Molecular Vision 2002; 8:130-7 <http://www.molvis.org/molvis/v8/a18>
Received 15 March 2002 | Accepted 22 April 2002 | Published 26 April 2002

Technical Brief

Evaluation and optimization of procedures for target labeling and hybridization of cDNA microarrays

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Purpose: To evaluate and optimize methods of target labeling and microarray hybridization using eye gene microarrays. Standardized protocols that consistently produce low background and high intensity hybridization with small amounts of starting RNA are needed to extract differentially expressed genes from a pool of thousands of unaltered genes.
Methods: Two identical aliquots of RNA from P19 cell line were labeled with Cy3 or Cy5 dyes using four different methods and self-against-self hybridization was performed on mouse eye gene arrays. The validity and reproducibility of these protocols were further examined using target RNAs isolated from wild-type or neural retinal leucine zipper (*Nrl*) knockout mouse retinas. Hybridizations were also carried out on human gene array slides with different amounts of starting RNA from human retina.
Results: Using self-against-self hybridization, we optimized the protocols for direct labeling (R-square = 0.93), aminoallyl indirect labeling (R-square = 0.97), Genisphere 3DNA labeling (R-square = 0.96), and for microarray hybridization and washing. Although small amounts of initial RNA can be used in TSA method, inconsistent labeling was encountered under our experimental conditions. When retinal RNA targets from $Nrl^{+/+}$ and $Nrl^{-/-}$ mice were tested by direct and aminoallyl indirect labeling protocols, both produced varying hybridization results with low intensity spots and non-uniform backgrounds. However, the Genisphere 3DNA labeling procedure consistently yielded strong hybridization and R-square values of 0.92 or higher. Furthermore, expression profiles were compatible with prior knowledge of this mouse model. Serial analysis of hybridizations with various starting amounts of RNA showed that the Genisphere 3DNA protocol could produce reliable signal intensity with 3 μg of total RNA.
Conclusions: We have systematically evaluated and optimized methods for target labeling, microarray hybridization and washing. These procedures have been used for expression profiling with 3 μg of starting RNA. Our studies should encourage further use of microarray technology for gene profiling during eye development and in retinal diseases.

Microarray technology is widely utilized for disease diagnostics [1,2], candidate gene identification [3,4], expression profiling [5,6], and pathway constructions [7,8]. Applications of this relatively new approach in vision research are rapidly growing with exciting prospects. Downstream targets of the photoreceptor homeobox gene *Crx* have been described by applying a small set of cDNA microarrays on $Crx^{+/+}$ and $Crx^{-/-}$ mouse models [9]. Expression profiles of specific eye tissues [10], retinal diseases [11], or biological processes [12,13] have been examined. To enlarge the repertoire of eye (particularly retina) expressed genes and to enhance the potential use of cDNA microarray technology in vision research, a large amount of ESTs expressed in eye and retina have been sequenced [14-17] and eye gene microarrays generated [18].

Owing to its high-throughput nature, cDNA microarray technology is vulnerable to systematic variations introduced during experimental processes [19]. Although a number of statistical algorithms have been developed to normalize microarray data and to control experimental variations [20-22], high quality input images are still the prerequisite for obtaining significant new output. This requires reproducible procedures for labeling of cDNA targets, prehybridization, hybridization, and washing of slides to consistently generate high intensity and low background images (high signal-to-noise ratios). A number of protocols, including direct and indirect labeling of cDNA targets, have been utilized in different laboratories [23] or by various vendors (Genisphere, Hatfield, PA; Perkin-Elmer, Boston, MA). However, a careful and systematic evaluation of these protocols has not been described, especially using slide microarrays. Furthermore, many of these techniques require over 10 μg of starting RNA, which makes it difficult to perform multiple microarray experiments when using eye tissues.

We have produced mouse eye and human retinal pigment epithelium (RPE) gene microarrays at the Sensory Gene Microarray Node, Kellog Eye Center, University of Michigan. Using several batches of slide microarrays, we have evaluated available protocols for labeling, hybridization and washing. In addition, we investigated the minimum amount of starting RNA needed to yield reproducible results using the preferred Genisphere 3DNA labeling method.

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METHODS

Generation of eye gene microarrays: Two cDNA libraries were constructed from mouse eyes at embryonic day 15.5 and postnatal day 2.5, respectively, and a third from adult retinas. The cDNA clones were isolated, amplified, and printed onto Corning CMT-GAPS slides (Corning Inc., Corning, NY), as previously described [18]. Two sets of mouse eye gene arrays, M2500 and M6000, were generated, containing nearly 2500 or 6000 cDNAs, respectively, that were randomly printed in duplicate. M2500 arrays were used to optimize microarray procedures by self-against-self hybridization, while M6000 arrays were hybridized with two different RNA targets to validate the optimized methods in identifying differential expression. To generate human microarrays, ESTs were obtained from two cDNA libraries constructed from native human RPE [14]. Slide arrays containing over 2500 of these clones (called H2500) were printed in duplicate as replicated super-grids. A range of starting amounts of RNA targets were hybridized to H2500 slides to estimate the minimum amount of RNA required for use with the Genisphere 3DNA labeling method.

