capillaries originating from the central retinal artery at the level of the retina, supply blood to the optic nerve head and non-myelinated region of the optic nerve (May and Lütjen-Drecoll, 2002). These vessels contain vascular endothelial cells, surrounded by a sheath containing pericytes. In mammals with larger eyes, the optic nerve head is supported by beams of collagen, which form holes that the axon bundles pass through. Astrocytes line the outside of the beams, while vessels are buried within them. A subset of astrocytes has also been described in the myelin transition zone directly adjacent to the optic nerve head in mice. This region contains astrocytes that have macrophage-like behavior, which phagocytose axonal evulsions (Nguyen et al., 2011), which contain mitochondria (Davis et al., 2014). Further distal to this the axons become myelinated, and the optic nerve is populated by oligodendrocytes.

Dramatic changes in cell behavior and population have been documented in models of experimental glaucoma and in the human condition (Dai et al., 2012; Hernandez, 2000;

Hernandez et al., 1990; Kirwan et al., 2009; Neufeld, 1999; Pena et al., 1999; Tezel et al., 2001), although the most detailed observations have been derived from mouse models. Astrocytes in the glial lamina show thickening processes and a decrease in overall coverage across the nerve head in response to ocular hypertension (Lye-Barthel et al., 2013). The astrocytes in the myelination transition zone upregulate the phagocyte marker Mac-2, but it is not certain that there is an increase in phagocytic activity (Nguyen et al., 2011). Oligodendrocytes, on the other hand, become depleted (Nakazawa et al., 2006). Interestingly, IBA-1(AIF1)-positive microglia begin to cluster in and around the optic nerve head of young DBA/2J mice, reaching peak activation by 3 months of age (Bosco et al., 2011). This is well in advance of an increase in IOP in this model, and it is not certain that this is a function of glaucoma in general, or unique to the DBA/2J model of glaucoma. Certainly, activation of microglial in the optic nerve has been documented in other models (Ebnetter et al., 2010; Taylor et al., 2011). Changes in cells comprising the vasculature in this tissue have not been accurately reported, but evidence of endothelin upregulation by optic nerve head astrocytes and microglia (Grieshaber et al., 2007; Howell et al., 2011; Yorio et al., 2002) in ocular hypertensive models, suggests that molecular changes are surely contributed by the vasculature as well. Finally, as will be discussed below, newer evidence suggests that infiltrating monocytes may also become part of the cellular population of the optic nerve head in glaucoma (Howell et al., 2012), and make their own unique contribution to the gene expression profile of this tissue.

2.2. The retina

The retina is a laminar structure of neurons and supporting glia. The outer nuclear layer is comprised of photoreceptors that exhibit differential sensitivities to photons and wavelengths of light (rods and cones). These cells are supported by retinal pigmented epithelia, which surround the photoreceptor outer segments and receive vascular support from the blood-rich choroid. Photoreceptors synapse with interneurons that comprise the inner nuclear layer. Principally, these connections are made with bipolar neurons, but multiple contacts are also made with horizontal cells and amacrine cells to create a neural network that modulates and processes much of the neurotransmitter activity that originates from photon detection by the photoreceptors.

The ganglion cell layer of the retina is comprised of a mixture of amacrine cells (called displaced amacrine cells) and RGCs. Morphometric studies suggest that the rodent retinal ganglion cell layer contains an equal proportion of each neuronal cell type (Schlamp et al., 2013).

In addition to neuronal populations, the retina contains specialized macroglia, including astrocytes residing in the nerve fiber layer adjacent to the ganglion cell layer, and Müller cells which are centered in the inner nuclear layer, but have processes that span from the nerve fiber and ganglion cell layer to the outer limiting membrane near the outer nuclear layer. Bone marrow derived immune cells also populate the retina, and are mostly classified as CD45+ microglia. These cells are found throughout the retina, principally in both plexiform layers and the ganglion cell and inner nuclear layers.

During retinal pathology due to IOP-induced damage to the optic nerve, subtle and dramatic changes take place with respect to the cell populations in this tissue, and any of these may make significant contributions to changing its gene expression profile. The greatest focus has traditionally been placed on the RGC population, since these cells are selectively lost during the disease process. While other neurons do not typically die off, there is a growing body of literature suggesting that cells of the inner and outer nuclear layer are affected, at least functionally (Hernandez et al., 2009; Kielczewski et al., 2005; Lei et al., 2008; Nork et al., 2000; Pelzel et al., 2006; Agudo-Barriuso et al., 2013; Vidal-Sanz et al., 2012).

Next to RGCs, retinal glia undergo the next most dramatic changes in cellular and molecular behavior, entering a damage-response state known as activation. Similarly, microglia also show changes consistent with activation (Bosco et al., 2011), which is typified from a phenotypic change of a ramified appearance to a more ameboid configuration. It is generally accepted that activated microglia take on a more phagocytic role, but they also alter their gene expression profile to participate in the innate immune response of the damaged retina. In addition, they may also enter a proliferative phase that will reflect an alteration in gene expression.

Neuroinflammatory responses may also be attributed to CD11c-positive dendritic monocytes, which have been reported to infiltrate the retina after acute optic nerve damage (Heuss et al., 2014; Lehmann et al., 2010). It is not clear where these cells enter the eye, but their generally uniform distribution throughout the retina, suggest that they come from vasculature rather than from the optic nerve. Additionally, there is no current evidence that dendritic cells infiltrate retinas in ocular hypertensive models, but they may play a role in optic nerve pathology.

3. Overview of technologies used to assess gene expression

Techniques used to assess gene expression in the retina and optic nerve fall into the broad categories of qualitative and quantitative, with the latter providing the most informative information when used to interpret the critical pathways being turned on or off in response to ocular hypertension. Qualitative methods provide information on localization of gene expression and are critical complements to evaluating data obtained from large gene profiling screens. For the most part, qualitative methods provide minimal information on the levels of changes in gene expression, while quantitative methods provide minimal information on what cells are effecting the change in gene expression.

