

High-Sensitivity Detection of DNA Hybridization on Microarrays Using Resonance Light Scattering

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The application of resonance light scattering (RLS) particles for high-sensitivity detection of DNA hybridization on cDNA microarrays is demonstrated. Arrays composed of ~2000 human genes ("targets") were hybridized with colabeled (Cy3 and biotin) human lung cDNA probes at concentrations ranging from 8.3 ng/ μ L to 16.7 pg/ μ L. After hybridization, the arrays were imaged using a fluorescence scanner. The arrays were then treated with 80-nm-diameter gold RLS Particles coated with anti-biotin antibodies and imaged in a white light, CCD-based imaging system. At low probe concentrations, significantly more genes were detected by RLS compared to labeling by Cy3. For example, for hybridizations with a probe concentration of 83.3 pg/ μ L, ~1150 positive genes were detected using RLS compared to ~110 positive genes detected with Cy3. In a differential gene expression experiment using human lung and leukemia RNA samples, similar differential expression profiles were obtained for labeling by RLS and fluorescence technologies. The use of RLS Particles is particularly attractive for detection and identification of low-abundance mRNAs and for those applications in which the amount of sample is limited.

Advances in microarray technology have enabled the massively parallel analysis of gene expression and the genotyping of mutations and polymorphisms.^{1–3} It is envisioned that this technology will significantly advance our understanding of disease and assist in the generation of novel and effective targets for drug discovery.⁴ In a typical gene expression experiment, RNA from two sources (reference and sample) is labeled with different fluorophores, mixed together, and hybridized to a DNA array. The ratio of fluorescence signals from the sample and reference is used to determine the over- or underexpression of each gene. Though powerful, one limitation of this technology is the relatively large amount of sample required per hybridization: typically 50–200 μ g of total RNA (or 2–5 μ g of poly(A) RNA) is labeled and used in a single experiment.⁵ For many applications, however, the

amount of genetic material available for analysis is extremely limited. Therefore, methods to increase the sensitivity of microarray-based analyses have been investigated. These methods can be broadly classified into two approaches—those based on the use of novel substrates such as planar waveguides⁶ and those based on the use of alternative labels such as near-infrared dyes,⁷ upconverting phosphors,^{8–10} DNA dendrimers,¹¹ and quantum dots.^{12,13} Although most of these technologies offer some advantages over traditional fluorescence (e.g., increased photostability, ability to multiplex) they have yet to afford the improvements in sensitivity required by many users. We describe and demonstrate here the use of resonance light scattering particles—RLS particles—as high-sensitivity labels for cDNA microarray applications.

RLS Particle labels are colloidal metal particles (generally between 40 and 120 nm in diameter) that efficiently scatter light. When illuminated with white light, RLS Particles scatter light of a specific color and intensity that is predictably determined by the size, shape, and composition of the particles.^{14,15} Thus, under appropriate illumination conditions, 80-nm-diameter gold particles scatter yellow light whereas 40-nm-diameter gold particles scatter green light. As detailed by Yguerabide and Yguerabide,¹⁴ the light-emitting power of a single RLS Particle label is orders of magnitude greater than a single fluorescent label. For example, the light-scattering power (product of the extinction coefficient and light-scattering yield) of an 80-nm-diameter gold RLS Particle is equivalent to $\sim 1 \times 10^6$ fluorescein molecules in light-producing power. Under dark-field illumination conditions, a single RLS Particle can be seen by eye using low-power (40 \times) magnification. On solid surfaces, RLS Particles can be detected by eye and

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integrated intensities accurately measured with simple instrumentation at surface densities as low as 0.003 RLS Particles/ μm^2 . Unlike fluorescent labels, the light signal generated by RLS Particle labels is not prone to quenching and does not photobleach with repeated or continuous exposure to light, allowing experimental results to be permanently archived. The surface of RLS Particles can be functionalized with proteins, nucleic acids, and other biomolecules using a variety of attachment and bioconjugate chemistries to impart specific binding of these labels for use in bioanalytical assays.