Cell culture, tissue preparation and RNA isolation: P19, a teratocarcinoma cell line derived from an embryonic carcinoma induced in a C3H/He strain mouse, was cultured in alpha MEM (Sigma, St. Louis, MO) with ribonucleosides and deoxyribonucleosides adjusted to contain 1.5 g/L sodium bicarbonate, supplemented with 10% fetal bovine serum and 0.5 µM retinoic acid (Sigma) at 37 °C in a humidified atmosphere of 5% CO_2. Cells in tissue culture plates were washed with ice-cold PBS, and homogenized in Trizol (Invitrogen, Carlsbad, CA) for RNA isolation.

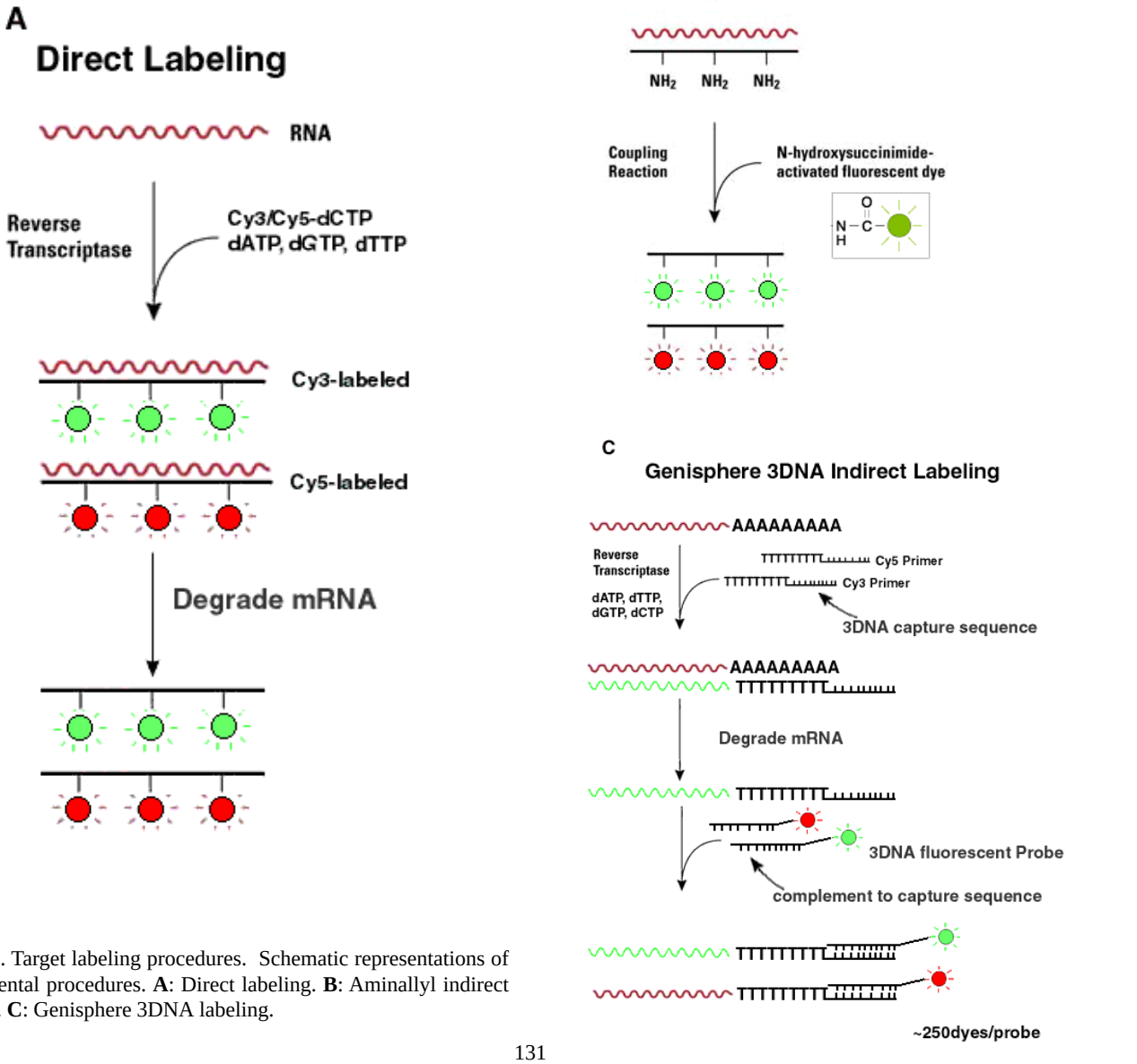

Figure 1. Target labeling procedures. Schematic representations of experimental procedures. **A**: Direct labeling. **B**: Aminallyl indirect labeling. **C**: Genisphere 3DNA labeling.