A critical caveat to many of the studies that have been conducted in the past is that glaucoma researchers have been limited to assessment of gene expression changes in whole tissues, such as retina, where gene expression changes may be inherent in only a few cells at a time, and in populations of cells that are underrepresented in the tissue as a whole. Consequently, interpretation of data, especially with respect to the importance of physiological pathways that are activated, may be suspect. Some of this limitation can be overcome by conducting studies where cells have been dissociated and then selectively sorted prior to interrogation (see for example, (Ivanov et al., 2008)). In general, however, a combination of both

quantitative, and qualitative/localization technologies, should be considered in the process of data interpretation.

3.1. Immunostaining/indirect immunofluorescence

More comprehensive reviews of the technology underlying immunostaining can be found elsewhere (Luongo de Matos et al., 2010; Ramos-Vera, 2005) and will not be discussed in detail here. The advantages of immunostaining in the evaluation of gene expression changes, however, are that it offers a highly selective method to localize where new gene products are being produced. This is enhanced by the ability to stain sections with antibodies from multiple sources (such as rabbit, goat, and mouse, etc) allowing investigators to use selective secondary antibodies with different fluorochromic labels. Thus, the expression of a target gene product can be localized to a specific cell type, or even cellular region, by virtue of co-labeling with a known cell-type- or organelle-specific antigen. The power of this ability to co-localize products of gene expression is especially relevant to studies of a progressive degenerative disease involving a minority cell population such as retinal ganglion cells in experimental glaucoma. When so few cells may actually be executing important pathologic processes at any given instant in time, during the course of the disease, immunostaining offers perhaps the only granular way to evaluate these changes. For example, retinal degeneration generally occurs during a 4-month window in the progression of the DBA/2J form of glaucoma (Libby et al., 2005; Schlamp et al., 2006). Assuming a starting population of about 60,000 RGCs in the DBA/2J retina (Williams et al., 1996), and that a desired gene expression event lasted 3 days in an affected or dying cell, that would equate to a total of ~1500–4500 cells/retina/day that exhibited the change. In a pool of total retina, this change would be imperceptible. Immunostaining can identify these cells, especially if co-localized with a marker for apoptosis, such as γ H2AX (Pelzel et al., 2012) or activated caspase 3 (Harder and Libby, 2011).

Quantification of immunostained tissue is limited by several factors. First, the staining reaction itself is influenced by antigen exposure, buffer factors (ionic strength, pH etc), reaction time, temperature, and antibody binding affinity and specificity. Second, fluorescent intensity is greatly affected by photobleaching and exposure time during image capture. Therefore, a comparison of staining intensity between two different samples is often not accurate. The standard unit of measure for quantifying immunostaining intensity is pixel density, which can be achieved by nearly all image analysis software packages. To overcome all the possible confounding variables that could influence this measurement, it is advantageous to have an internal standard that can be measured in the same field as the region of interest. For example, Pelzel et al. (Pelzel et al., 2010) was able to measure the decrease in histone H4 acetylation in RGCs after optic nerve damage by first measuring the average pixel density (pixels/ μm^2) of indirect fluorescence in nuclei stained with an antibody against acetyl-H4. The density value of the ganglion cell layer cells was corrected by the pixel density of nuclei in the inner nuclear layer, which was expected to remain unchanged during the experiment. Using normalized values, which corrected for variable labeling and photography conditions, data from any one slide/section could be directly compared to others.

3.2. Western blotting and enzyme linked immunosorbent assays (ELISA)

Although indirect immunofluorescent methods can be adapted to quantify changes in protein levels (see Section 3.1), greater quantitative precision can be obtained from immunoblotting (westerns) and ELISAs. In western blots, proteins are electrophoretically separated, usually under denaturing conditions, transferred to a solid membrane and probed with a primary and then secondary antibody for detection. Labeled bands can then be quantified to provide an estimate of change due to different conditions. Variations of the method can also be used to assess protein–protein interactions (cross-linking followed by immunoprecipitation and western blotting), de novo synthesis (incorporation of radioactive precursor amino acids by pulse-chase labeling), and proteolytic processing, to name a few. This is the principal method used to test antibody specificity, since cross-reactivity is obvious in blots of complex protein mixtures. A number of factors influence successful western blotting, most notably how the proteins were originally extracted, the amounts of protein loaded onto gels, and the efficiency of some proteins over others to transfer and bind solid phase support membrane (Taylor and Posch, 2014). Quantification of protein bands is controversial, however (Marcus and Oransky, 2012). Early methods of quantification utilized secondary antibodies linked to enzymes like alkaline phosphatase, which was used in colorimetric assays to stain the position of the band on the membrane. Greater sensitivity of this method was achieved using substrates that generated photons when cleaved (i.e., chemiluminescence using substrates like CDP-Star), but were limited by the narrow linear range of X-ray film. Newer technologies to increase the dynamic range of detection (fluorimaging), or the use of infrared sensitive dye-labeled secondary antibodies (LiCor imaging) have overcome this problem. Additionally, the traditional use of normalizing the protein load to a housekeeping protein (HKP normalization) has come into question, since many reference proteins, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), also exhibit changes in expression under changing conditions (Nickells and Browder, 1988). Experiments using the HKP method should have validated that the reference protein of choice does not change in the dynamic range of the experiment (reflecting protein load required and the experimental condition). There is a growing trend to quantify target proteins using total protein normalization (TPN), which utilizes dyes to stain all the proteins in a gel lane to estimate the amount of total protein present (Ghosh et al., 2014; Gilda and Gomes, 2015; Taylor and Posch, 2014).

In addition to the technical issues with using western blots, there is also a serious and increasing problem with data manipulation of published results. Conservative estimates indicate that a majority of cases of scientific fraud involve western blot data (Taylor and Posch, 2014). Examples of this are gels that are spliced together or cropped to only show the bands of interest. Several journals are now requiring that supplementary material showing entire gel blots be submitted with manuscripts (Editorial-comment, 2004).