The use of colloidal metal particles for biological applications is not new. Gold- and silver-staining techniques based upon optical absorbance properties of small (typically <20 nm) metallic particles have been utilized for many years in the fields of microscopy and immunochemistry.¹⁶ Colloidal silver particles have been employed previously for in situ hybridization and immunocytology applications,¹⁷ and the use of silver-enhanced colloidal particles on DNA oligonucleotide arrays using optical absorbance has recently been reported.¹⁸ Detection measuring light scattering of RLS Particle labels provides dramatically enhanced sensitivity over optical absorbance (typically >3 orders of magnitude) in various bioassay applications. The potential for RLS Particles as highly sensitive tracers in cell biology has been previously documented.¹⁵ Mirkin and co-workers recently reported the use of light-scattering particles for enhanced discrimination of single-nucleotide polymorphisms in a model oligonucleotide system.¹⁹

In this report, we compare the performance and sensitivity of fluorescent and RLS Particle labels in hybridization experiments using a complex probe and high-density cDNA microarrays. We demonstrate that RLS Particle labels can provide a significant increase in sensitivity over fluorescent labels.

EXPERIMENTAL CONSIDERATIONS

Reagents. Human lung and leukemia mRNA (Clontech); Superscript II reverse transcriptase, DTT, RNase H, formamide, human Cot 1 DNA (Life Technologies); Cy3-dCTP, Cy5-dCTP, biotin-11-dCTP (Perkin-Elmer); random 9-mers (Stratagene), RNase A (USB), QIAquick PCR purification kit (Qiagen); and bovine serum albumin (BSA), dextran sulfate, nuclease-free water, and poly A (Sigma) were all used as received. RLS Particles and reagents were provided by Genicon Sciences Corp. under a collaborative research agreement.²⁰ The RLS Particles employed in this study had diameter CVs of <10%. Modification of the particles with anti-biotin antibodies was accomplished by passive adsorption.

Array Fabrication. The arrays employed in this study were fabricated by a novel, high-throughput contact printing technology developed at Corning Inc. that allows for the simultaneous

deposition/printing of 1024 elements. Briefly, an extruded glass serves as the DNA reservoir. A matching pinplate containing ~100- μm -tall, ~100- μm -diameter circular pins is "inked" with the DNA from the reservoir and then printed onto an aminosilane-modified glass substrate (Corning CMT-GAPS slides). Two 1024-element subgrids were printed on each slide; each subgrid consisted of 960 human genes, 9 *Bacillus subtilis* genes, and 55 blanks in which no DNA was printed.

RNA Labeling. A 22- μL solution containing 2 μg of human poly A RNA and 2.5 μg of random 9-mers was incubated for 5 min at 70 °C, briefly chilled on ice, and then added to a 20- μL solution containing (i) 8 μL of 5 \times Superscript II buffer; (ii) 4 μL of 100 mM DTT; (iii) 2 μL of a dNTP mixture consisting of 10 mM each dGTP, dATP, and dTTP and 1 mM dCTP; (iv) 2 μL of Cy3 or Cy5 dCTP (1 mM); (v) 2 μL of biotin-dCTP (1 mM); and (vi) 2 μL of reverse transcriptase. This combined 42- μL solution was incubated for 10 min at room temperature followed by a 1-h incubation at 42 °C. After addition of 1 μL of reverse transcriptase, the mixture was incubated for an additional hour at 42 °C. The RNA was degraded by the addition of 1 μL of RNase H and 0.25 μL of RNase A followed by incubation for 15 min at 37 °C. Probes were purified using a QIAquick PCR purification kit according to the manufacturer's instructions. The cDNA concentration and the amount of Cy3/Cy5 incorporation was measured on an Agilent 8453E UV-visible spectrometer.

Hybridization. Arrays were prehybridized by soaking in the following solutions: (i) 2 \times SSC/0.05% SDS/0.2% BSA at 42 °C for 10 min; (ii) 1 \times SSC for 2 min; (iii) 0.2 \times SSC for 2 min. After the final wash, the slides were spin-dried in a centrifuge at 2000 rpm for 2 min. Each array was hybridized with a solution consisting of 29% formamide, 2.25 \times SSC, 6% dextran sulfate, 0.17 $\mu\text{g}/\mu\text{L}$ poly A, 0.10 $\mu\text{g}/\mu\text{L}$ Cot 1 DNA, 0.2% BSA, and a given amount of labeled cDNA. For hybridization, 60 μL of this solution was spotted onto the array and then spread over the entire surface using a 24 mm \times 60 mm coverslip. The arrays were incubated overnight at 42 °C, immersed for 1 min in 2 \times SSC/0.05% SDS at 42 °C to remove the coverslip, and then washed by soaking in the following solutions: 2 \times SSC/0.05% SDS at 42° (5 min); fresh 2 \times SSC/0.05% SDS at 42° (5 min); 1 \times SSC at room temperature (5 min); fresh 1 \times SSC at room temperature (5 min); 0.2 \times SSC at room temperature (2 min); fresh 0.2 \times SSC at room temperature (2 min); fresh 0.2 \times SSC at room temperature (2 min). The slides were spin-dried in a centrifuge at 2000 rpm for 1 min.

