Molecular Vision 2003; 9:482-96 <http://www.molvis.org/molvis/v9/a61>
Received 29 August 2002 | Accepted 30 September 2003 | Published 6 October 2003

Technical Brief

Comparing the use of Affymetrix to spotted oligonucleotide microarrays using two retinal pigment epithelium cell lines

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Purpose: The present study was designed to compare the results obtained from two different microarray platforms: spotted cDNAs using a two-color system (Clontech, Atlas Glass Human 3.8) and the Affymetrix platform. We evaluated the internal consistency within each of the platforms, and compared the results across the two platforms.
Methods: RNA was isolated from two retinal pigment epithelial (RPE) cell lines, D407 cells and ARPE19 cells. Each microarray system requires a specific RNA isolation and target preparation procedure. To compare the results between the two platforms, the intensity values for each platform were standardized and scaled. This allowed for a direct comparison of the data between two very different microarray platforms. Real-time RT-PCR was used as an independent conformation of expression levels for selected transcripts. The protein levels for some of these genes were determined using a quantitative immunoblot method.
Results: First, we compared the transcriptome of the D407 cell line to itself. Within each of the platforms there was a high degree of consistency. However, when the data from the Atlas Glass Human 3.8 microarray platform was compared to that of the Affymetrix platform there was a dramatic lack of agreement. The second step was to compare the mRNA profile of the ARPE19 cell line to the D407 cell line. Again there was good agreement within each platform. When the results of the Atlas Glass Human 3.8 platform were compared to the Affymetrix platform, there was a surprising lack of agreement between the two data sets. Real-time RT-PCR was used as independent means of defining RNA levels in the two cell lines. In general, the real-time RT-PCR results were in better agreement with the Affymetrix platform (85%) than the Atlas Glass platform (33%). In addition, we also examined the levels of 11 proteins in these two cell lines using a quantitative immunoblot method. The results from this protein analysis had a higher degree of concordance with the results from Affymetrix platform.
Conclusions: In both the Atlas Glass Human 3.8 system and the Affymetrix platform, there is a high degree of internal consistency. However, comparisons between the two platforms show a lack of agreement. In general, the real-time RT-PCR confirmed the results on the Affymetrix system more often than those from Atlas Glass arrays. However, in both cases, conformation by an independent method proves to be of considerable value.

The emerging technology of DNA microarrays is revolutionizing our approach to science by combining the power of genomics with the experimental questions asked by basic and clinical scientists. Microarray technology allows for the monitoring of thousands of genes, measuring the relative abundance of mRNA transcripts. There are a number of different microarray platforms available. These include cDNAs [1] or oligonucleotides [2] that are spotted on nylon membranes [3] or glass slides [4]. There are in-house and commercially manufactured microarrays. All of these platforms work on a similar principle with a probe immobilized to a surface and a target made from RNA isolated from cells or tissues. All of the microarray platforms can be used to analyze pattern of gene expression. However, fundamental differences exist between the methods. Some of these differences include: methods used to attach the probes to the surface; the length of the probes; whether the spotted material is a cDNA or an oligonucleotide; the different methods used to isolate RNA; the synthesis and the labeling of the targets.

In the present study, we chose to examine and compare two microarray platforms using two different RPE cell lines, D407 [5] and ARPE19 [6]. The Atlas Glass Human 3.8 microarray platform is a spotted microarray with each probe consisting of a single “long oligo” (an 80mer) spotted on a glass slide. The targets were produced for the Atlas Glass Human 3.8 platform by labeling cDNA from each cell line with either Cy3 or Cy5. After the targets were hybridized to the slide, the relative intensity of each fluorescence signal was determined using a two-color laser scanner. The data was transformed to minimize non-linearities [7]. The second platform that we used was the Affymetrix (Affymetrix, Inc., Santa Clara, CA) system. Each gene is represented by a set of short sequences (typically 11-20 individual spots in a single probe set with each oligonucleotide being a 25mer). Individual chips

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are hybridized with the cRNA from only one cell line and then the comparison of RNA profiles from each cell line is made post hybridization using the Affymetrix analysis system (MAS 5.0). The present study examines both of these microarray methods to determine their internal consistency. Then we make a direct comparison between systems.

METHODS

Cell cultures: Two human RPE cell lines were used throughout the study. The D407 was a gift from Malinda Fitzgerald, and the ARPE19 was purchased from American Type Culture Collection (ATCC). Cells were plated at low density and grown in Basal Medium Eagle (GIBCO, Carlsbad, CA). The medium also contained 2 mM L-glutamine solution (Gibco BRL), 5 g glucose/L (Sigma) and 10% fetal bovine serum (Hycolone). Cells were maintained at 37 °C in 5% CO_2. The cells were plated in T75 or T150 flasks, and allowed to grow until the cultures were confluent. For microarray, real-time RT-PCR and immunoblot analysis cells were grown independently for each experiment.

Target preparation and hybridization for Atlas Glass Human 3.8 microarrays: In this study two different methods were used to isolate RNA: one for labeling targets for the Atlas Glass system and a second for the Affymetrix. RNA samples were isolated independently for each experiment. The Atlas Glass system used total RNA extracted by the RNAeasy Midi Kit (Qiagen Inc.). After the RNA was isolated, genomic DNA was removed using DNAse (Qiagen Inc.). The targets were prepared using the Atlas Glass Fluorescent Labeling Kit (Clontech Laboratories, Inc.). This kit provides for indirect, "two-step" labeling of the target cDNA. Two-step labeling typically incorporates higher levels of label than direct, single-step procedures that directly incorporate fluorescently tagged nucleotides during cDNA synthesis. Target preparation began with 20 μg of total RNA. Aminoallyl-dUTP was incorporated during first-strand cDNA synthesis. Fluorescent dye (Cy3 or Cy5) was covalently coupled to aminoallyl-dUTP in the first-strand cDNA. The resulting labeled cDNA was purified using the Atlas NucleoSpin Extraction Kit. The absorbance of each target was determined by optical density measurements at 260 nm (DNA) and either 550 nm (Cy3) or 650 nm (Cy5). The total dye content (pmoles), amount of probe (ng), and specific activity (number of Cy molecules incorporated/number of bases) was calculated for each target synthesized. The optimal incorporation of dye ranged from 20 to 50 covalently linked dye per 1000 nucleotides. We also checked the quality and size range of the labeled cDNA by electrophoresis. Labeled cDNA fragments were resolved in 2% agarose gel and the images were acquired using 532 nm excitation for Cy3 dye and 633 nm excitation for Cy5 dye using the Typhoon System (Amersham Biosciences, Piscataway, NJ). Once the target quality was determined to be appropriate, the targets were hybridized to the probes immobilized on the glass slide. The slides were hybridized overnight at 50 °C. Following hybridization, slides were washed, dried and then scanned using a ScanArray 5000XL laser scanner (PerkinElmer LAS, Inc., Shelton, CT). The images were analyzed using QuantArray Microarray Analysis Software, Version 3.0 (Packard BioChip Technologies). The fluorescence intensity of each spot was calculated using the histogram quantitation method, which has the major advantage of being simple and stable.

Target preparation and hybridization for Affymetrix Human GeneChips U95A and U133A: The isolation procedures for the Affymetrix analysis were conducted using TRIzol Reagent (GIBCO BRL) according to the manufacturer's instructions. Initially, the quality of total RNA was assessed by electrophoresis through a 1% agarose gel, then the Agilent Bioanalyzer System (Agilent Technologies, Palo Alto, CA) immediately prior to cRNA synthesis. The procedures for the Affymetrix gene chips, beginning with first strand cDNA synthesis, were conducted by Genome Explorations (Memphis, TN). The Human Genome U95Av2 GeneChip contains 12,500 full-length annotated genes together with additional probe sets designed to represent EST sequences. The Human Genome U133A GeneChip contains 22,283 genes together with EST sequences. The RNA (isolated using TRIzol) was run over a G50 spin column. First and second strand cDNAs were synthesized from 15 μg of total RNA using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) and oligo-dT24-T7 (5'-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG-3') primer according to the manufacturer's instructions. cRNA was synthesized and labeled with biotinylated UTP and CTP by in vitro transcription using the T7 promoter coupled double stranded cDNA as template and the T7 RNA Transcript Labeling Kit (ENZO Diagnostics Inc.). The fragmented cRNA was hybridized to the olygonucleotide array, washed, stained with phycoerythrein conjugated streptavidin (Molecular Probes) and scanned. Intensities were determined using a laser confocal scanner (Affymetrix, Inc.). The scanned images were analyzed using Microarray Suite Version 5.0 (MAS 5.0, Affymetrix). The MAS 5.0 statistical algorithms calculate signal intensity, probe set detection, probe set (gene expression) change, and signal log ratio. We arbitrarily chose the D407 cell line to be the baseline sample for the differential expression analysis. Increases or decreases in expression of genes relative to this sample were calculated. The Expression Report Files are available in Appendix 1. These files provide quality control information for the mRNA quality (3' to 5' ratio), and the quality and consistency of the hybridization of the target to the probes (number present call).