ELISAs provide a high-throughput option for measuring protein levels in samples. The basic principal of the assay utilizes antibody recognition of the target protein, but in a well-based format (i.e., microtiter plate) rather than on a transfer membrane. Antigens in the form of complex protein mixtures are adsorbed onto the surface of the wells and probed with antibodies that are conjugated to a detector enzyme, such as alkaline phosphatase.

Quantification of the target protein is made by measuring product generated by the detector enzyme in either colorimetric or fluorescent-based spectra. Variations of ELISAs include using secondary antibodies for detection, much like western blotting. Greater specificity can also be obtained in Sandwich ELISAs in which a specific capture antibody is first adsorbed to the surface, the antigen is captured, and then detected by a second, conjugated antibody, also selective for the target antigen. Major advantages of ELISAs over westerns are that (i) the detection process offers a greater dynamic range so that lower concentrations of target protein can be measured, (ii) actual numbers of molecules of target can be quantified because it is often easier to incorporate a standard curve of antigen in the same 96-well microtiter plate format, and (iii) employing the TPN method of normalization is easier in a solution-assay based protocol. ELISAs are subject to the same drawback as indirect immunofluorescence methods, however, in not being able to separate out specific from non-specific protein-antibody interactions.

3.3. In situ hybridization (ISH)

ISH is a powerful tool to localize RNA molecules in cells, and makes use of the same principals of RNA:DNA/RNA hybridization kinetics that govern hybridization of probes to targets bound to solid substrates. Earlier methods utilized radioactively labeled probes, which were applied to tissue sections and allowed to bind to specific targets. Like any hybridization reaction, non-specific binding of excess probes to low affinity sites required the introduction of low salt/high heat reaction conditions. This often created extensive damage to tissue sections. Hybridization reactions now often utilize a high concentration of formamide, which lowers the T_m for specific complementary binding of two nucleic acid strands, allowing for reaction conditions that require less damaging temperatures. A significant problem with radioactive probes was that the tissue section had to be coated in photographic emulsion in order to detect bound probe. This often obscured pertinent histology of the sections when viewed, but was useful to provide a quantitative metric in the form of the number of silver grains that could be counted.

A dramatic improvement in histologic resolution was made by using non-radioactive labeling methods, such as digoxigenin-labeled rRNA probes (riboprobe), combined with whole mount hybridization and staining procedures (Harland, 1991; Li et al., 1994). For the retina, these probes can be used on 1×1 mm blocks of tissue that have not been previously sectioned and the entire series of reactions can be conducted in a vial rather than on a section affixed to a slide. The digoxigenin is detected by a Fab antibody fragment that is conjugated to alkaline phosphatase. After histochemical staining, retina blocks can be embedded in glycol methacrylate resin and sectioned to yield high-resolution images of the retina, with blue staining revealing the position of the bound riboprobe. Probes can now be made with fluorescently labeled tags, which allow for multiple transcripts to be localized in the same tissue of ISH experiments using sectioned material, a process that was effectively employed to assess RGC-specific gene expression changes in DBA/2J glaucoma (Soto et al., 2008). A draw-back of the use of non-radioactive methods is that they do not yield reliable quantitative data unless there is an all-or-none type of effect. Nevertheless, in situ hybridization was instrumental in studies showing that RGCs exhibit silencing of cell-type specific mRNAs as an early pathologic response in experimental glaucoma (Schlamp et al.,

2001; Soto et al., 2008), and was an effective tool to demonstrate depletion of red/green cone opsin transcripts in both non-human primate experimental ocular hypertension and human glaucoma (Pelzel et al., 2006).

3.4. Quantitative reverse transcriptase polymerase chain reaction (RT-qPCR)

Prior to the advent of PCR technologies, mRNA abundance was measured by Northern blotting, ribonuclease protection assays, and RNA probe excess titration (Davidson, 1986). All of these methods have yielded important information in the study of gene expression changes, but have been limited by having relatively low sensitivity (especially for Northern blotting) and requiring large amounts of input sample. The discovery of Taq polymerase and its application to cycling amplification (Mullis and Faloona, 1987; Saiki et al., 1988) dramatically overcame the need for a lot of input sample. End-stage Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) was not a reasonable technology for quantification, however, because of the plateau effect that was achieved in a typical reaction, regardless of the amount of input target template (Morrison and Gannon, 1994). To overcome this, “real-time” technology was developed, which allows direct measurement of the concentration of newly synthesized DNA at the completion of every extension cycle. Quantification is assessed from a measurement of the cycle at which any given target DNA crosses a common threshold of concentration (termed the C_t value).

Any experiment utilizing RT-qPCR should adhere to the “Minimal Information required for Quantitative PCR Experiments” (MIQE) guidelines (Bustin, 2010; Bustin et al., 2009, 2010), to ensure accurate and informative data collection. These guidelines govern the quality of the starting total RNA, synthesis of first strand cDNA to minimize genomic DNA contamination, the design of primers and amplicon sizes to yield products that are synthesized with similar efficiencies, and the validation of amplicons by sequence analysis to ensure they are actually the products of the desired target sequence. Additionally, the selection of appropriate reference genes is paramount since all data obtained is typically normalized by these internal standards (Bustin et al., 2010; Edmunds et al., 2014; Jacob et al., 2013). Reference genes should exhibit stability across the conditions of the experiment.

Generally, data obtained from RT-qPCR reactions can be analyzed using absolute quantification or relative quantification. Absolute quantification requires that a standard curve of decreasing template input of the target sequence be run at the same time as the unknown samples. Sometimes this can be overcome when multiple targets with similar amplification efficiencies are run simultaneously. Fig. 1 shows a graph of the average standard curve generated from 15 different cDNAs that are commonly assayed in studies examining for gene expression changes in mouse RGCs (see Table 1). Calculation of each standard curve is shown in Table 2. The similarity of curves for all these molecules enables reasonable quantitative estimation for all these gene products using a single representative standard curve. This effectively allows multiple targets to be analyzed from one or two samples on a single plate.