RLS Particle Binding. Prior to treatment with RLS Particles, the arrays were first blocked for 10–15 min in a blocking solution (150 mM NaCl, 50 mM sodium phosphate, 1% BSA, 0.001% Proclin 5000, pH 7.4). Excess blocking solution was shaken off, and the arrays were then covered with 200 μL of a solution containing 100 μL of anti-biotin antibody-coated RLS Particles and 100 μL of RLS diluent buffer (final particle concentration of 3 o.d. units). RLS Particle binding was allowed to proceed for 1 h at room temperature, after which the slides were rinsed in buffer and then dried.

Imaging. A GenePix 4000A (Axon Instruments) fluorescence scanner (sensitivity of ~0.1 fluorophores/ μm^2 for Cy3 and Cy5) was used to obtain the Cy3/Cy5 fluorescence images using a PMT setting of 750–950 V. A prototype 14-bit CCD-based, white light illumination system (Genicon Sciences) was used to obtain the

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(20) RLS Particles and RLS Technology-related reagents, chemistries, instrumentation, and software are proprietary to Genicon Sciences Corporation (U.S. Patent 6,214,560 and patents pending) and are anticipated to be commercially available early in 2002. The research community will have "open access" to the technology via product label license rights and research collaborations.

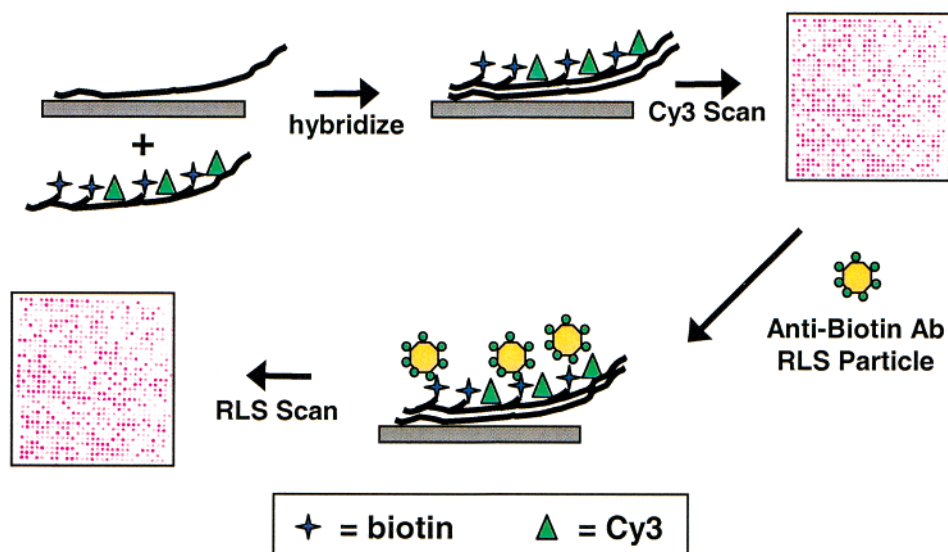


Figure 1. Outline of experimental protocol. cDNA probes generated by the colabeling of lung RNA with Cy3 and biotin are hybridized to an array. Following several wash steps to remove unbound probe, the array is scanned in a fluorescence scanner to capture the Cy3 fluorescence image. Treatment of the array with RLS Particles is accomplished by incubating the slides with anti-biotin antibody-coated 80-nm-diameter gold RLS Particles. The same array is then imaged in a CCD-based white light imaging system to generate the RLS image.

RLS images. The exposure times used were 0.2–1 s. All images were analyzed using GenePix Pro 3.0 analysis software (Axon Instruments).