Retinas from *Nrl*$^{+/+}$ and *Nrl*$^{-/-}$ mice [24] were dissected at postnatal day 21. Animals utilized in this study were handled as approved by the University Committee on Use and Care of Animals (UCUCA, Ann Arbor, MI). Dissected retinas were immediately frozen on dry ice and kept in -80 °C freezer until use.

Two pairs of human eyes, one from a 46-year-old donor and the other from a 24-year-old donor, were obtained few hours after death from the Michigan Eye Bank (Ann Arbor, MI) and the National Disease Research Interchange (Philadelphia, PA), respectively. The donor eyes were acquired for research purposes with family consent and processed in compliance with University of Michigan regulations. The retina was dissected from the eye, rapidly frozen on dry ice, and kept at -80 °C.

Total RNA was isolated using Trizol reagent (Invitrogen) and further purified by RNeasy kit (Qiagen, Valencia, CA). Purity and RNA integrity were evaluated by absorbance at 260 nm and 280 nm, and by denaturing formaldehyde agarose gel electrophoresis. High quality RNAs with A260/A280 ratio over 1.9 and intact ribosomal 28S and 18S RNA bands were utilized for the microarray experimentation.

Direct labeling of cDNA targets: The direct labeling methods for fluorescent cDNA targets were reported previously [18] (Figure 1A) and are briefly summarized as follows. A mixture of 10 µg total RNA and 2 µg oligo-dT in a total volume of 22 µl was heated to 70 °C for 10 min and chilled on ice for 4 min. A reverse transcription labeling mixture of 18 µl was added to RNA to provide a final concentration of 25 µM dATP, 25 µM dGTP, 25 µM dTTP, 12.5 µM dCTP, 10 mM DTT, 1X first-stand buffer, 400 U SuperScript II, 40 U RNase inhibitor, and 12.5 µM Cy3-dCTP or 25 µM Cy5-dCTP (Amersham, Piscataway, NJ). The reaction was incubated at 42 °C for 2 h to generate fluorescent-labeled cDNA. Starting RNA template was removed by adding 2 U RNase H and 10 µg RNase A, followed by incubation at 37 °C for 15 min. Cy3