An important variable that is obtained from a standard curve analysis of any product is its efficiency (E) of amplification. This variable is an important component of the second

method of analysis known as relative quantification (RQ). Efficiency is calculated from Equation 1:

$$E=10^{\left(-\frac{1}{\text{slope}}\right)} \quad (1)$$

Where the slope is the value generated from a graph of C_t vs $\log_{10}(\text{template input})$ such as shown in Fig. 1. An amplimer with ideal efficiency should double in amount with every cycle, yielding a value of $E = 2$.

Once known, RQ evaluation of qPCR results can be made using the C_t method with the Pfaffl correction (Pfaffl, 2001). This method compares the difference in C_t values obtained for a target cDNA between two conditions (i.e., control and experimental) and adjusts that value using a reference gene that has been amplified from the same two samples. The calculation for RQ is depicted in Equation 2:

$$RQ=E_{\text{target}}^{(C_{tX}-C_{tY})} \div E_{\text{reference}}^{(C_{tX}-C_{tY})} \quad (2)$$

Where C_{tX} is the cycle threshold value of each sample for condition 1, and C_{tY} is the cycle threshold value of each sample in condition 2.

Inherent issues with variability are other important factors to consider when applying any PCR based technology to evaluate changes in quantitative gene expression. Technical factors that yield variability include comparing data sets collected by different users or the attempt to quantify very low abundant transcripts (Bustin, 2002). The level of technical variation for qPCR experiments using the primers described in Table 1 was determined empirically in separate experiments in which the same batch of cDNA was run on different days, or two different cDNA batches were made from the same total RNA sample and run independently. Data from these experiments were used to generate MA plots (Log Ratios versus Mean Values) to assess variability (Fig. 2). In each circumstance, the level of variability between the samples was at or below a level equivalent to 1 cycle of amplification (the exception to this were low abundant transcripts, which exhibited variation of 1.5 cycles in approximately 50% of the runs examined). This level of technical variation is also widely taken into consideration in assignments of “fold-change” thresholds applied to analysis of differential gene expression in microarray studies (McCarthy and Smyth, 2009) (see below). Probably more of a concern is biological variability. Animal–animal variability (a subclassification of biological variability) is typically greater than technical variability (Chen et al., 2004; King et al., 2005; Novak et al., 2002). Fig. 3 shows MA plots of RT-qPCR runs comparing the expression of RGC target genes in 3 different mouse retinas obtained from age and sex-matched independent mice of the same strain (male C57BL/6J). In these experiments, the level of variation was considered acceptable when the same target from different samples did not exceed the level of technical variation (dotted lines). Mouse retinas, in this experiment, demonstrate a wide range of variation that cannot be attributed to technical variation. These data are demonstrative of an important consideration when designing any study to quantify differential gene expression. Investigators should first

determine the biological variation inherent in their target tissues in order to develop an experiment that is adequately powered to detect significant changes in their datasets.

3.5. Microarray platforms

High throughput gene expression profiling using microarrays is now extensively used to investigate gene expression changes in glaucomatous tissue (Jakobs, 2014; Yang and Zack, 2011). Micro-arrays represent an advance over filter based hybridization reactions, such as “dot blots” where cold cDNA fragments were spotted onto a membrane and probed with labeled cDNA made from a complex RNA mixture. Microprinting of cDNAs onto glass surfaces was able to increase the numbers of genes examined in the same experiment by an order of magnitude (from several hundred to several thousand). Printed or spotted cDNA arrays are typically probed with labeled cDNA from both the control and test conditions, with each cDNA carrying a different fluorescent dye (i.e., Cy3 and Cy5). Differential expression is detected by the ratio of one dye to the other at individual spots.

A further advance over the printed array is the gene chip. This is comprised of a solid silica surface onto which specific oligonucleotides (generally 25 nucleotides in length) are covalently linked. Because the array is very small, more genes can be interrogated on a single gene chip, with typically two chips needed to cover the entire complement of the genes and expressed sequence tags in the mammalian genome. Affymetrix gene chips, which have most often been used in glaucoma studies (Table 3), utilize a probe-pairing strategy. Each gene, on a single chip, is represented by 16–20 different perfect matching (PM) oligonucleotides and 16 matching oligonucleotides with a single miss-matched (MM) nucleotide in its sequence. Non-specific binding of labeled cDNA material is predicted to be similar for each PM and MM pair of oligos, therefore the actual specific binding is determined by the difference in label between the two. The binding intensity for any given gene product is calculated as the mean of the difference between all 16–20 oligo pairs (PM-MM) for that particular target, which is then normalized to up to 100 reference genes in the array. Normalization of hybridization signal to probe sets is still an ongoing and improving process. The intensity of hybridization signal across probe sets for a single transcript can vary by two orders of magnitude, and simple averaging may not provide accurate normalization for a substantial number of genes being analyzed. Consequently, more sophisticated algorithms are being applied to take into consideration the probe hybridization characteristics and intensity levels across multiple chips (Bolstad et al., 2003; Irizarry et al., 2003). Currently, “Robust Multiarray Average” (RMA) is used to normalize Affymetrix data. This employs quantile normalization, making it useful in comparisons of multiple batches of arrays, provided they were done on the same chip set and the data is available in raw form (see below).

Early gene chips could accommodate up to 400,000 different oligonucleotides, therefore the pair-wise strategy used by Affymetrix limited the interrogation to 12,000 targets per chip and two or more chips were required per sample to ensure adequate coverage of the genome. Improvements in chip builds has allowed for an increase in the number of targets that can be interrogated. New whole transcriptome chips, for example, contain up to 10 primer pairs per exon, and include primers that can detect alternative splice variants for many genes. A

limitation of the oligo-based gene chip is that only a single sample of labeled cDNA can be hybridized to the array, so comparison of different samples requires duplicate runs on separate chips. Alternatively, this overcomes some bias that has been reported in preparing samples by incorporating different fluorescent labels.