RESULTS AND DISCUSSION

Enhanced Sensitivity of RLS versus Cy3. The performance and sensitivity of the RLS technology for microarray applications was investigated as outlined in Figure 1. Human lung mRNA was colabeled with Cy3 and biotin using equimolar amounts of the correspondingly labeled dCTP during reverse transcription. This experimental protocol utilizing colabeled probes was designed to allow the *direct* comparison of Cy3 and RLS labeling on the same cDNA strand. The incorporation level of Cy3 for a typical labeling reaction was ~3 dye molecules/100 bases; although the incorporation efficiency of biotin is not directly measurable, we assume it is as good as, if not better than, the incorporation of Cy3.²¹ These colabeled probes were hybridized to cDNA arrays composed of ~2000 human genes. Following several wash steps to remove unbound probes, the arrays were scanned in a fluorescence scanner to obtain the Cy3 image. Treatment of the arrays with RLS Particles was accomplished by incubating the slides with a buffer solution containing 80-nm-diameter gold RLS Particles coated with anti-biotin antibodies. A CCD-based white light imaging system was used to capture the RLS image.

The higher sensitivity of RLS compared to Cy3 fluorescence was demonstrated in a series of probe titration experiments. Figure 2 shows the results of these hybridizations in which the labeled probe amount was varied from 500 (8.3 ng/ μ L) to 1 ng (16.7 pg/ μ L). Representative Cy3 and RLS images of a 1000-element subgrid of the arrays after hybridization are shown in

Figure 2A. For hybridizations performed using 500 ng of labeled probe, approximately the same numbers of genes were detected by both methods. As the amount of probe was decreased, RLS detection significantly outperformed Cy3 fluorescence in terms of the number of genes giving positive signals. This enhanced sensitivity is most dramatically seen by comparing the Cy3 and RLS images obtained after hybridization with 1 ng of probe. Essentially no detectable signal is observed in the Cy3 image whereas significant signal for many genes is observed in the RLS image. We quantify this enhanced sensitivity by determining the number of genes on the entire 2000-element array with a signal-to-background ratio of >1. In this analysis, the signal-to-background ratio is defined as

$$(S - B)/B \quad (1)$$

where S is the mean raw signal intensity of the spot and B is the median local background intensity of the spot. The results of this comparative analysis are presented in Figure 2B. While a similar number of genes was detected with Cy3 and RLS at high (500 ng) probe concentrations, RLS significantly outperformed Cy3 at low probe concentrations. Specifically, for hybridizations performed with 5 ng (83.3 pg/ μ L) of probe, the RLS technology detected 10 times as many genes compared to Cy3, and with 1 ng of probe, the RLS technology detected >300 times as many genes. Moreover, as seen in Figure 2C, the number of genes detected by Cy3 declines rapidly for each 10-fold decrease in probe concentration; in contrast, the decrease in the number of genes detected with decreasing probe concentration is significantly more gradual for RLS. While the observed sensitivity of RLS is much higher than Cy3, especially at low probe concentrations, it is lower than that predicted by theory.¹⁴ We hypothesize that there are three primary reasons for this discrepancy: (i) steric effects due to the relatively large size of the RLS Particles; (ii) the use of passively adsorbed antibodies on the RLS Particles that are prone to desorption; if present, these free antibodies compete for binding

(21) Due to the size of the RLS Particles, it is highly unlikely that every molecule of biotin binds a separate RLS Particle. Moreover, lateral steric hindrance²² prevents the binding of all the biotin groups in the surface-bound probe DNA to the anti-biotin antibody-coated RLS Particles. Therefore, the incorporation of biotin is not the limiting factor in the determination of the differential sensitivity of RLS versus Cy3.