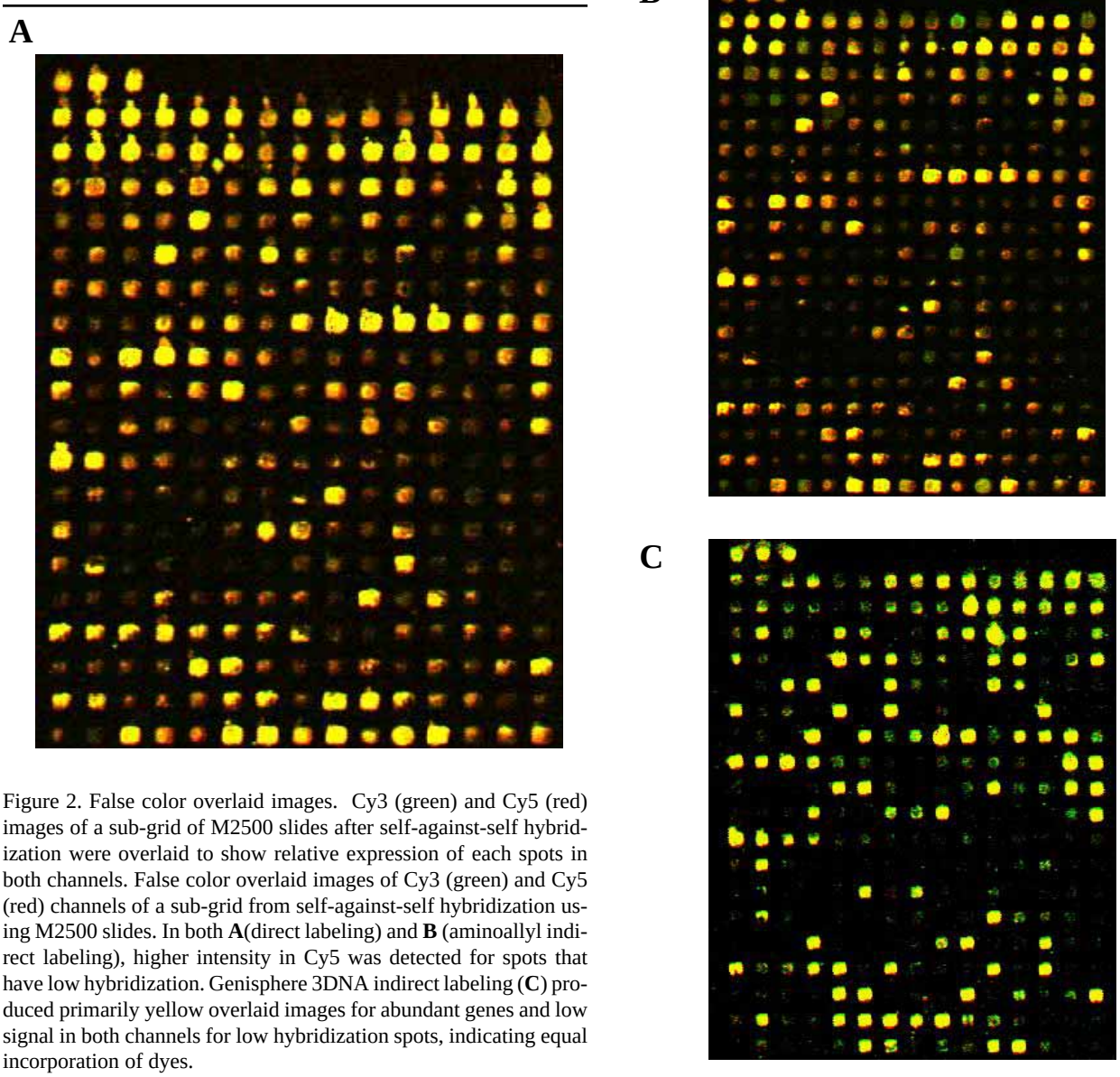

Figure 2. False color overlaid images. Cy3 (green) and Cy5 (red) images of a sub-grid of M2500 slides after self-against-self hybridization were overlaid to show relative expression of each spots in both channels. False color overlaid images of Cy3 (green) and Cy5 (red) channels of a sub-grid from self-against-self hybridization using M2500 slides. In both **A**(direct labeling) and **B** (aminoallyl indirect labeling), higher intensity in Cy5 was detected for spots that have low hybridization. Genisphere 3DNA indirect labeling (**C**) produced primarily yellow overlaid images for abundant genes and low signal in both channels for low hybridization spots, indicating equal incorporation of dyes.

or Cy5-labeled cDNA targets were mixed together, purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and concentrated to 10 µl. Blocking reagents, including 1 µg poly(A) RNA (Sigma), 2 µg mouse Cot-1 DNA (Invitrogen), 1 µg yeast tRNA (Invitrogen), and 10 µg salmon sperm DNA (Invitrogen) were added to the labeled cDNA, followed by the addition of an equal volume of 2X hybridization buffer (50% formamide, 10X SSC, 0.2% SDS).

Aminoallyl indirect labeling of cDNA targets: A number of protocols for aminoallyl indirect labeling (Figure 1B) were evaluated. The method we used is as follows. Briefly, 10 µg total RNA and 5 µg oligo-dT primers were mixed to a final volume of 18.4 µl, incubated at 70 °C for 10 min and snap-cooled in ice. Reverse transcription labeling mixture (11.6 µl) was then added to RNA to obtain a labeling reaction, containing 0.5 mM dATP, dCTP, dGTP, 0.3 mM dTTP, 0.2 mM aminoallyl-dUTP (aa-dUTP), 400 U SuperScript II, 10 mM DTT, and 1X first strand buffer. This mixture was incubated at 42 °C for 3 h or overnight to generate aminoallyl-labeled cDNA. To hydrolyze RNA template, 10 µl 1 M NaOH and 10 µl 0.5 M EDTA were added to the reaction and incubated at 65 °C for 15 min. The reaction was neutralized by 25 µl 1 M Tris-HCl (pH 7.5). Unincorporated aa-dUTP and free amines were removed by QIAquick PCR purification kit (Qiagen) and the sample was then vacuum dried. Aminoallyl-cDNA pellet was resuspended in 4.5 µl 0.1 M sodium carbonate buffer (pH 9.0) and coupled with Cy3 or Cy5 monoreactive dye (Amersham) prepared in DMSO for 1 h at room temperature in the dark. Uncoupled dyes were removed by QIAquick PCR purification kit (Qiagen). Cy3 and Cy5 labeled cDNA targets were mixed, vacuum dried and resuspended in 45 µl GlassHyb (Clontech, Palo Alto, CA).

MICROMAX Tyramide Signal Amplification (TSA) labeling of cDNA targets: This labeling was carried out using MICROMAX TSA Labeling and Detection Kits (Perkin-Elmer, Boston, MA) as previously described [25], except that 2 µg total RNA was used for each of the biotin or dinitrophenyl labeling.