Determining significant differences in microarray experiments has also been a challenging and complex problem in the area of bioinformatics. Early studies used simple t-test derived evaluations of normalized gene expression datasets. These tests reflect significance relative changes from zero (i.e., the difference between the control and experimental level of a transcript), but may not reflect actual biological significance for the change (McCarthy and Smyth, 2009). More commonly, significant changes in microarrays are required to satisfy a level of significance using conventional statistical methods combined with a threshold cut-off for an acceptable fold-change. Generally the threshold is set at 1.5-fold, 2.0-fold, or higher. Thresholds lower than 1.5 have proven unreliable at reducing background noise, and are especially meaningless for transcripts at low copy number. Stringent criteria typically use a P value of 0.02 and a fold-change of 2.0 as cut-offs (Dalman et al., 2012). Care should be taken in selecting the stringency of the criteria, however, since small changes can dramatically change the overall interpretation of differentially expressed genes.

In 2001, the Minimal Information About a Microarray Experiment (MIAME) guidelines were established to help create data-sets that conformed to a set of standards for presenting and exchanging data (Brazma et al., 2001). Of principal importance was the recommendation that data-sets should be deposited in a central data-base in a common format. Currently, most studies describing microarray data are deposited in the NIH/NLM Gene Expression Omnibus (GEO) in both raw data formats (.cel files) or after normalization of the data.

Microarrays create large amounts of quantitative information, which has created a separate need for powerful bioinformatics approaches to be able to analyze it all. To date, the most useful tools have taken gene products that show quantitative differences between samples and determined functional pathways that appear to be principally affected in a disease like glaucoma. Bioinformatics resources like the Database for Annotation, Visualization, and Integrated Discovery (DAVID) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) are often employed for these types of analyses. Genes that are differentially expressed are stratified on the basis of descriptions attributed to them regarding function, pathways, etc (Gene Ontology, or GO, terms). A second powerful method of analysis is gene clustering, where clusters of probes sets, which exhibit similar patterns of change, are ordered together. Molecular clustering was effectively employed by Howell and colleagues (Howell et al., 2011), in a longitudinal analysis of disease progression in the optic nerve head and retina of DBA/2J glaucoma. Initially, samples were stratified from diseased mice using conventional anatomical criteria. After molecular clustering, this group found distinct gene expression profiles that stratified samples further, showing disease-related changes in gene expression that preceded obvious cellular pathology. Additionally, this group has created an interactive data-base to allow users to view changes in individual gene products during disease progression in both the retina and optic nerve head (<http://glaucomadb.jax.org/glaucoma>).

3.6. RNA-seq

An alternative to microarray analysis, utilizes “next-generation” DNA sequencing technology to directly sequence cDNA fragments generated from RNA isolated from target tissues/cells. RNA-seq has important advantages over microarray studies in that it has greater power to identify quantitative changes in specific allelic variants, and to identify changes in the expression of alternatively spliced RNAs (Wang et al., 2009). Because there is no requirement for a hybridization reaction, there is also no upper or lower limit to the number of molecules that can be measured. Therefore, the dynamic range of RNA-seq is substantially higher than a typical microarray.

Initially evaluating RNA-seq data sets was limited by the ability to align sequence data to reference genomic sequence data to identify differentially expressed genes (Rehrauer et al., 2013). This was particularly problematic for transcripts containing repetitive sequence elements or short reads of highly abundant targets. As sequence information has become more comprehensive, alignment algorithms have dramatically improved. A second drawback to the technology is the overall cost, especially in samples from complex genomes that require “deep sequence” information to get adequate coverage of the transcriptome. Estimates of how many independent reads are necessary to get complete coverage vary, but the number of unique start sites in mouse embryos begins to plateau at depths of 80 million reads (Wang et al., 2009). In yeast, a depth of 4 million reads is sufficient to cover 80% of the genome. In an analysis of data from 127 RNA-seq experiments, Hart and colleagues found that a depth of 10 million reads provided 90% coverage of both human and zebrafish genomes, with a minimum of 10 reads per gene (Hart et al., 2013). Cost for a single experiment, then, is a function of the depth of sequence analysis an investigator is willing to go.

As with microarrays, defining differentially expressed genes from RNA-seq data is challenging (Rehrauer et al., 2013). Simple count-based methods, which can be adequate for some isoforms, take into account proportional changes in gene expression between samples, and not the level of expression. These types of analysis are being replaced with more powerful methods that take advantage of empirical Bayesian updating to define differentially expressed genes (EBSeq: (Leng et al., 2013)).

To date, RNA-seq has not been employed to evaluate the transcriptome of the retina or optic nerve in models of glaucoma, but a recent study of retinal gene expression changes in an acute optic nerve crush model has been reported (Yasuda et al., 2014). Two days after optic nerve injury in mice, this study reported significant up-regulation of genes involved in endoplasmic reticulum (ER) stress, antioxidative response, and the complement cascade. Genes normally expressed in RGCs were significantly down-regulated.

3.7. Transgenic mouse models

Transgenic mouse models have been used on a limited basis to study glaucomatous changes in gene expression. The most useful lines carry reporter transgenes that are controlled by promoters that are cell-type specific. The expression of the reporter gene then provides a direct, often visual, assessment of activity of that promoter sequence. The three lines most

prominently used in glaucoma studies are B6.Cg-TgN(*Thy1-CFP*)23Jrs/J, *Fem1c*^{Rosa3}, and *hGFAPpr-GFP*. All three reporter transgenes have been congenically bred onto the DBA/2J inbred background to allow for gene specific reporter gene expression studies during development of glaucoma that is specific to the DBA/2J mouse (Lye-Barthel et al., 2013; Raymond et al., 2008; Schlamp et al., 2006; Tsuruga et al., 2012). A variant of the *Thy1-CFP* mouse, in which YFP has been inserted after the *Thy1* promoter, has also been studied in this model (Williams et al., 2013).