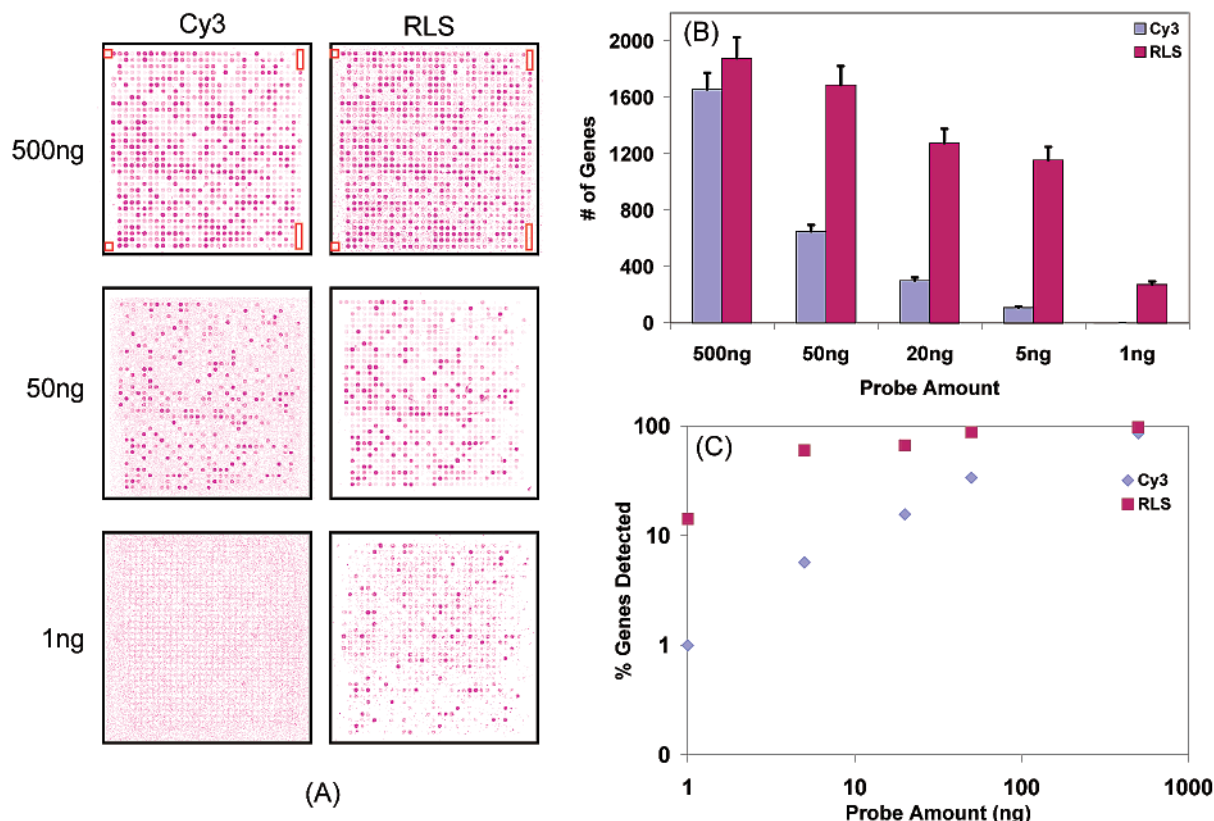


Figure 2. Hybridization results comparing RLS and Cy3 signal intensity as a function of input probe concentration. (A) Representative Cy3 and RLS images of a 1000-gene subgrid. The red boxes in the top two images show the location of printed *B. subtilis* control genes which serve as negative controls. No significant Cy3 or RLS signal was observed for these control genes. (B) Plot of the number of genes on the entire 2000-element array with a signal-to-background ratio of >1 as detected by Cy3 and RLS labeling for different amounts of probe. (C) log-log plot of the percentage of genes on the entire 2000-element array with a signal-to-background ratio of >1 as a function of probe amount.

to the biotin-labeled probe. It is possible that covalent immobilization of the antibodies on the RLS Particles would further increase the sensitivity. (iii) Slow kinetics of binding²² of RLS Particles to the surface-bound biotin groups.

Specificity and Reproducibility of RLS and Correlation with Cy3. The specificity of the hybridization signal detected by RLS was examined by measuring the signal intensity of several control spots on the array. These control spots consisted of (i) 18 *B. subtilis* genes as negative controls and (ii) 110 "blank" spots in which no DNA was printed; the array locations outlined in red in the two images of Figure 2A show the location of the *B. subtilis* controls. No significant signal was observed by RLS detection in any of these control spots, and we thus infer that the binding of RLS Particles to the array is hybridization specific.

The correlation between RLS and Cy3 detection was analyzed by comparing the net Cy3 and RLS signal intensities (i.e., signal - background) obtained for each gene in a 1000-element subgrid of the array after hybridization with 100 ng of colabeled probe. The results of this analysis (data not shown) show a good correlation between Cy3 and RLS detection ($R^2 = 0.83$), indicating that either detection technology would reveal the same relative expression pattern.

To investigate the reproducibility of RLS Particle binding, hybridizations were performed on two different slides using the

same amount of probe (100 ng). As described for previous experiments, human lung mRNA was colabeled with Cy3 and biotin, and both Cy3 and RLS images were obtained for each slide. The results of these experiments are presented in Figure 3, which shows plots of the net hybridization signals obtained on the first slide against the net hybridization signals obtained on the second slide, for both Cy3 and RLS. In each case, the data are clustered around the diagonal with a similar correlation, suggesting that the reproducibilities of the two labeling technologies are comparable. Specifically, the average coefficient of variation (CV) for each gene is 22% and 27% for the Cy3 and RLS curves, respectively. An analysis of Figure 3B also reveals that the linear dynamic range of the RLS signal is >2 log units.