Genisphere 3DNA indirect labeling of cDNA targets: Labeling of total RNA using this method is outlined in Figure 1C, and was performed using 3DNA Submicro Expression Array Detection kit according to manufacture's protocol (Genisphere, Hatfield, PA). Briefly, total RNA was reverse transcribed using reverse transcription (RT) primers tagged with either Cy3 or Cy5 specific 3DNA capture sequence. The synthesized tagged cDNAs were then fluorescent labeled by Cy3-3DNA or Cy5-3DNA based on the complementary of capture sequence with 3DNA capture reagents.

Microarray hybridization and signal detection: Microarray slides were prehybridized in buffer containing 5X SSC, 1% Bovine Serum Albumin and 0.1% SDS at 42-50 °C for 1 h and washed by dipping five times in distilled water. The slides were then dipped in isopropanol for 1 s and centrifuged at 1000 rpm for 2 min to dry in 50 ml un-capped centrifuge tubes.

The direct or aminoallyl indirect labeled targets were heated at 95 °C for 5 min, snap-cooled on ice for 30 s, and applied to prehybridized slide in a CMT-Hybridization chamber (Corning Inc., Corning, NY). Genisphere 3DNA labeled targets were incubated at 75-80 °C for 10 min, followed by 50 °C for 20 min before applied to prehybridized slide. A 22 x 60 mm coverslip (Grace Bio-Lab, Bend, OR) was cleaned with compressed air and then gradually placed on the slide to form a thin layer of labeled targets. To maintain humidity inside the

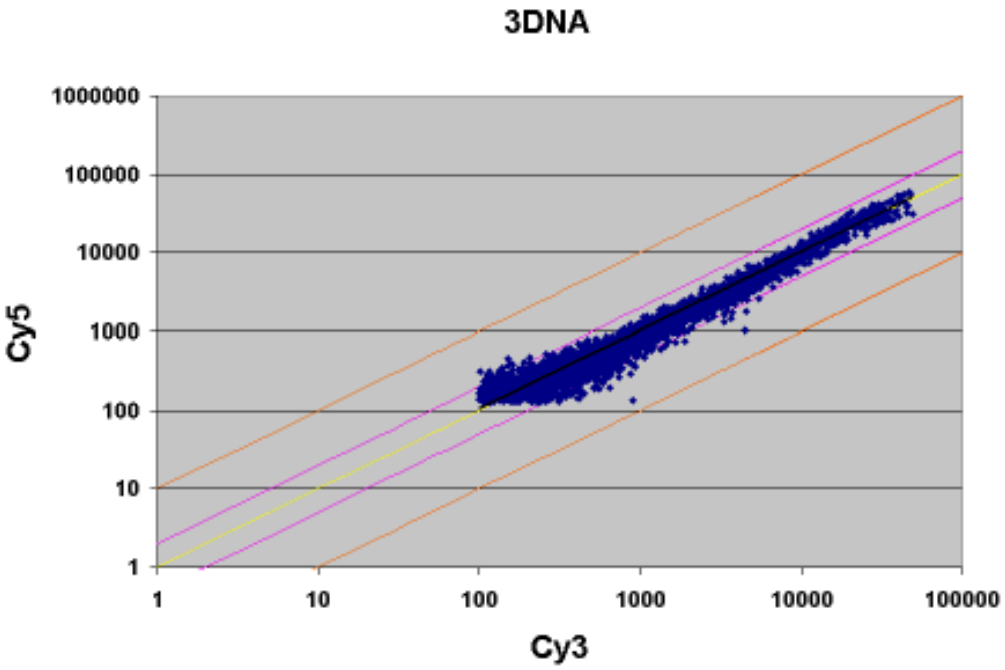

Figure 3. Scatter plot of self-against-self hybridization. Genisphere 3DNA labeled targets were hybridized to M2500 slides, containing over 5000 spots. The scatter plot indicates a majority of spots lie within 2-fold lines. Linear regression of data shows an R^2 value of 0.96.

chamber, 10 µl DEPC water was added to the two reservoir wells. The chamber was then tightly sealed and incubated at 42-50 °C water bath overnight for 16-20 h. Slide was then removed from the chamber, washed for 10 min sequentially in 2X SSC/0.2% SDS buffer, 2X SSC buffer and 0.2X SSC buffer, rinsed in distilled water for 5 s, and dried by centrifugation at 1000 rpm for 2 min.

The hybridized slides were scanned with Affymetrix 428 scanner (Affymetrix, Santa Clara, CA) using appropriate gains on the photomultiplier tube (PMT) to obtain the highest intensity without saturation. A 16 bit TIFF image was generated for each channel, Cy3 and Cy5.