Statistical analysis: Since the present study compares two very different microarray platforms, we made every attempt to make the data comparable. For the Atlas Glass microarray data, the signal intensities were converted to logarithmic scale, base 2. To compensate for non-linearity in the data, MA plots (the log intensity ratio $M=\log_2[R/G]$ vs. the mean log intensity $A=[1/2]\log_2[R*G]$) [7,8] were used with a smoothing process to correct the biases linked to the scanned data. The user-defined parameter f used for smoothing at each point was 20%. For the Affymetrix microarray, the signal intensities for one experimental sample were plotted against the second sample in logarithmic scale, base 2. To facilitate the analysis within each platform and between the two different

platforms, the data were scaled using a Z-score transformation. Using the formula 2(z-score of $\log_2$[intensity+1])+8, we set the mean of each population to 8 and the standard deviation to 2. This standardization procedure has several desirable effects. The first benefit is the stabilization of variance within and between the Affymetrix platform and the Atlas Glass platform. The second benefit is that a one-unit change on this scale is similar to a two-fold change in intensity. The third benefit of this procedure is that all of the values remain in the positive range. Thus, for the purpose of the present study we used the z-transformation to focus on the relative expression levels across the two microarray platforms.

Real-time RT-PCR, primer design: Gene-specific primers were designed using the MacVector 6.5 program (Accelrys, Inc., Burlington, MA). Whenever possible, primers were selected with a melting temperature T_m of 58-60 °C. Both primers had equal T_m and an amplicon size of 75-150 bases [9]. The presence of contaminating genomic DNA in the RNA

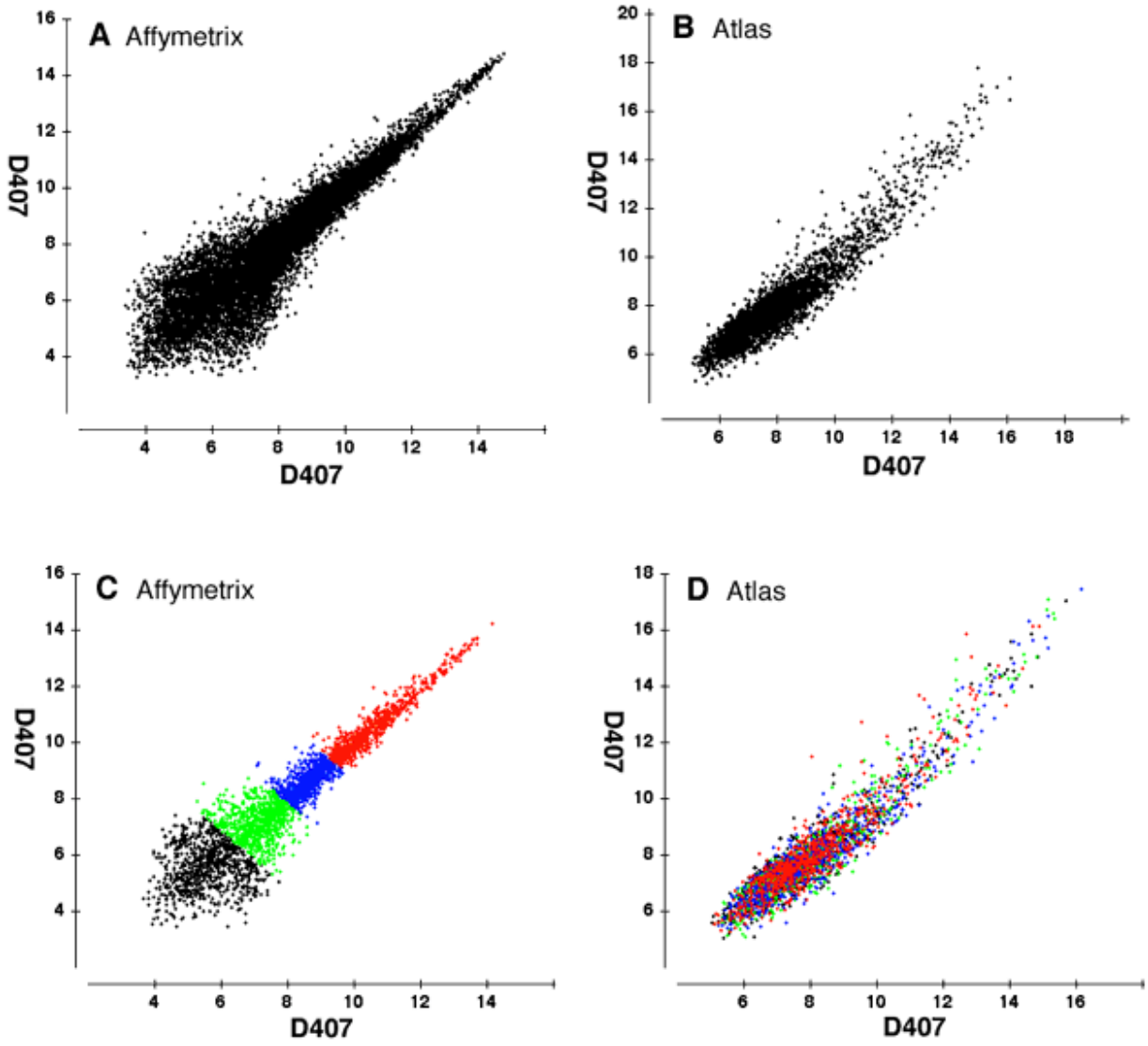

Figure 1. Internal consistency in the Affymetrix and Atlas Glass microarrays. The results from the control experiments using only RNA from the D407 cell line are illustrated in four scatter plots. To compare the data between the two microarray platforms the data was scaled using the formula 2(z-score of $\log_2$[intensity+1])+8. This set the mean intensity of each experiment to 8 and the standard deviation to 2. The results from a single Affymetrix HG-U95Av2 experiment are shown in **A**. Affymetrix chips hybridized with D407 targets reveal a good correlation between these two samples (Pearson correlation coefficient 0.924). For the data of the Atlas Glass Human 3.8 slide, an average of two separate experiments is shown in **B**. Notice that, on average, there is a good correlation between the genes on the two channels (Pearson correlation coefficient 0.937) with the majority of genes clustering around a line running through an intensity ratio of 1. To make a direct comparison between the two platforms, the data was filtered to include only genes in common with both platforms (**C** and **D**). The data on the Affymetrix platform was then divided into quartiles and colored. Notice the disconcordance between signal intensities on the Affymetrix platform as compared to those observed on the Atlas Glass platform (**D**). For example, genes having very high signal intensity on the Affymetrix chip (upper quartile colored red) are spread throughout the distribution on the Atlas Glass platform. Some of the genes even have very low signal intensity on the Atlas platform.

preparation was checked using primers for housekeeping genes, that spanned exon-intron junctions. We checked the gene expression of two housekeeping genes: β-actin and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). In two samples of total RNA (D407 and ARPE19 cells), we found that only the β-actin expression is constant. All of our quantifications were normalized to the endogenous control β-actin. The acquisition temperature was set 2 °C below the T_m of the specific product.

Real-time RT-PCR: Real-time RT-PCR was used as an independent method to quantify the relative levels of transcripts. Total RNA was extracted using TRIzol Reagent (GIBCO BRL). The mRNA was isolated by the polyATract mRNA Isolation Kit (Promega Corp., Madison, WI). Real-time RT-PCR was performed using an iCycler Real-time Detection System (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Reactions were performed in a 25 µl volume with primers and $MgCl_2$ concentration optimized for each pair of primers. Nucleotides, AmpliTaq Gold DNA polymerase, MultiScribe Reverse Transcriptase and buffer were included in the SYBR Green RT-PCR Kit (Applied BioSystem, Warrington, UK). For real-time RT-PCR, the protocol included an initial incubation of the reaction mixture for 30 min at 48 °C. Following 10 min at 95 °C the AmpliTaq Gold DNA Polimerase was activated. The amplification program consisted of 40 cycles with a 95 °C denaturation for 15 s and a 55-60 °C annealing and extension for 1 min. Detection of the fluorescent product was carried out either at the end of