Differential Gene Expression Using RLS. We performed a differential gene expression experiment to demonstrate and validate the use of RLS for expression analysis and to further verify that the extra genes detected by RLS were due to specific hybridization and not due to nonspecific binding of the RLS Particles to the array. This experiment compared the expression profiles of two different RNA samples (human lung tissue and a leukemia cell line). Half of each RNA sample was colabeled with Cy3 and biotin; the other half was labeled with Cy5 only.

Three different arrays were employed for this study. One array was hybridized with a mixture containing 100 ng of lung and 100 ng of leukemia cDNA probes. These probes were obtained from the labeling of leukemia RNA with Cy3 and the labeling of lung

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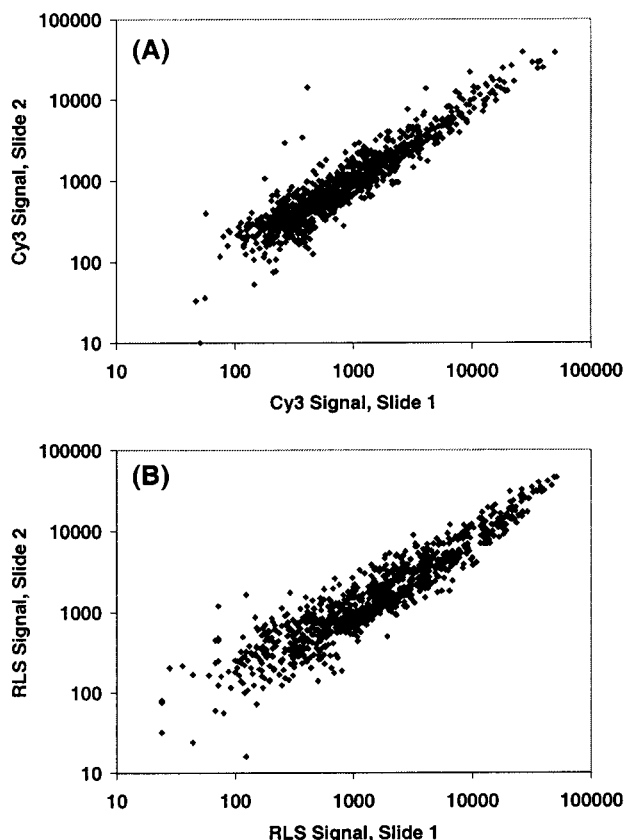


Figure 3. Reproducibility of Cy3 and RLS. Plots of the hybridization signals from replicate hybridizations performed on two different slides using 100 ng of labeled probe generated from lung mRNA. The graphs show the net hybridization signal obtained for slide 1 plotted against the net hybridization signal obtained for slide 2, for both Cy3 and RLS detection. Both labeling technologies possess comparable reproducibility as indicated by the similarity in shape and width of the two scatterplots.

RNA with Cy5. A composite image of a 1000-element subgrid for this hybridization is shown in Figure 4A. Here, the Cy3 and Cy5 fluorescence signal intensities are represented in green and red, respectively. Accordingly, those genes that are more highly expressed in leukemia appear green, those that are more highly expressed in lung appear red, and those that are equally expressed appear yellow. For RLS detection, differential expression was analyzed by hybridization assays on two different arrays because of the current availability of only single-color RLS.²³ Figure 4B shows the RLS images for these hybridizations (the corresponding Cy3 fluorescence images are not shown). A comparison of the two RLS images with the Cy3/Cy5 fluorescence image reveals that, in general, those genes that appear differentially expressed by fluorescence also appear to be differentially expressed by RLS.

To evaluate and compare the performance of RLS against fluorescence for gene expression analysis, a differential gene expression (DGE) ratio was calculated for each gene with a $s/b > 1$. This was done by taking the net signal intensity after hybridization with the leukemia cDNA probes and dividing by

(23) Single-color detection (i.e., one particle type of a particular size and composition) using RLS was used for sensitivity comparison and differential gene expression analysis. Development of additional RLS particle sizes and compositions is in progress; this development will allow "multicolor" detection.