Studies using *Thy1* driven reporters have principally been utilized to evaluate changes in RGC numbers and structure during the progression of glaucoma, and have not been used as a resource to study changes in RGC-specific gene expression changes. This is in part due to the phenomenon that *Thy1* gene expression is silenced prior to cell loss in models of experimental glaucoma (Huang et al., 2006; Schlamp et al., 2001). Alternatively, *Fem1c*^{Rosa3} mice, which carry the β GEO reporter coding region, inserted into the first intron of the mouse *Fem1c* gene (Friedrich and Soriano, 1991; Schlamp et al., 2004) have been used to evaluate RGC gene expression changes as a function of β -galactosidase enzyme activity. RGC expression changes in the DBA/2J glaucoma model have been visualized both histochemically and in solution assays (Pelzel et al., 2012; Schlamp et al., 2006) (Fig. 4).

Transgenic mice expressing GFP under control of the human *GFAP* promoter (*hGFAPpr-GFP*) exhibit dramatic labeling of retinal and optic nerve astrocytes. In glaucoma studies, these mice have been used to elegantly document astrocytic changes in the glial lamina of the DBA/2J mouse model (Lye-Barthel et al., 2013; Sun et al., 2009, 2010). Even though GFAP expression increases in the retina in glaucomatous eyes from multiple species (Lam et al., 2003; Rojas et al., 2014; Savagian et al., 2008; Tanihara et al., 1997; Wang et al., 2000), mice expressing GFP exclusively in macroglia have not been utilized for quantitative gene expression studies.

3.8. Proteomics

In addition to advances in large-scale evaluation of mRNA levels, new advances in high-throughput evaluation of proteins have also been applied to the study of gene expression changes in glaucoma (Tezel, 2014). Collectively, these technologies fall under the category of proteomics research. Early studies were gel-based, and involved separation of complex protein mixtures by 2-D gel electrophoresis followed by digestion of individual protein spots and peptide analysis by mass spectrometer based technology (typically MALDI-TOF). Like early methods to quantify mRNA levels, this early proteomics approach required a large amount of input material. Subsequent studies have shifted to gel-free approaches, in which the complex protein mixtures are enzymatically digested and the peptides are first separated by liquid chromatography and then analyzed by mass spectrometry. The advent of gel-free based approaches has reduced the required amount of input material dramatically (Tezel, 2014).

Analysis of quantitative changes in proteins between control and glaucomatous material has revealed important signaling networks that focus on molecules like TGF β , TNF α , and p53 in purified LC cells from human glaucoma samples (Rogers et al., 2012). Analysis of retinal tissues from human cadaver eyes, as well as from isolated astrocytes from rat retinas with

experimental glaucoma, also reveal important up-regulation of TNF α signaling molecules, and proteins associated with mitochondrial and endoplasmic reticulum (ER) stress, and apoptotic signaling (Tezel et al., 2012; Yang et al., 2011). A similar study comparing retinas from eyes of non-human primates with normal, low, and high IOP yielded a different spectrum of protein changes, mostly centered around cytoskeletal elements and glycolytic energy metabolism (Stowell et al., 2011). Proteomic interrogation of purified RGCs isolated from rats with experimental glaucoma also showed increased levels of proteins signaling mitochondrial dysfunction, ER stress, and energy metabolism, as well as an increase in ubiquitin, suggesting increased involvement of debris clearing pathways in damaged cells (Crabb et al., 2010). Interestingly, two independent studies showed that hemoglobin levels were modulated in glaucomatous retinas (Stowell et al., 2011; Tezel et al., 2010), suggesting important changes in the regulation of oxygenation as part of the disease process. Finally, proteomics studies have added further elucidation to the potential neuroinflammatory changes associated with glaucomatous damage.

In addition to quantitative changes in protein levels, proteomic technologies can be adapted to evaluate changes in targeted modification of proteins (functional proteomics). This approach has documented increases in protein citrullination and decreased arginyl methylation (Bhattacharya et al., 2006) of proteins in the glaucomatous optic nerve head, consistent with other studies showing upregulation of neuroinflammatory proteins. In the retina, phosphorylation-specific changes have been documented in RGCs isolated from glaucomatous rats, principally involving activation networks of the *Bcl2* gene family of apoptotic proteins (Yang et al., 2008).

4. Important pathways identified by microarray gene expression studies

Table 3 shows a summary of microarray-based gene expression profiling using glaucomatous tissues. Although each study has reported changes in gene expression for multiple different gene networks and functional pathways, the table illustrates pathways that have repeatedly been identified in different models of glaucoma.

One of the most often reported functional pathway that exhibits differential gene expression in glaucoma is neuroinflammation, particularly the complement cascade. This functional pathway change is evident in both the optic nerve head and retina, and represents one of the earliest changes detected in both tissues, even before evidence of cellular pathology (Howell et al., 2011). In the optic nerve head, neuroinflammatory responses have been associated with infiltration of monocytic cells in DBA/2J glaucoma (Howell et al., 2012), and may also be a function of metabolic changes in the resident microglial population, which have also been reported very early in the disease process of this model (Bosco et al., 2011). The contribution of complement in retinal pathology may be associated with synaptic remodeling associated with RGC dendritic arbor retraction from connections with amacrine cells, since this remodeling process is regulated by the complement cascade (Perry and O'Connor, 2008). Clearly, however, there is evidence that inflammatory responses have additional involvement in glaucomatous retinal pathology. The functional pathway centered around the inflammatory cytokine TNF α has been identified in quantitative studies assessing both mRNA (Table 3) and protein changes (Yang et al., 2011) in different glaucoma models.