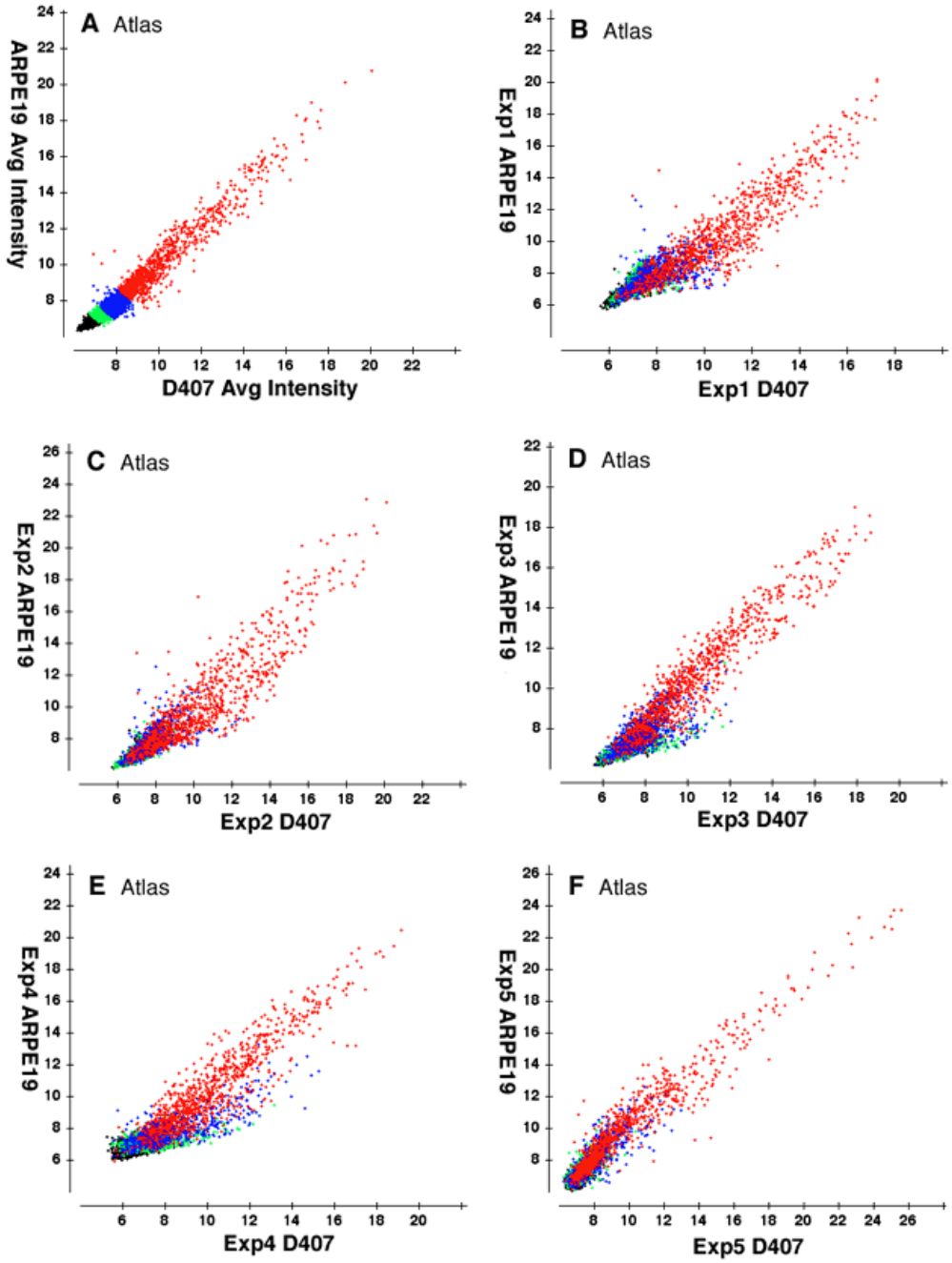

Figure 2. Comparison of D407 to ARPE19 cells using Atlas Glass microarrays. The comparison between the D407 cell line and the ARPE19 cell line using the Atlas Glass Human 3.8 platform is shown. Panel **A** is the average of all five individual experiments (**B**, **C**, **D**, **E**, and **F**). The averaged data was divided into quartiles and colored, allowing for a visual comparison to the individual experiments. In general, there is a similar distribution of intensities across experiments with the most intensively labeled probes being heavily labeled on all experiments. These data reveal the internal consistency of the Atlas Glass Human 3.8 platform. The data is scaled as in Figure 1.

the extension period or after an additional 2 s step at 2 °C below the product T_m. To confirm amplification specificity, the PCR products from each primer pair were subjected to a melting curve analysis and agarose gel electrophoresis. The data were analyzed with the iCycler analysis software. The key parameter for quantification is the cycle threshold (Ct, determined by iCycler software version 3.0). To obtain relative gene expression data (fold change) between two samples, we used the comparative ΔΔCt method, previously described by K. J. Livak [10]. In our experiments, the standard curves had correlation coefficients (r) of -1 with efficiencies ranging from 90 to 110% (i.e., slopes between -3.6 and -3.1). The fold change was determined by the formula: fold change=2-Δ(ΔCt), where ΔCt=Ct,target-Ct,β-actin; Δ(ΔCt)=ΔCt,ARPE19-ΔCt,D407; and where "target" is the gene of interest. The experiments were performed in triplicates for each pair of primers. All of the defined changes were significant with a 95% confidence level (student t test, $p<0.05$).

Protein immunoblot methods: Protein samples taken from the human RPE cell lines, ARPE19 and D407, were balanced (an equal concentration of protein in each sample) and then dissolved in non-reducing sample buffer (2% sodium dodecylsulfate, 10% glycerol in 0.05 M Tris-HCl buffer, pH 6.8). The balanced samples were separated by SDS polyacrylamide gel electrophoresis using a 4-16% gradient mini-gel. The proteins were transferred to nitrocellulose. The blots were then blocked in 5% nonfat dry milk and probed with the primary antibodies in borate buffer in 2% skim milk overnight at 4 °C. The primary antibodies were diluted at 1:250. The primary antibodies used against human proteins were as follows: goat anti-α-actinin (Chemicon, Temecula, CA); mouse anti-integrin α1 (Chemicon), anti-integrin α2 (Chemicon), anti-integrin α3 (Chemicon), anti-integrin α4 (Chemicon), anti-integrin α5 (Chemicon), anti-integrin αv (Chemicon), anti-integrin β1 (PharMigen) and anti-integrin β7 (Chemicon); goat anti-fibronectin (Chemicon); rabbit anti-tenascin (Chemicon); rabbit anti-connexin43 (Zymed, South San Francisco, CA); mouse anti-pan cadherin (Sigma); mouse anti-A-CAM (Sigma); rabbit anti-vitronectin (Chemicon), rabbit anti-keratin (Accurate Chemicals, CA), mouse anti-CD9 (a gift from Lisa Jennings, University of Tennessee, Memphis, TN) and anti-CD81 (a gift from Shoshona Levy, Stanford, CA). Then, the blots were rinsed in borate buffer (pH 8.5) and incubated in horseradish peroxidase labeled secondary antibodies (1:500) for 2 h at room temperature or overnight at 4 °C. The blots were then rinsed and reacted with 0.5 mg/ml 3,3' diaminobenzidine tetrahychloride (Sigma) in 0.05 M Tris buffer (pH 7.3) and 3 μl/ml of 3% hydrogen peroxide. To quantify the level of protein immunoreactions, the blots were scanned with a HP ScanJet 5370C, and the intensities of the

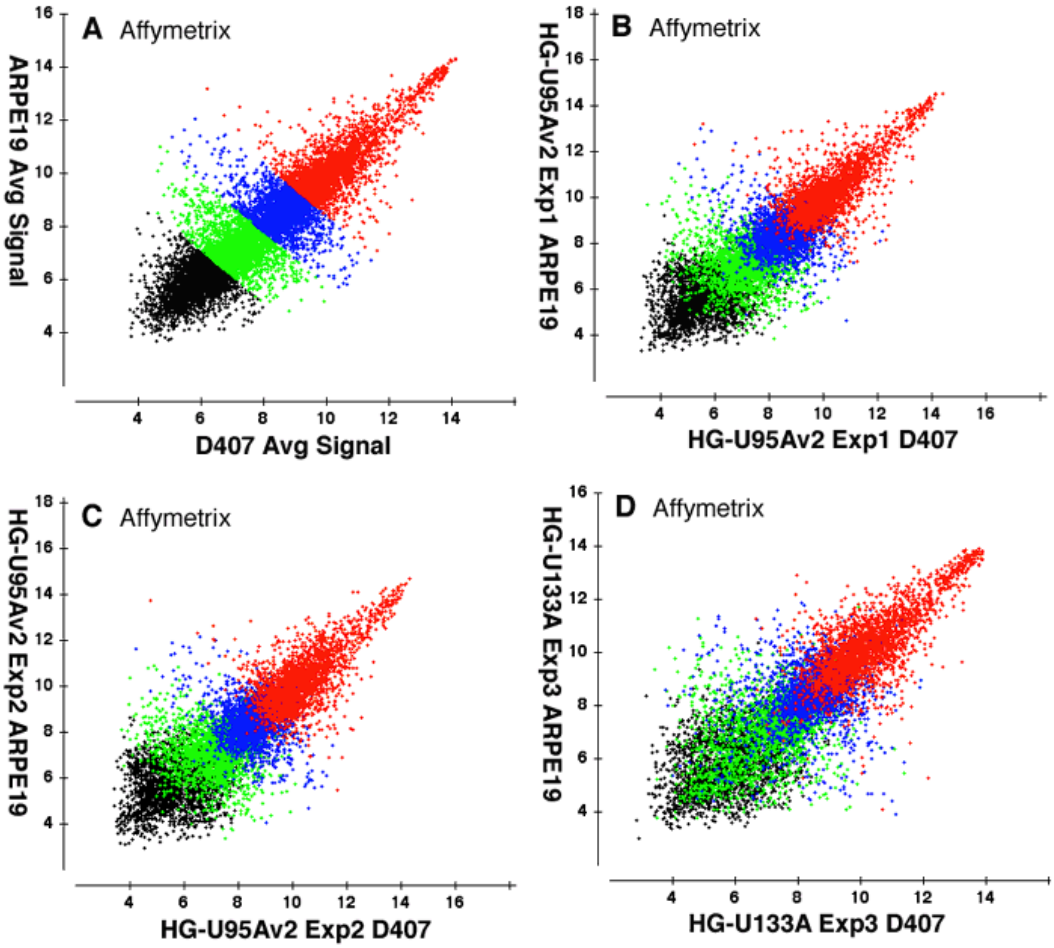

Figure 3. Comparison of D407 to ARPE19 cells using Affymetrix microarrays. The comparison of the D407 cell line and the ARPE19 cell using the Affymetrix microarray platform is illustrated. The average of three separate experiments is shown in **A**. The HG-U95Av2 microarray chip was used for the data represented in **B** and **C**, while the HG-U133A chip was used for the data in **D**. All of the data is normalized and scaled to allow for between experiment comparisons. The average of the three experiments was divided into quartiles and colored. Notice the overall agreement between the distributions of the signal intensities across the different individual experiments. The data is scaled as in Figure 1.

labeled bands were measured and analyzed with the NIH Image Software (version 1.62).