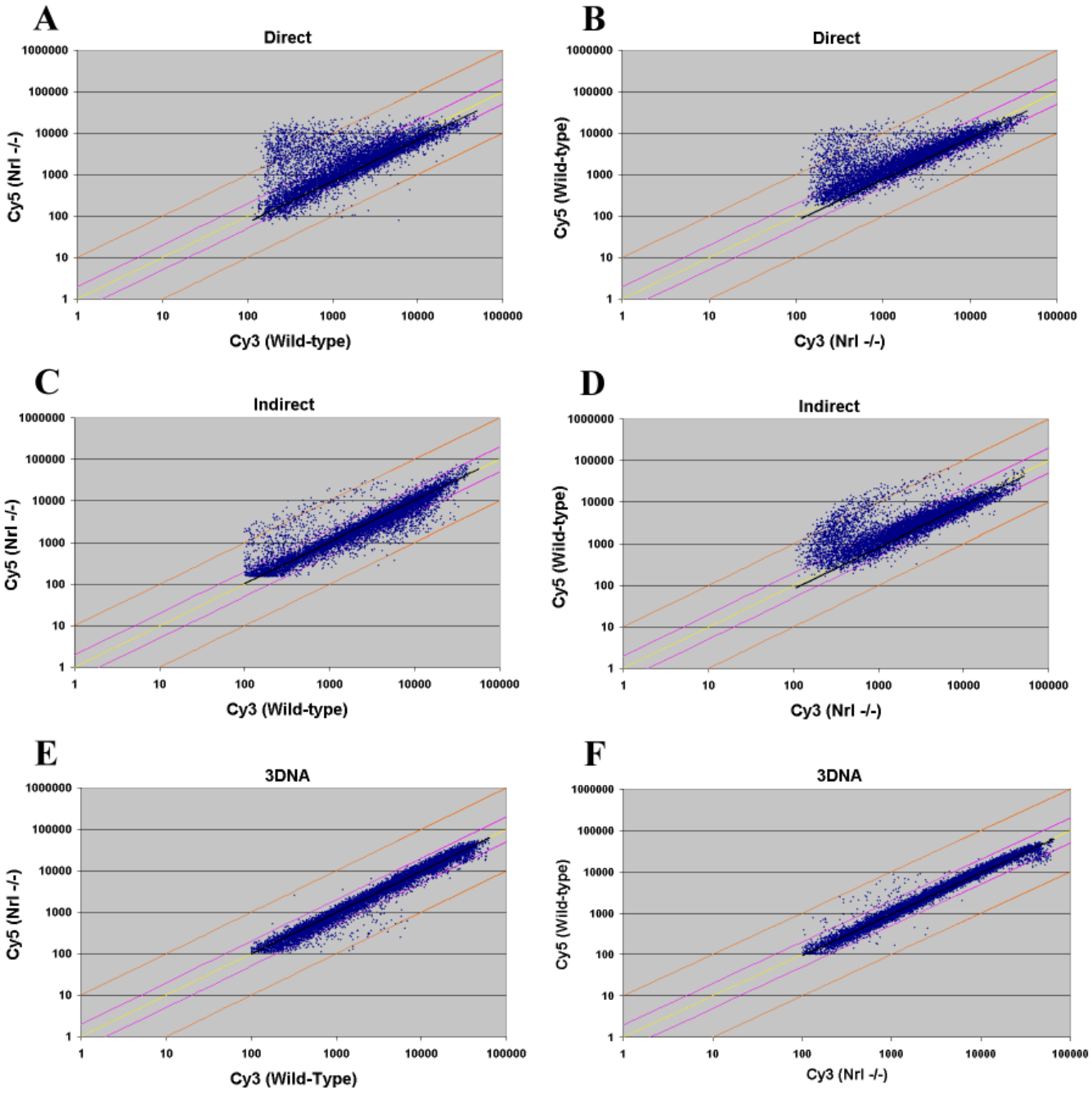

Figure 4. Scatter plots of two target hybridization. Two different targets, labeled using RNA either from *Nrl*$^{+/+}$(Wild-type) or *Nrl*$^{-/-}$ mice retina, were hybridized to M6000 slides, which contains over 13000 spots. The graphs on the left represent data generated by labeling RNA from *Nrl*$^{+/+}$ mouse retina by Cy3 and RNA from *Nrl*$^{-/-}$ mice retina by Cy5, while the graphs on the right was created by dye flip-over hybridizations. Similar scatter plots were observed between **A** and **B**(direct), and **C** and **D** (aminoallyl indirect), although a reverse pattern was expected for dye flip-over experiments (as shown in **E** and **F** with Genisphere 3DNA method). Furthermore, the Genisphere 3DNA method results in a higher R^2 value of 0.92 in both **E** and **F**, compared to 0.4 in **A**, 0.6 in **B**, 0.8 in **C**, and 0.6 in **D**.