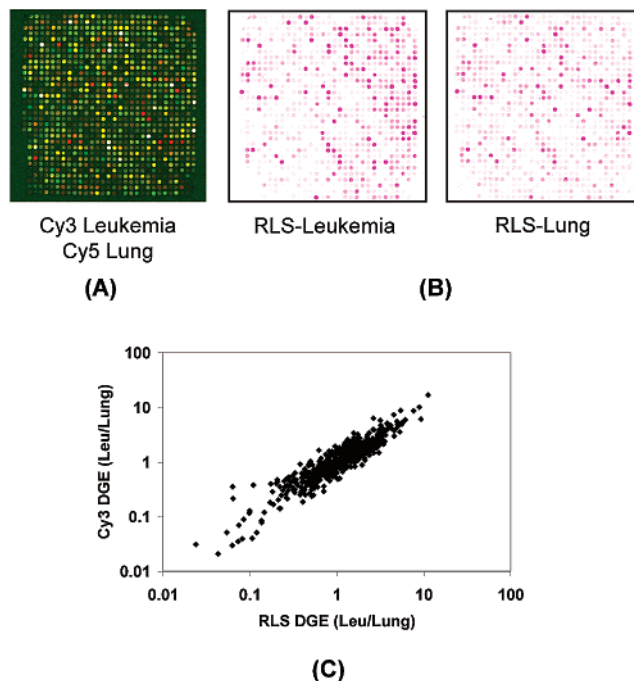


Figure 4. Differential gene expression profile as determined by fluorescence and RLS. The expression profiles of human lung and leukemia RNA samples were examined. These samples were labeled with either (i) Cy3/Cy5 or (ii) Cy3/biotin followed by staining with anti-biotin antibody-coated RLS Particles. Three different arrays were employed for this study. One array was hybridized with a mixture of 100 ng of Cy5-labeled lung and 100 ng of Cy3-labeled leukemia cDNA probes. For RLS analysis, differential expression was determined by hybridization on two different arrays, one each for lung and leukemia samples colabeled with biotin and Cy3. (A) Composite fluorescence image of a 1000-element subgrid. The Cy3 and Cy5 fluorescence signal intensities are represented in green and red, respectively. (B) RLS images of a 1000-element subgrid. (C) Correlation of the DGE ratios as determined by RLS versus the DGE ratios for the same slides as determined by Cy3.

the net signal intensity after hybridization with the lung cDNA probes. Traditionally, DGE is carried out using two-color fluorescence on a single slide, which removes artifacts associated with slide-to-slide variability. Nevertheless, for the differential gene expression analysis presented here, we used the results from Cy3 fluorescence with the same two slides used for differential expression analysis by RLS. This comparative analysis between the two detection methods removes any bias associated with analyzing data from one slide for fluorescence detection and two slides for RLS detection. Figure 4C shows the results of this analysis for a 1000-element subgrid of the array. In this figure, the DGE ratios as determined by RLS using two slides are plotted against the DGE ratios for the same two slides as determined by Cy3. A strong correlation between RLS and Cy3 is observed in the plot ($R^2 = 0.83$). Out of the 1000 genes in the subgrid, 141 were found to have a DGE ratio greater than 3 or less than 0.3. Among the genes observed to be overexpressed in leukemia were putative oncogenes and several genes encoding for different ribosomal proteins. Many genes that encode for antibodies were identified as being underexpressed in the leukemia RNA sample relative to the lung RNA sample. This was an expected result because the leukemia RNA employed in this study was derived from a K-562 cell line (ATCC number CCL-243) that consists of

multipotential, hematopoietic cells; these cells do not express genes encoding for antibodies.²⁴⁻²⁶

CONCLUSIONS

We have demonstrated that the RLS labeling technology is significantly more sensitive than Cy3 labeling for DNA microarray applications. This higher sensitivity allows for (i) the identification of very low expressors not detectable by conventional (Cy3/Cy5) fluorescence and (ii) the utilization of less sample per assay while

maintaining a strong signal-to-background ratio. The reproducibility of single-color RLS detection was similar to that observed for single-color fluorescence. Despite the large differences in the size of the labels, there was a good correlation between differentially expressed genes detected by RLS and those detected by fluorescence. We anticipate that the implementation of two-color RLS labeling, currently under development, would enable the measurement of smaller changes in differential gene expression relative to multislide, single-color RLS.

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