These data are corroborated by studies using rodent models of experimental glaucoma, in which genetic deletion of the R2 receptor for TNF α , or therapeutic application of the receptor trap Etanercept, are able to ameliorate the severity and progression of the disease (Nakazawa et al., 2006; Roh et al., 2012).

In addition to neuroinflammation, several groups have reported an increase in cell proliferation in the optic nerve head, which may reflect an increase in astrocyte and possible macrophage-like cell number in this tissue as the disease progresses. Concomitant with this, changes in TGF β signaling pathways and extracellular matrix remodeling have been detected. In the retina, in addition to neuroinflammatory pathways, changes in RGC-specific gene expression are commonly reported. This latter phenomenon illustrates separate categories of changes in RGCs, including a decrease in normal expression with an increase in stress-response related gene expression. Finally, RGCs appear to exhibit an increase gene expression associated with cellular disruption, loss of metabolic processes, and an increase in signaling for apoptosis.

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Fig. 1.

Combined standard curve analysis of 15 target genes commonly used to examine gene expression changes in mouse RGCs by RT-qPCR. Standard curves were generated from cloned target cDNA amplicons of 15 genes that are commonly interrogated in RGCs (see Tables 1 and 2). The data from all 15 targets was plotted (mean $\pm$ SD), yielding a best-fit straight line of $y = -3.46x + 37.8$. The two cDNA targets with the most similar individual best line fits were *Tubb3* and *Gapdh*.

Fig. 2.

MA plots of the technical variability inherent in RT-qPCR reactions. Each MA plot (Log Ratios versus Mean Values) was calculated using quantitative data from (A) two separate RCR runs on the same batch of cDNA, run on different days or (B) two separate batches of cDNA prepared from the same total RNA. In both sets of control experiments, the majority of comparable target genes fall within the ± 1.0 level for $\log_2(X/Y)$ value, which effectively reflects a single cycle of PCR amplification (dotted lines). The exceptions to this are low abundant targets, such as *Bim* mRNA, which sometimes approach a level of 1.5.

Fig. 3.

MA plots of the variability between different mouse samples of retinal cDNA. Each MA plot (Log Ratios versus Mean Values) was calculated using quantitative data from two individual mouse retinas as the two variables. Data was collected by a single user. Three different eyes (from 3 different age- and sex-matched mice) were compared against each other (A–C), and then plotted as a grand mean of the data (D). There was a high degree of variability between different mouse eyes, with 9/13 targets falling outside of a 1 cycle level of variation (dotted line).

Fig. 4. *Fem1c^{Rosa3}* reporter gene expression in DBA/2J mouse glaucoma. (A–C) Retinal whole mounts of *Fem1c^{Rosa3}* DBA/2J mice processed at the ages indicated. The retinas were stained with X-Gal to reveal β-galactosidase activity of the βGEO reporter gene. Young mice exhibit a gradient of staining across the retina that corresponds to the distribution of RGCs in the mouse retina (Dräger and Olsen, 1981). Older mice exhibit reduced staining, which often presents in sectors (arrows in B). (D) βGEO activity can be quantified using either a solution assay, or by measuring pixel density using filters specific to the blue range of X-Gal precipitate formed (J. A. Ver Hoeve and R. W. Nickells, unpublished data). The graph shows pixel density corrected to retinal area for young and old DBA/2J mice (minimum of 10 retinas per group – mean and standard error shown).

Table 1

Primers used for mouse RGC transcript amplification in RT-qPCR experiments. Primers are designed to cross at least one intron-exon boundary, have a CG content of 60%, and generally have a 3' G or C at the end. For RT-qPCR, amplimers in the range of 200–300 bp yield similar amplification efficiencies.

Gene name		Primer sequence (5' → 3')	Exon	Size (bp)
<i>BclX</i>	Fwd	TTG GAC AAT GGA CTG GTT GA	2	780
	Rev	GTA GAG TGG ATG GTC AGT G	3	
<i>Bim</i>	Fwd	TCT GAG TGT GAC AGA GAA GG	2	378
	Rev	CTC CTG AGA CTG TCG TAT GG	3	
<i>Brn3b</i>	Fwd	TCT TCC AAC CCC ACC GAG C	1/2	157
	Rev	GTG GTA AGT GGC GTC CGG CTT G	2	
<i>Fem1c</i>	Fwd	GAA GTG TCC AAC CGC CAT GG	2	292
	Rev	TTG TCT GGG CAT GGT GCG	3	
<i>Gad67</i>	Fwd	TCT TCC ACT CCT TCG CCT GC	1	279
	Rev	GGA GAA GTC GGT CTC TGT GC	3	
<i>Gap43</i>	Fwd	TGA GCA AGC GAG CAG AAA	1	199
	Rev	GCA GCC TTA TGA GCC TTA	2	
<i>Gapdh</i>	Fwd	GGC CGG TGC TGA GTA TGT CG	2	291
	Rev	TTC TGG GTG GCA GTG ATG GC	4	
<i>Gfap</i>	Fwd	CAA ACT GGC TGA TGT CTA CC	1	269
	Rev	AGA ACT GGA TCT CCT CCT CC	3	
<i>Hsp27</i>	Fwd	CGC AAC AGC AGT CAT GTC GG	1	257
	Rev	GGC TCA CAT CCA GAA ACG CC	2	
<i>Nefl</i>	Fwd	AGC ACG AAG AGC GAG ATG GC	2	173
	Rev	TGC GAG CTC TGA GAG TAG CC	3	
<i>Nrn1</i>	Fwd	TTC ACT GAT CCT CGC GGT GC	1	238
	Rev	TAC TTT CGC CCC TTC CTG GC	3	
<i>Sncg</i>	Fwd	GAC CAA GCA GGG AGT AAC GG	1	240
	Rev	TCC AAG TCC TCC TTG CGC AC	3/4	
<i>Thy1</i>	Fwd	CTT GCA GGT GTC CCG AGG GC	3	379
	Rev	CTG AAC CAG CAG GCT TAT GC	4	
<i>TrkB</i>	Fwd	GTC TGA CCT GAT CCT GAC GG	5/6	280
	Rev	CCC AAC GTC CCA GTA CAA GG	7	
<i>Tubb3</i>	Fwd	GTT CTG GGA GGT CAT CAG CG	2	207
	Rev	TCG GGC CTG AAT AGG TGT CC	3	
<i>Sl6</i>	Fwd	CAC TGC AAA CGG GGA AAT GG	2	198
	Rev	TGA GAT GGA CTG TCG GAT GG	4	