Image extraction and data analysis: Scanned images for Cy3 and Cy5 were then overlaid with GLEAMS software (NuTec, Atlanta, GA). This software utilizes auto-segmentation and edge detection to calculate spot intensities and backgrounds. Signal-to-noise ratios (SNR) were calculated as the mean pixel intensity over the intensity standard deviation for all pixels in a spot. For each slide, an Excel-type spreadsheet was generated for further analyses. Spots with background-subtracted intensity lower than 100 in either Cy3 or Cy5 channel were filtered out. Global normalization was then applied to correct artifacts caused by different dye incorporation rates or scanner settings for two dyes. Scatter plots in log scale were performed to visualize fold changes between two channels by plotting background-subtracted Cy5 intensity against Cy3, with parallel fold lines across data points. A linear regression (using the data without the logarithmic transformation) trend-line with intercept at origin was applied to the scatter of background-subtracted Cy5 and Cy3 intensities and the coefficient of determination (R^2 value) was calculated to indicate how well Cy5 and Cy3 intensities fit in this linear relationship.

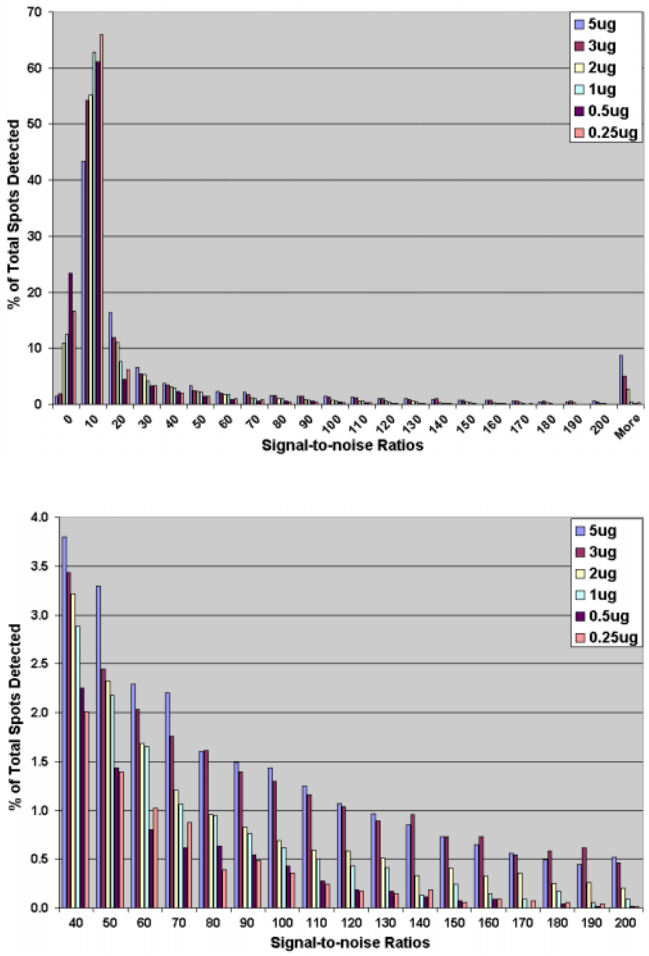

Figure 5. Signal-to-noise ratio (SNR) for serial hybridization. Histogram of SNR were constructed for all spots detected in a series of hybridizations performed with different amounts of starting RNA: 5, 3, 2, 1, 0.5, and 0.25 µg. **A**: With higher amounts of RNA, fewer spots have SNR lower than 20. **B**: Targets derived from 3-5 µg of total RNA resulted in more spots with higher SNR.

RESULTS & DISCUSSION

Optimizing microarray procedures using self-against-self hybridization: In order to perform self-against-self hybridization, total RNA isolated from P19 cell line [26] was divided into 2 aliquots that were labeled with Cy3 and Cy5 dyes, respectively. Since both dye-labeled samples were identical, these hybridizations should ideally produce similar intensities in both channels for every spot. Global normalization was applied to normalize Cy5 intensity against Cy3, based on the assumption that total intensity of Cy5 channel is equal to that of Cy3. This approach was adapted to account for different dye incorporation ratios, various scanning scales, and other systematic variations. The normalized data should produce a Cy3 and Cy5 overlaid false color image of primarily yellow and a scatter plot with the majority of spots having Cy3 intensity similar to Cy5. The four labeling methods were tested multiple times using M2500 slides and modified repeatedly to achieve the best possible results with slide microarrays.

The TSA method gave inconsistent labeling with either Cy3 or Cy5 in 3 trials of self-against-self hybridizations. We opted not to use this method because of time consuming post-hybridization manipulations and bias generated by signal amplification that may cause inconsistency.