RESULTS

Microarray Analysis: The overall goal of the present study was to compare two commercially available microarray platforms: the Affymetrix system and the Clontech Atlas Glass system. The first experiment was designed to test the internal consistency of each platform. For this analysis, we tested the expression profiles of the D407 RPE cell line against itself. The two platforms have different methods for mRNA isolation, target preparation, hybridization and scanning. These differences prohibited us from using a single batch of RNA to compare the two microarray platforms. For this reason the D407 cell line was the starting material. On the Atlas Glass system, the signals were balanced for the Cy3 and Cy5 intensities immediately before the slides were scanned. After the signals were standardized, the Cy3 and Cy5 labeling was approximately the same on each spot (Figure 1B). For these experiments, two different microarrays were used and the data were averaged. Our analysis reveals equal labeling of the probes with the two different targets (Pearson correlation coefficient r=0.94). This demonstrates that the platform is internally consistent, presenting reproducible results. On the Affymetrix platform, two samples of total RNA were used to produce the cRNA targets in two separate reactions. Each of the cRNA targets was run on a separate U95Av2 Affymetrix chip. There was a high correlation between the labeling of the two chips (Pearson correlation coefficient r=0.92, Figure 1A). This demonstrates a high degree of internal consistency of the Affymetrix system. To make direct comparisons between the two microarray platforms, we selected probe sets (genes) that were on both platforms (Figure 1C,D). To allow for a direct comparison of the results we standardized the data using the formula 2(z-score of $\log_2$[intensity+1])+8. Surprisingly, when the expression level of specific genes on the Affymetrix platform was compared to that observed on the Atlas Glass slide, there was a dramatic disconnection between the two systems (Figure 1C,D). Many of the genes with very high signal detection on Affymetrix chip have very low signal levels on Atlas chip and vice versa. To illustrate this point, the average intensity of the Affymetrix platform was divided into four equal parts (quartiles) and colored. Each gene was linked to its homolog in the Atlas Glass data (Figure 1C,D). As can be seen, the upper quartile in the Affymetrix platform is scattered throughout the distribution on the Atlas Glass system. Thus, there is an internal consistency within each platform but very little agreement between platforms.

The next step in our analysis examined the differentially expressed genes between the ARPE19 and the D407 cell lines. For the Atlas Glass system, a total of five chips were used. On three chips the cDNA from the D407 cell line was labeled with Cy3, while the cDNA from ARPE19 cell line was labeled with Cy5. On the remaining two slides, the dyes were reversed. In each experiment, the $\log_2$ of the intensity of the Cy3 signal was plotted against the $\log_2$ of the intensity of the Cy5 signal. The results from all five chips are illustrated in Figure 2. The average of all the experiments is shown in Figure 2A. Again the average signal was used to divide the data set into quartiles and each was colored to aid in evaluating the overall distribution of the relative intensities of the genes. As seen in Figure 2, the genes in the upper quartile on the aver-

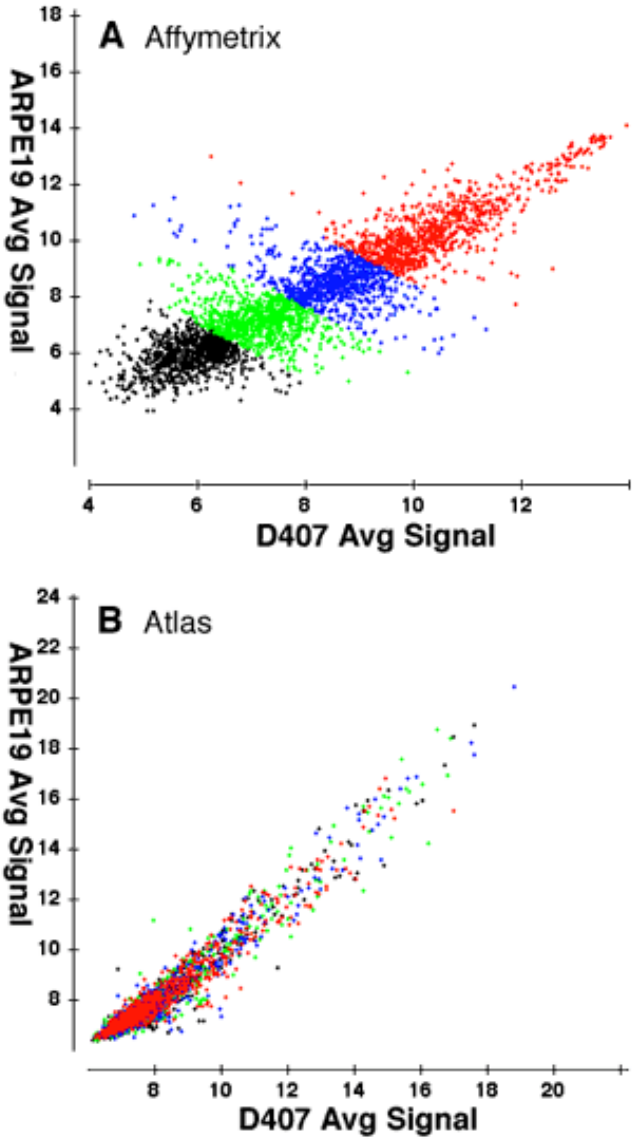

Figure 4. Average signals: Affymetrix vs. Atlas Glass. The differential gene expression between ARPE19 cells and D407 cells on the Affymetrix platform (**A**) and the Atlas Glass platform (**B**) were compared. To make a direct comparison between the two platforms, the genes in the average summations of the data were filtered to exclude genes not common to both platforms. The data on the Affymetrix platform (**A**) was then divided into quartiles and colored. Notice the disconcordance between signal intensities on the Affymetrix platform as compared to those observed on the Atlas Glass platform (**B**). For example, genes having very high signal intensity on the Affymetrix chip (upper quartile colored red) are spread throughout the distribution on the Atlas Glass platform. The data is scaled as in Figure 1.

age of all experiments also show high intensities on the individual chips. Of the 939 genes in the top quartile of the averaged data, an average of 697 (74%) were found in the top quartile of individual experiments. Thus, when using the Atlas Glass system to monitor expression levels between the ARPE19 cell line and the D407 cell line, there was good internal consistency between individual experiments. When the Affymetrix platform was used to examine the differences between ARPE19 and D407 cells a high degree of internal consistency was observed (Figure 3). For the Affymetrix platform, three sets of chips were used: two pairs were the HG-U95A chip and the third pair of chips was the HG-U133A chip. The average of all three comparisons is shown in Figure 3A. The intensities on the averaged data were divided into quartiles and colored. As can be observed there was a similar distribution across all three individual experiments. There was good internal consistency within each of the microarray platforms. On the Affymetrix platform 2,676 genes were in the top quartile of the averaged data (Figure 3). Of these 2,676 genes, an average of 2,340 (87%) were found in the top quartile of each of the three individual experiments. Although both platforms generate data that is internally consistent, the Affymetrix system was significantly better than that of the Atlas platform (Mann-Whitney U test P=0.018). In stark contrast to the internal consistency, there was a dramatic difference in the results across platforms. If we compare the differences in intensity values for the averaged Affymetrix experiments to those of the averaged Atlas experiments, there was a complete lack of agreement. The genes that one would expect to be the most consistent between experiments were the genes with the high-