Table 2

Calculation of amplification efficiency for mouse transcripts expressed in RGCs (see Table 1 for primer design). Efficiency is determined from the slope of a standard curve generated from increasing template copies for each target cDNA (Fig. 1). Efficiency values greater than 2 generally reflect non-specific priming or primer-dimer formation in the reaction. Efficiency values under 2 reflect less than optimal efficiency of the amplimer, which can often be a function of larger amplimer size.

Gene name	Line equation	R ² value	Efficiency
<i>BclX</i>	$y = -4.59x + 44.13$	0.73	1.65
<i>Bim</i>	$y = -3.24x + 38.33$	0.99	2.04
<i>Brn3b</i>	$y = -3.75x + 39.29$	0.98	1.84
<i>Fem1c</i>	$y = -3.73x + 39.94$	0.99	1.85
<i>Gap43</i>	$y = -2.74x + 42.68$	0.90	2.26
<i>Gapdh</i>	$y = -3.50x + 38.16$	1.00	1.93
<i>Gfap</i>	$y = -3.40x + 38.34$	0.98	1.97
<i>Hsp27</i>	$y = -3.31x + 36.49$	0.99	2.01
<i>Nefl</i>	$y = -2.78x + 33.37$	0.97	2.29
<i>Nrn1</i>	$y = -3.08x + 33.86$	0.97	2.11
<i>Sncg</i>	$y = -3.15x + 34.56$	0.99	2.08
<i>Thy1</i>	$y = -3.78x + 41.54$	0.99	1.84
<i>TrkB</i>	$y = -3.63x + 38.89$	0.99	1.89
<i>Tubb3</i>	$y = -3.52x + 37.00$	1.00	1.92
<i>SI6</i>	$y = -3.15x + 34.13$	0.99	2.08
<i>All genes</i>	$y = -3.46x + 37.8$	0.99	1.95

Table 3

Summary of microarray experiments involving models of experimental and human glaucoma.

Reference	Model (tissue)	Array platform	Major functional cluster (Increase) ^a	Major functional cluster (Decrease) ^a
(Hernandez et al., 2002)	Human Glaucoma (ONH astrocyte cultures)	U95A and U95Av2 human array (Affymetrix)	Signal transduction/proliferation/cell adhesion/metabolic enzymes	Cell–cell interactions/cell migration
(Miyahara et al., 2003)	NHP EG (retina)	UniGEM Human V 2.0 (Incyte Genomics)	Cytoskeleton, ECM remodeling, neuroinflammation (mild)/neuroinflammation (severe)	Cytoskeleton (severe)
(Ahmed et al., 2004)	Rat EG (retina)	U34A rat array (Affymetrix)	Neuroinflammation and apoptosis (early and late)/Glial activation (late)	Cytoskeleton
(Naskar and Thanos, 2006)	Rat CG (retina)	Rat 10k spotted array (MWG Biotech)	Neuroinflammation/cell surface signaling/angiogenesis/ECM	Cytoskeleton/cell adhesion/G-protein signaling
(Yang et al., 2007)	Rat EG (retina)	Rat genome 230 2.0 (Affymetrix)	Neuroinflammation (complement cascade)/cell death	Connective tissue disorders
(Johnson et al., 2007)	Rat EG (Severe – ONH)	Mouse cDNA spotted array SMCmou660A SMCmou840A (OHSU)	Cell proliferation/neuroinflammation (complement cascade)/Lysosome	Lipid metabolism/cytoskeleton
(Wang et al., 2010)	Rat EG (RGCs from LCM)	Rat genome 230 2.0 (Affymetrix)	Complement cascade/cell death (Fas pathway/caspases)	Metabolism (citrate cycle)/G-protein signaling
(Panagis et al., 2010)	DBA/2 CG ^b (intraretina)	Mouse genome 430 2.0 (Affymetrix)	Cellular assembly/growth/proliferation/complement cascade	Phototransduction
(Panagis et al., 2011)	DBA/2J CG (retina)	Mouse genome 430 2.0 (Affymetrix)	Complement cascade	RGC specific genes/complement cascade
(Johnson et al., 2011)	Rat EG (Mild – ONH)	Mouse cDNA spotted array SMCmou660A SMCmou840A (OHSU)	Cell proliferation/cytoskeleton/neuroinflammation	Lipid metabolism/energy metabolism
(Guo et al., 2011)	Rat EG (RGCs from LCM)	Mouse cDNA spotted array SMCmou660A SMCmou840A (OHSU)	Protein metabolism/stress response	Energy metabolism/protein synthesis
(Howell et al., 2011)	DBA/2J CG (ONH)	Mouse genome 430 2.0 (Affymetrix)	Neuroinflammation/cell proliferation/endothelin system/ECM	None specified
(Howell et al., 2011)	DBA/2J CG (retina)	Mouse genome 430 2.0 (Affymetrix)	Complement cascade	RGC specific genes/

Abbreviations: CG, congenital glaucoma; ECM, extracellular matrix; EG, experimental glaucoma; LCM, laser capture micro-dissection; NHP, non-human primates; ONH, optic nerve head; OHSU, Oregon Health Sciences University; RGCs, retinal ganglion cells.

^a Note that results given for functional pathways is only a partial list and is restricted to the major reported finding, and/or pathways that commonly recur in different studies.

^b This study compared gene expression changes within different regions of the same mouse retina.