A number of direct cDNA fluorescent labeling protocols, including those developed by Microarrays Inc. (Nashville, TN), Corning microarray technology (Corning Inc.), Amersham (Piscataway, NJ), and The Institute for Genomic Research (TIGR, Rockville, MD) [23], were evaluated by hybridizing P19 (Cy3) against P19 (Cy5) RNA targets using M2500 slides. Various blocking reagents, hybridization buffers, and washing conditions were also tried to produce optimal hybridization with highest intensity and lowest background. Overlaid images of Cy3 and Cy5 showed mostly yellow spots, although for low intensity spots preferential incorporation of Cy5 was observed (Figure 2A). The scatter plots demonstrated that 95% of spots lie within -2 and +2 fold lines [18].

Since Cy3 and Cy5 have different incorporation rates in direct labeling methods, aminoallyl indirect labeling protocols were examined. A protocol from TIGR consistently yielded low background and high intensity hybridizations in 4 self-against-self experiments using M2500 arrays; however, for low hybridization spots, signals from Cy5 channel were still slightly higher than Cy3 (Figure 2B). Scatter plot analysis of these slides showed regression with R^2 of 0.97 [18].

3DNA indirect fluorescent labeling method utilizes DNA dendrimer probes that include a “capture sequence,” which is complementary to 5'-end sequence of either Cy3 or Cy5 tagged RT primers. Self-against-self hybridizations using cDNA targets labeled by 3DNA method produced mostly yellow overlaid images of Cy3 and Cy5 (Figure 2C) with tight scatter plots (Figure 3). Furthermore, for low hybridization spots, it produced low signals for both channels, reflecting equal incorporation of dyes (Figure 2C).

Validation of labeling methods using two different RNA targets: Optimized protocols for direct, aminoallyl indirect, and Genisphere 3DNA methods were used to label retinal RNAs from *Nrl*$^{+/+}$ mice by Cy3 and *Nrl*$^{-/-}$ mice by Cy5. The labeled targets were then hybridized to M6000 arrays to identify differentially expressed genes. Since only a specific set of genes are altered in the *Nrl*$^{-/-}$mice retina relative to *Nrl*$^{+/+}$ [24], a tight scatter plot with few outlier spots is expected. Scatter plot analysis was applied to normalized data from three hybridizations utilizing the same batch of RNA labeled with different techniques (Figure 4A,C,E). Both direct and aminoallyl indirect labeled targets generated hybridization results with uneven background and scatter plots showed regression with R^2 less than 0.9. On the other hand, Genisphere 3DNA labeling protocol consistently produced results with 0.92 R^2 values. Furthermore, outlier spots generated by this method identified genes that were shown to be differentially expressed in the two RNAs [24]. Flip-over hybridizations, with RNA from *Nrl*$^{+/+}$ mice retina labeled by Cy5 and RNA from *Nrl*$^{-/-}$ mice retina by Cy3, showed that both direct and aminoallyl indirect labeling have signals biased to Cy5 (Figure 4B,D). Dye flip-over experiments using Genisphere 3DNA method generated flip-over scatter plots indicating equal incorporation of Cy3 and Cy5 dyes (Figure 4F).

Hybridization with different amounts of total RNA: To empirically estimate the lowest amount of RNA required for high quality hybridization, a series of self-against-self hybridizations were performed using H2500 slides with 0.25, 0.5, 1, 2, 3, and 5 µg starting RNA isolated from two pairs of human retinas. The target RNAs were labeled by the Genisphere 3DNA method. In spite of the six different RNA amounts used, high R^2 scatter plots were consistently obtained indicating equal incorporation of Cy3 and Cy5 dyes. However, hybridizations with less than 2 µg of RNA produced relatively low and variable spot intensities. In addition, the use of 3 µg or more total RNA resulted in higher signal-to-noise ratios (Figure 5).

In summary, we have evaluated and optimized protocols for fluorescent labeling of cDNA targets and hybridization conditions for cDNA microarray experiments. Four major labeling techniques, including direct, aminoallyl indirect, TSA, and 3DNA method, were examined using mouse eye arrays or human RPE arrays. The Genisphere 3DNA labeling method produced superior and consistent results in both self-against-self and *Nrl*$^{+/+}$ versus *Nrl*$^{-/-}$ mice retina RNA hybridizations. This procedure was found to be less time consuming and more robust. We believe that these protocols can serve as templates for researchers that intend to use slide microarrays for investigating expression changes during eye development and disease.

ACKNOWLEDGEMENTS

We thank Dr. Alan Mears for constructive discussions and Ms. Sharyn Ferrara for administrative assistance. Thanks are also due to the staff of the Michigan Eye Bank (Ann Arbor) and the National Disease Research Interchange (Philadelphia) for their help in obtaining human donor eyes. This research was supported by grants from the National Institutes of Health (EY11115, including administrative supplement, EY07961, and core EY07003), the Foundation Fighting Blindness, the Macula Vision Research Foundation, and Research to Prevent blindness (RPB). A.S. is a recipient of a Lew R. Wasserman Merit Award from RPB.

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