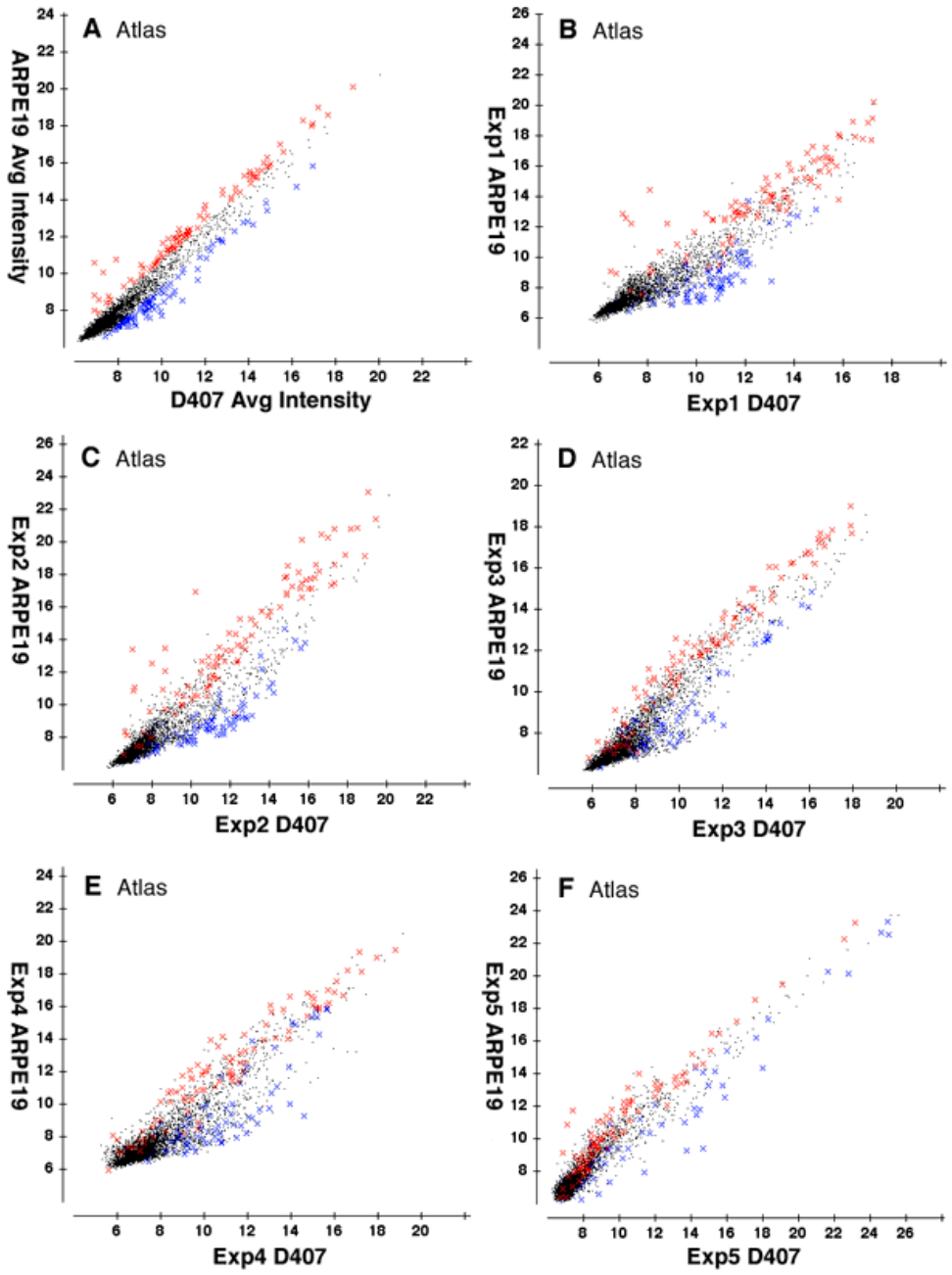

Figure 5. Significant differences on the Atlas Glass microarrays. The data presented in this figure is similar to that presented in Figure 2, a comparison between the D407 cell line and the ARPE19 cell line as defined by the Atlas Glass Human 3.8 array. Panel **A** is the average of all five individual experiments (**B-F**). The averaged data was used to identify genes that were 1.7 fold up-regulated (red) and 1.7 fold down-regulated (blue). In general, there is a similar distribution of up-regulated and down-regulated genes across experiments. These data again reveal the internal consistency of the Atlas Glass Human 3.8 platform. The data is scaled as in Figure 1.

est level of expression. When we examine these highly expressed genes in the Affymetrix system (in the upper quartile), we see these same genes are scattered throughout the plot of the Atlas Glass data (Figure 4).

The next step was to examine the distribution of differentially expressed genes in each of the platforms. To allow for comparison across microarray platforms, we treated each data set in a similar manner and used an arbitrary cutoff of 1.7 fold change to define differentially expressed genes. We acknowledge that there may be better statistical approaches to define changes on each of the platforms [11,12]. However, this analysis provided a simple and effective approach to compare the data between the two platforms. If we examine the changes in gene expression on the Atlas Glass Human 3.8 slides and use an average fold change of 1.7 to define genes that are differentially express, then 95 genes (2.5% of the genes) are more highly expressed in ARPE19 cells relative to the D407 cells and 110 genes (2.9% of the genes) appear to be down-regulated in the AREP19 cells. If we look at the distribution of these genes across all individual experiments (Figure 5), a considerable amount of variability is observed between experiments. The results on the Atlas Glass Human 3.8 system stand in sharp contrast to those observed on the Affymetrix system. When we set the fold change to 1.7 on the Affymetrix data, 1,495 genes (14.0% of the genes) are more highly expressed in ARPE19 cells relative to the D407 cells and 1,575 genes (14.7% of the genes) appear to be down-regulated in the ARPE19 cells. When these genes were examined across each individual set of experiments, there was a consistent distribution of these changes (Figure 6).

To compare the results obtained with the two platforms, we compared the 1.7 fold changes defined on the three experiment average on the Affymetrix platform to the changes defined by the five chip average on the Atlas Glass platform (Figure 7). As can be seen in Figure 7, many of the genes that were up-regulated on the Affymetrix platform did not change on the Atlas platform. If we examine the genes in common between the two platforms, there are 1005 genes on the Affymetrix platform with a fold change of 1.7, 491 up and 514 down. If we add the additional criterion that the change in expression must have a p value of 0.05 (student t test), then 337 genes (167 up-regulated and 170 down-regulated) exhibit change. The false discovery rate [13] of the 337 probe sets that demonstrate a significant difference and a fold-change of 1.7 is 14.9%. Futhermore, of the 337 genes with a 1.7 fold change and a p value of 0.05 on the Affymetrix platform, only 78 (14.2%) had a 1.7 fold change on the Atlas Glass Human 3.8 platform.

Quantification of mRNA by real-time RT-PCR: There was no immediate explanation for the dramatic differences ob-

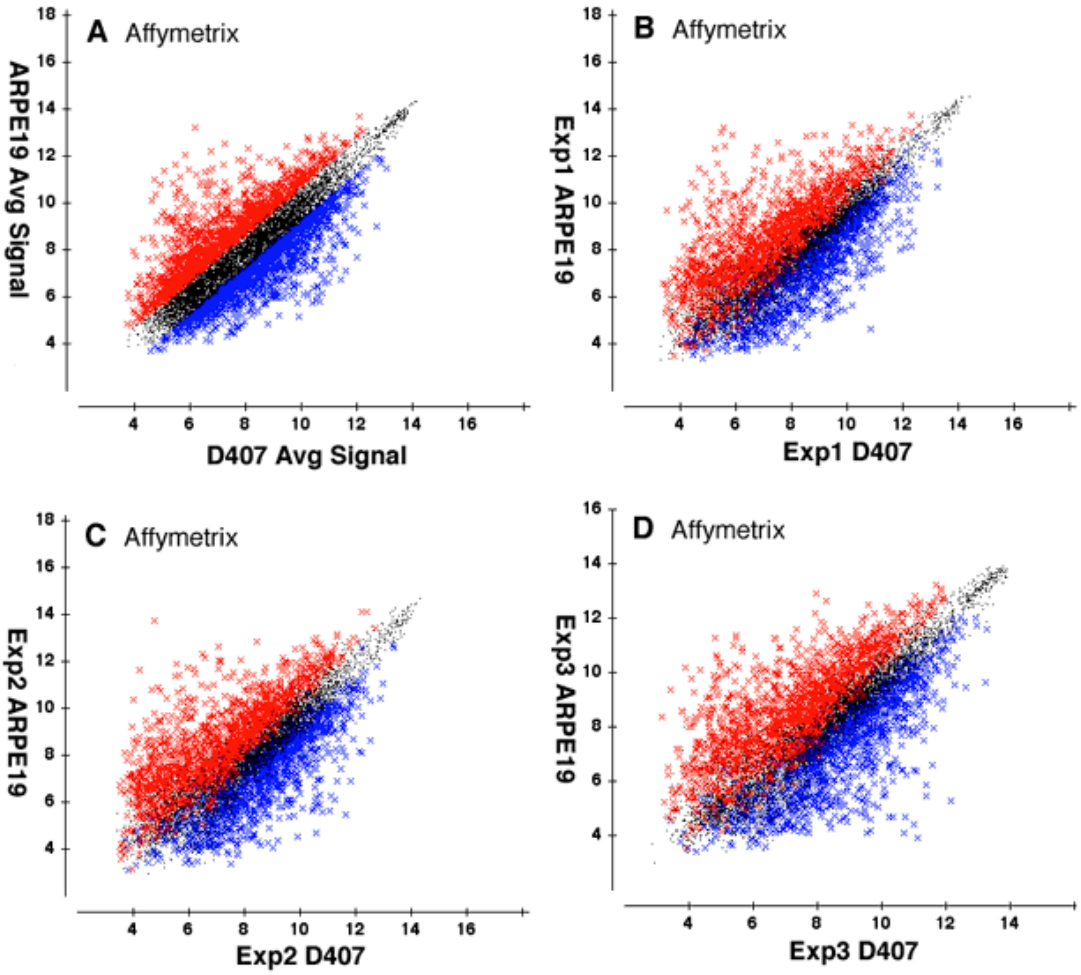

Figure 6. Significant differences on the Affymetrix microarrays. This figure presents a comparison of the D407 cell line and the ARPE19 cell line using the Affymetrix microarray platform, similar to Figure 3. However, in this figure the average of three separate experiments (shown in **A**) was used to define genes with a 1.7 fold up-regulation (red) and a 1.7 fold down-regulation (blue). The data from the two sets of HG-U95Av2 chips is shown in **B** and **C**; while the data from the HG-U133A is in **D**. All of the data is normalized and scaled to allow for between experiment comparisons. Notice the overall agreement between the distributions of the signal intensities across the different individual experiment. The data is scaled as in Figure 1.

served on the two microarray platforms when the D407 cell line was compared to the ARPE19 cell line. The only way to reconcile these differences is to attempt to define the differences in gene expression using an independent method. A separate series of experiments were conducted using real-time RT-PCR. We selected 22 different genes to test by real-time RT-PCR. Some of these genes are of specific interest to our laboratory and others were selected due to their differential expression on the two microarray platforms. A total of 22 PCR primer pairs were made and tested (Table 1). We analyzed 22 genes from the Affymetrix platform (Table 2). Of the 22 genes selected on the Affymetrix chip, 10 were represented by multiple probe sets. For example, CaM kinase II gamma is represented by 3 different probe sets. Thus, we analyzed 22 genes that were represented by a total of 34 probe sets. Of the 34 probe sets on the Affymetrix platform, 14 of the probe sets were up-regulated, 15 of probe sets were down-regulated, and 4 probe sets were unchanged. Our analysis demonstrated a relatively good correlation between real-time RT-PCR and the Affymetrix platform, with 85% of the probe sets changing in the same direction by both methods. If we look at genes instead of probe sets the correlation was approximately the same, with an 86% agreement between the two methods. If we limit our analysis to genes with relative high levels of expression, there are 12 probe sets with high signal intensities (data not shown). The changes observed in these 12 probe sets showed complete agreement between the Affymetrix platform and the real-time RT-PCR method. When we examined probe sets with very low signal intensities (less than 5% of the mean intensity), the correlation between Affymetrix and real-time RT-PCR was not as good: only 57% (4 of 7). Overall, real-time RT-PCR confirmed Affymetrix-determined relative expression differences for 29 of 34 probe sets (85%) tested. A similar comparison was made between the real-time RT-PCR results and those obtained using the Atlas Glass platform. On the Atlas Glass microarray, each gene was represented by a single probe. Of the 15 probes on the Atlas platform that were tested, 4 were up-regulated, 5 were down-regulated, and 6 were unchanged. Seven probes had signal intensities above the mean. Of these seven, 4 (57%) were confirmed by real-time RT-PCR. When we examined the 6 genes with low signal intensities, none (0%) showed a change that was similar to that of the real-time RT-PCR. Overall, on the Atlas Glass platform, only 5 of 15 genes (33%) were confirmed by real-time RT-PCR (Table 3).

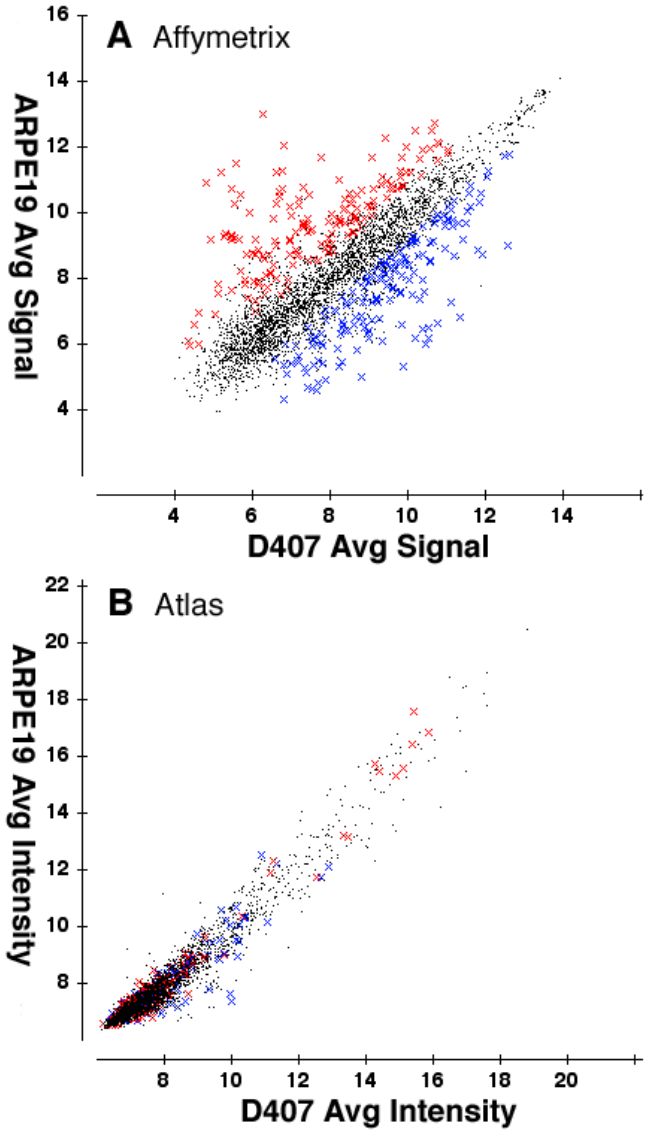

Figure 7. Average significant differences: Affymetrix vs. Atlas Glass. The differential up-regulation or down-regulation between ARPE19 cells and D407 cells on the Affymetrix platform (**A**) and the Atlas Glass platform (**B**) were compared to each other. To make a direct comparison between the two platforms, the genes in the average summations of the data were filtered to include only genes in common with both platforms. The genes on the Affymetrix platform (**A**) were categorized as changed if there was a 1.7 fold difference on the Affymetrix platform and a p value of 0.05. The up-regulated genes are shown in red and the down-regulated genes in blue. Notice the dramatic difference between platforms. For example, genes that were up-regulated on the Affymetrix chip (colored red) appear to be down-regulated on the Atlas Glass platform. The data is scaled as in Figure 1.

Gene Expression and Protein Levels: To understand the overall meaning of the microarray data, the expression levels of selected proteins were quantified by immunoblot analysis. For this analysis, we selected 11 proteins that were of interest to our laboratory and to which we already had antibodies that would recognize the proteins on western blots. The protein samples from D407 and ARPE19 cells were balanced, separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies. The relative intensity of the immunopositive bands was determined. The difference in the expression of the proteins was expressed as a fold change. The protein sample from the D407 cells served as the baseline level and increases or decreases in the ARPE19 levels were indicated as positive or negative fold changes respectively. The changes in protein levels were compared to fold changes from the Affymetrix microarray and the Atlas Glass microarray (Table 4). When

comparing the levels of protein expression to the changes in mRNA levels (Affymetrix platform), the direction of the fold change for 7 of the 11 proteins analyzed was similar to the mRNA level observations. This was not the case for 4 proteins. The relative levels of these 4 proteins did not correlate with the mRNA changes observed on the Affymetrix platform (Table 4). When the protein levels were compared to the data from the Atlas microarray, only 5 proteins were present on the Atlas Human 3.8 slide. Furthermore, there was no correlation between the levels of protein expression with the results of the Atlas microarray. Of the 5 proteins analyzed, none changed in the direction observed on the Atlas microarray.

DISCUSSION

The developing technology of DNA microarrays is giving investigators a tool to examine a large portion of the transcriptome in a single experiment. This monitoring of the RNA profile opens the door to define the effects of new drugs on cellular function [14-18], characterize changes in pathological states within a tissue [19,20] or develop expanded diagnostic protocols [21-25]. At the present time, there are a number of different types of commercially available microarray platforms: Affymetrix, Inc. (GeneChip Expression arrays); SuperArray, Inc. (Frederick, MD; GEArray); Clontech (Palo Alto, CA; Atlas Arrays), Incyte Genomics (Palo Alto, CA; GEM series of microarrays), Agilent (Agilent custom oligo-

Table 1. Oligonucleotide primer sequences and amplification conditions

Gene number	Title	Accession number	Primer sequences (5'-3') (Forward and Reverse)	Tm primer	Product Size (bp)
1	Integrin, alpha 1	XM_032902	TGCCAGTGAGATTTCAGAGACC	62.1	117
			GTGATTTCCTGTGTTTTCGTCG	62.1	
2	Integrin, alpha 2	X17033/M28249	AACTCTTTGGATTTGCGTGTG	60.5	82
			TGGCAGTCTCAGAATAGGCTTC	61.2	
3	Integrin, alpha 3	XM_008432	ACTGTGAAGGCACGAGTGTG	60.3	103
			TGCTGGTTCGGAGGAATAG	59.2	
4	Integrin, alpha 4	XM_039011	AGAGAGACAATCAGTGGTTGG	57.2	168
			TCAGTTCTGTTCGTAAATCAGG	57.4	
5	Integrin alpha 5	X06256	TGCCTCCCTCACCATCTTC	61.6	171
			TGCTTCTGCCAGTCCAGC	61.7	
6	Integrin, alpha V	NM_002210	TTGTTGCTACTGGCTGTTTTG	59.4	89
			TCCCTTTCTTGTTCTTCTTGAG	58.1	
7	Integrin, beta 1	XM_058357	CAAAGGAACAGCAGAGAAGC	58.2	168
			ATTGAGTAAGACAGGTCCATAAGG	58.1	
8	Connexin 43	NM_000165	TCTGAGTGCCTGAACTTGC	58	188
			ACTGACAGCCACACCTTCC	59.1	
9	CD81	NM_004356	ATGTGAAGCAGTTCTATGACCAG	58.8	100
			TCAAGCGTCTCGTGGAAG	59	
10	Syntaxin 16	NM_003763	ACAAGGCAGAACAGTATCAAAAG	58	117
			ACGAGGACAACAATGAGCAC	59.1	
11	Arg/Abl-interacting protein	NM_003603	TCAAATAAGCCACAGCGTC	57.8	161
			AACCAGCCGTCATCACAC	58.4	
12	MHC class 1 related sequence B	NM_005931	ACTTTCCCTCTGTTTCCTGAC	57.7	91
			AGCAGTCGTGAGTTTGCC	57.8	
13	Actinin, alpha 1	NM_001102	TGAGGAGTGGTTGCTGAATGAG	63	63
			AACTTCTCTGCCAGGTGGTCC	63.2	
14	Ras homolog, C	NM_005167	ACCTGCCTCCTCATCGTCTTC	63.4	105
			CACCTGCTTGCCGTCCAC	64.4	
15	Agouti (mouse) related protein	NM_001672	GTCTTCCTCTGCTTCTTCACTGC	62.3	149
			ATCGGTTTGGATTTCTTGTTCAG	61.9	
16	Activator of S phase kinase	NM_006716	TTCACAAGAGCACCTAACTGTTCAG	62.3	53
			CAGGAGGAGTATGGAATGGAGC	62.6	
17	ATPase, Cu++ transporting, alpha	NM_000052	AGAGTCCTTCAGAAATCAGCGTTC	62.7	136
			AGGGAGCGTTTATCAGACAGTAGTG	62.5	
18	Beclin 1	NM_003766	AGGATGATGTCCACAGAAAGTGC	63	99
			AGTGACCTTCAGTCTTCGGCTG	63.5	
19	CaM kinase, II gamma	XM044349	CTTGTCTCCAGGAACTTCTCAGC	62.1	83
			TTGTTGTTGCTCTGTGGCTTG	62.7	
20	Cyclin-dependent kinase 8	NM_001260	AGGTGTGGCTTCTGTTTGACTATG	62.1	94
			AACTGAACTGGCTTCTTGTTTGC	62.3	
21	Actin, beta	X00351	AAAGACCTGTACGCCAACAC	57	219
			GTCATACTCCTGCTTGCTGAT	57.3	
22	Glyceraldehyde-3-phosphate dehydrogenase	X01677	GTGAAGGTCGGAGTCAACG	58.8	113
			TGAGGTCAATGAAGGGGTC	59	

The oligonucleotide sequence for the primer pairs used for RT-PCR.

nucleotide microarrays), Sequenom (San Diego, CA; SpectroCHIP), Nanogen (San Diego, CA; NanoChip Arrays), PerkinElmer LAS, Inc. (Shelton, CT; MICROMAX) [26]. The present study compared two of these commercially available systems: the Atlas Glass oligonucleotide platform to the Affymetrix system. The Affymetrix system was used for it is clearly becoming the industry standard. The Atlas Glass system was chosen for two reasons. The first is that the price of the DNA chip is relatively inexpensive. The second reason is that the Atlas Glass chips were readily available and contained a good number of probes representing 3,800 genes. Each gene on the Atlas Glass Human 3.8 microarray is represented by a single 80-base oligonucleotide. Two samples (the control and experimental) are analyzed on a single chip. The cDNA targets are labeled with fluorescent dyes and are hybridized to the Atlas Glass Human 3.8 microarray. The use of simultaneous hybridization of two labeled targets allows for the direct comparison of mRNA profiles from two specimens. The major theoretical advantage to this approach is that the hybridization conditions for each of the targets are always identical, allowing for perfect within-slide comparisons of the two samples. The disadvantage to this approach is that the between slide comparisons are difficult to make without additional normalization steps. One type of normalization is to use a standard RNA sample as one of the targets and expressing the intensity levels of the experimental target relative to the standard.

Table 2. Affymetrix fold changes relative to RT-PCR

Gene number	Title	Affymetrix ProbeSet ID	Fold Change Affymetrix	Fold Change RT-PCR
1	Actinin, alpha 1	39329_at	3.3	2.4
	Actinin, alpha 1	39330_s_at	3.1	
2	Agouti (mouse) related protein	35068_at	1.2	3.2
3	Arg/Abl-interacting protein	39295_s_at	-7.8	-1.1
4	Ras homolog, C	1395_at	1.3	1.6
5	Activator of S phase kinase	31865_at	-4.8	-1.5
6	ATPase, Cu++ transporting, alpha	36523_at	-1.6	4.3
7	Beclin 1	39378_at	1.6	-2.2
8	CaM kinase,II gamma	31670_s_at	-1.5	-1.2
	CaM kinase,II gamma	32104_i_at	-1.2	
	CaM kinase,II gamma	32105_f_at	-2.9	
9	CD81 antigen	35282_r_at	1.6	1.9
10	Cyclin-dependent kinase 8	1189_at	1.1	1
	Cyclin-dependent kinase 8	35140_at	-1	
11	Glyceraldehyde-3-phosphate dehydrogenase	39482_at	-2.3	-2.1
12	Connexin 43	2018_at	1.5	14.6
	Connexin 43	32531_at	4.1	
13	Integrin, alpha 2	1978_at	-2.8	-1.4
	Integrin, alpha 2	41481_at	-7.4	
14	Integrin, alpha 3	884_at	3.5	1.9
	Integrin, alpha 3	885_g_at	4.6	
15	Integrin, alpha 4	2061_at	2.6	7.1
	Integrin, alpha 4	35731_at	5	
16	Integrin, alpha 5	39753_at	-2	-2.5
17	Integrin, alpha V	257_at	1.7	6.9
	Integrin, alpha V	39071_at	2.5	
18	Integrin, beta 1	2055_s_at	3.2	1.1
	Integrin, beta 1	33161_at	1.1	
	Integrin, beta 1	39483_s_at	1.2	
19	LDL receptor-related protein 8	32398_s_at	-2.9	-1.3
20	Syntaxin 16	39523_at	-4.4	-20
	Syntaxin 16	41121_at	-1.1	
21	ESTs integrin alpha 1	37484_at	-1.3	-4.6
22	ESTs MHC class1 related sequence B	35937_at	-6.4	-7.7

Comparison of the data obtained using the Affymetrix platform (HG-U95Av2 chips) with the results from real-time RT-PCR experiments. Affymetrix and real-time RT-PCR results are shown as a fold change of the level of mRNA from the ARPE19 cell line relative to the level of that transcript in the D407 cell line. A total of 22 genes were characterized by real-time RT-PCR. Out of these 22 genes, 10 were represented by several probe sets on the Affymetrix HG-U95Av2 chip. Real-time RT-PCR confirmed 85% (29 of 34) of the probe sets examined on the Affymetrix U95Av2 chip.

The results from the Clontech Atlas Glass platform were compared to those of the Affymetrix platform. There are considerable differences between the two systems. In the Affymetrix system, only one target is hybridized with an individual chip. This represents advantages in some cases and disadvantages in others. The major apparent disadvantage of Affymetrix is the cost. A single experiment now involves the use of at least two chips and the chips must be purchased from Affymetrix. However, in specific cases the use of one sample on a chip can be an advantage. If a single sample fails to label, then only that sample is rejected, unlike the dual labeling system where the data from both samples would be lost. Furthermore, since microarray requires between-group comparisons, Affymetrix has developed a method for standardizing the data output for each chip allowing for between chip comparisons [27]. By allowing for between chip comparisons, any sample in the experiment can be compared to any of the other experimental samples, potentially reducing the overall cost of experiments. Thus, with an experiment in which a single control

Table 3. Atlas Glass fold changes relative to RT-PCR

Gene number	Title	Unigene ID	Fold change Atlas	Fold change RT-PCR
1	Actinin, alpha 1	Hs.119000	-1	2.4
2	Agouti (mouse) related protein	Hs.104633	-1.2	3.2
3	Ras homolog, C	Hs.179735	1.3	1.6
4	Activator of S phase kinase	Hs.152759	-2.5	-1.5
5	ATPase, Cu++ transporting, alpha	Hs.606	2.3	4.3
6	Beclin 1	Hs.12272	1.3	-2.2
7	CaM kinase, II gamma	Hs.250857	-1.3	-1.2
8	CD81 antigen	Hs.54457	1	1.9
9	Cyclin-dependent kinase 8	Hs.25283	1	1
10	Glyceraldehyde-3-phosphate dehydrogenase	Hs.169476	-1.1	-2.1
11	Connexin 43	Hs.74471	-1.2	14.6
12	Integrin, alpha 3	Hs.265829	-1.5	1.9
13	Integrin, alpha V	Hs.295726	-1.1	6.9
14	LDL receptor-related protein 8	Hs.54481	1.1	-1.3
15	Syntaxin 16	Hs.102178	1.1	-20

Comparison of the results obtained on the Atlas Glass platform with real-time RT-PCR. Atlas and real-time RT-PCR results are shown as a fold change of the level of mRNA from the ARPE19 cell line relative to the level of that transcript in the D407 cell line. The changes of 5 of 15 genes analyzed (33%) were confirmed by real-time RT-PCR.

Table 4. mRNA fold changes relative to protein Levels

Gene number	Title	Fold change Affymetrix	Fold change Atlas	Fold change Western blot
1	Actinin, alpha 1	3.1*	-1	1.2*
2	CD81 antigen	1.6*	1	1.5*
3	Connexin 43	4.1	-1.2	-8.5
4	Integrin, alpha 2	-2.8	no gene	2.4
5	Integrin, alpha 3	3.5*	-1.5	2.2*
6	Integrin, alpha 4	2.6*	no gene	1.7*
7	Integrin, alpha 5	-2.0*	no gene	-1.2*
8	Integrin, alpha V	2.5*	-1.1	1.7*
9	Integrin, beta 1	1.1	no gene	1.5
10	Integrin, beta 7	1.4*	no gene	1.3*
11	Integrin, alpha 1	-1.3	no gene	1.1

The expression levels of selected proteins are compared to mRNA levels as defined by the Atlas Glass platform, the Affymetrix platform (HG-U95Av2 chips), and real-time RT-PCR. The fold changes in protein levels detected by immunoblot were compared to the fold changes in mRNA. When protein levels are compared to the Affymetrix data, seven of the proteins (*) change in the same direction as the mRNA and 4 proteins change in the opposite direction. For the Atlas Glass platform none of five mRNA levels was similar to the protein level change.

sample will be compared to multiple experimental samples, the use of the Affymetrix system becomes more cost effective.

The direct comparison between the results of the Atlas Glass platform and the Affymetrix platform reveals a surprising lack of agreement between the two platforms. The relative intensity of a gene on one platform was significantly different when examined on the second platform. For example, genes that had relatively high signal intensity on the Affymetrix microarrays were seen to have relatively low signal intensity on the Atlas microarrays (Figure 1 and Figure 3). This lack of agreement between the two platforms is best illustrated by examining genes with a 1.7-fold change (Figure 4). Others [28] have found a disconnection between Affymetrix data and the results from spotted cDNA microarrays (Stanford-type cDNA microarray). They found very little concordance between the clusters resulting from the different platforms [28].

It is tempting to speculate that the differences observed between the two platforms is due solely to the differences in their design. On the Atlas Glass microarray, all of the weight of detection rests on a single oligonucleotide. If cross-hybridization with an inappropriate target occurs, then the readout for that gene is incorrect. This could result in an erroneous quantification of mRNA levels. On the Affymetrix microarray, each gene is represented by a probe set (16 pairs of oligonucleotides on the HG-U95 chip and 11 pairs on the HG-U133 chip). If one spot does not hybridize the appropriate target, then other probes are present in the set to aid in the determination of the gene expression level. Furthermore, Affymetrix uses a statistical approach to compensate for outliers in the data set. The One-Step Tukey's Biweight Estimate decreases the weight of the "bad" probe pair relative to the other probe pairs. A second point is that the Affymetrix system appears to spread the data out further than the Atlas Glass system. (Figure 1, Figure 4, and Figure 7). Having given this accolade to the Affymetrix platform, there are several disconcerting aspects to their system. Some genes are represented on a single chip by several different probe sets and the levels of detection can be quite different for the different probe sets.

The use of microarrays is revolutionizing our studies of tissues [25], diseases [2] and effects of drug treatments [18]. Underlying this power is a pervasive belief that the changes in gene expression represent an accurate reflection of the state of the cell or tissue. In fact, the levels of mRNA are only a part of the cellular mechanisms that regulate function. The measurement of transcribed mRNA alone is not sufficient to characterize fully cell behavior. After the mature mRNA leaves the nucleus and enters the cytoplasm there are many different regulatory steps that can influence the production of a functioning protein. An early regulation of protein expression can occur at the translational level itself. Some mRNA has regulatory elements in the 5' and 3' untranslated regions (UTRs) [29,30]. For example, the translation of transferrin is regulated by very specific modulators (by iron-regulatory proteins) that bind to the iron-responsive element in the UTR of ferritin mRNA and inhibit its translation. The activity of iron-regulatory proteins is modulated by intracellular iron concentrations, regulating protein expression by the iron concentration within the cell [31,32]. In our limited study, we found that only 7 of the 11 proteins examined had changes in expression levels that mirrored the changes we observed in mRNA levels as defined by the Affymetrix gene chip. This difference in mRNA and protein expressions has been observed in other systems using a variety of methods: in human cancer [33], and in yeast [34,35]. In addition to the regulation of transcription, post-translational modifications of proteins can have dramatic effects on the cellular localization and function: protein folding, proteolytic cleavage, phosphorylation, glycosylation and lipid addition (such as palmitoylation). Microarray technology does not provide any data concerning the key regulatory events. The translation of mRNA into protein and the posttranslational modifications are often an ignored step in the functional consequences of regulating cellular function. When considering the functional implications of microarray data the ultimate function of the gene product must be taken into consideration.

Finally, there are functional differences in cells and tissues that may not be detectable by microarray technology. We chose to use the D407 and the ARPE19 lines to evaluate the microarray platforms because both RPE cell lines differ in well defined behaviors in tissue culture [5,6,36]. Both lines exhibit a typical cobblestone epithelial morphology and express keratin, but they differ in karyotype; the D407 cells contain 71 chromosomes and the ARPE19 cells contain 46 chromosomes. The D407 cells do not exhibit the extreme polarity of RPE cells like the ARPE19 cells. The ARPE19 cell line is capable of achieving senescence, while the D407 line is immortal. This immortality may be the result of the aneuploidy of the D407 cell line. The D407 cells have a higher mitotic rate than the ARPE19 cells (Geisert and Rogojina, personal observation) and one might expect that this difference would lead to significant differences in the cell-cycle genes. However, both cells are undergoing mitosis and require all of the genes that are necessary to cycle. Even though the cells are cycling at different rates, on average, these populations of cells express the same levels of the different cell-cycle genes. Microarray can tell us if a cell is cycling, however the specific changes in gene expression do not always relate directly to cell behavior. Many of the check points in the cell-cycle involve phosphorylation of existing proteins and not a change in the transcriptome. This degree of regulation is not detectable by miroarray methods. Therefore, the use of microarrays provide us with a powerful tool in evaluating changes in the cell's transcriptome, however this information alone does not allow us to fully describe the changes in cellular function. Our laboratory is currently preparing manuscripts defining the differential gene expression between the ARPE19 and D407 cell lines.

ACKNOWLEDGEMENTS

This research was supported by the grant PHS grant RO1 EY12369, NEI Core Grant P30 EY13080 and the Center of Excellence for Neurological Disease. Funding provided by

unrestricted grant from Research to Prevent Blindness. The authors would like to thank Mr. Jack Greenhaw for his help with RNA isolation and Mr. F.Vazquez-Chona for his help in analysis of the microarrays.

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Appendix 1. Expression Report Files

The full Affymetrix expression reports are presented in this appendix in the online version of this article, for the six chips: D407-1, D407-2, D407-3, ARPE-1, ARPE-2 and ARPE-3. These reports describe a series of measures related to the quality of target hybridization and target preparation. Several factors are worth specific attention. The scaling factor relates to the degree of hybridization; in the present study this ranges from 3.5 to 6.7 (an acceptable range). The number present call reflects the overall quality of each experiment; in the present study this ranges from 37.7 to 44.8, which is very consistent between experiments. The quality of the targets is reflected in the 3' to 5' ratio of housekeeping genes. For example, GAPDH (probe set HUMGAPDH/M33197) has a 3' to 5' ratio that ranges from a low of 0.8 to a high of 1.9. This indicates that the amount of 3' end of the target is very similar to the amount of 5' end of the target. All of these measures provide the investigator the quality controls necessary to evaluate individual microarray experiments.

To access this data, click or select the words "Expression Report Files" in the online version of this article. This will initiate the download of a compressed (zip) archive. This file should be uncompressed with an appropriate program (the particular program will depend on your operating system). Once extracted, you will have a folder (or directory) containing six files (one for each microarray). The files are tab delimited text. Most spreadsheet and word processing programs will import files in this format.

The print version of this article was created on 6 Oct 2003. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.