

One-Color Differential Gene Expression Profiling on Microarrays Using Ultra-Sensitive Resonance Light Scattering (RLS) Particles

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Abstract

Differential gene expression analysis is one of the most widely used applications of microarray technology. This approach relies on the ability to accurately compare disparate biological samples while minimizing experimental variability that might influence comparative data. There are a number of detection strategies currently employed for differential gene expression studies on microarrays. One approach is a one-color detection strategy, whereby two different samples are hybridized to two separate microarrays and detected with the same label. This approach eliminates the experimental variability introduced by the use of two different labels, but requires normalization for inter-slide variability. A two-color detection approach involves the labeling of the two different samples with separate labels and simultaneous hybridization of both samples to the same microarray. This approach minimizes inter-slide variability but is subject to the effects of inter-label variability and therefore requires normalization to address this label-induced inconsistency. A common approach to circumvent the problems associated with inter-label variability in a two-color detection system is to perform reciprocal labeling. However, because two microarrays are required for hybridization with this approach, microarray and sample consumption are increased, and the need to normalize for inter-slide variability is reintroduced. A differential gene expression analysis utilizing a one-color Resonance Light Scattering (RLS) Technology detection approach was performed. The results illustrate that the differentially expressed transcripts identified utilizing a linearly normalized RLS one-color detection method match well with those transcripts determined to be differentially expressed using a linearly normalized fluorescent two-color reciprocal labeling approach. It is shown that the increased sensitivity of the RLS Technology enables the identification of an additional 26 differentially expressed transcripts that fall below the threshold for fluorescence detection. Furthermore, the RLS data was generated using 5-fold less input RNA. The increased sensitivity of the one-color RLS detection approach allows for the ability to measure the expression of low abundance transcripts identifying more differentially expressed transcripts than fluorescence approaches with a reduced consumption of sample RNA.

Introduction

Gene expression microarray studies require sensitive, reproducible and easy to use labeling strategies. Commonly utilized fluorescent

labeling approaches lack the sensitivity required for detection of low abundance transcripts without signal or target amplification, especially when starting sample material is limited (1,2,3). Target amplification strategies are used to increase the sensitivity of fluorescence detection, but these methods introduce bias. Due to these sensitivity limitations, relatively large amounts of total RNA, typically 10 - 20 μg , are required to perform microarray experiments using a fluorescence approach without target amplification. This input requirement can be prohibitive for many investigators, including those studying clinical samples or homogeneous cell populations obtained using laser capture microdissection or FACS.

RLS Technology, an ultra-sensitive, reproducible non-fluorescent signal generation and detection technology enables the detection of low abundance transcripts using less sample input material without amplification. RLS is based on nano-sized metal colloidal particles (RLS Particles) of uniform dimension that generate highly intense scattered light signals when illuminated with configured white light (4,5). RLS Particles can be used as ultra-sensitive labels for a wide variety of analytical bioassays. The colored light signal generated by a single RLS Particle is 10^4 - 10^6 times greater than the signal obtained from the most sensitive fluorescent molecules commonly used as labels in bioanalytical assays. The optical light scattering behavior of the particles is predictable based on proprietary algorithms that relate the signal intensity and color spectrum of the particles to their size, shape and composition. In addition, RLS signals are not subject to the effects of photo-bleaching or quenching, and as such are archiveable, forming a permanent record. To effect specific binding in analytical bioassays, the surface of RLS Particles can be derivatized with a variety of biomolecules, including proteins, antibodies, small molecule ligands, nucleic acids and oligonucleotide probes.

The use of microarrays in differential gene expression studies requires that one addresses the experimental variability introduced by the use of either multiple labels or multiple microarrays. This paper describes the application of RLS Technology as a signal generation and detection platform for one-color differential gene expression microarray experiments. The results illustrate that RLS Technology offers accurate gene expression detection coupled with increased sensitivity, enabling the study of previously undetected differentially expressed genes.

One-Color Microarray Approach for Differential Gene Expression Analysis

To demonstrate the value of RLS Technology for differential gene expression analysis, an experiment was performed using a one-color labeling approach. Biotin-labeled target cDNAs from human normal lung and lung tumor RNA sources were hybridized to separate 1.7K human microarrays (see experimental protocol:Arrays) using 2 μ g of total RNA per sample. Anti-biotin 80 nm gold RLS Particles were introduced to the microarray assay during post-hybridization to bind to the hybridized biotin-labeled cDNA targets. The RLS signals on the microarray slides were collected as a TIFF image using the white-light/CCD based GSD-501 RLS Detection and Imaging Instrument. A standard fluorescent two-color direct incorporation reciprocal labeling approach using 10 μ g total RNA per sample was performed as a comparison. Fluorescent signals were collected as a TIFF image using the Axon GenePix 4000B laser scanner. Both the RLS and fluorescence experiments were performed in replicates of 10 arrays. RLS TIFF images were analyzed with ArrayVisionRLS image analysis software using Genicon Sciences' proprietary linear normalization feature. Fluorescence TIFF images were analyzed in the fluorescence mode of ArrayVisionRLS also using the linear normalization feature.

Genes were scored as expressed when their feature signal intensities were measured to be 2.5 times greater than the median gene intensity calculated for the 256 *A.thaliana* negative control features on the arrays. The median %CV was also calculated for each label and tissue sample as reported in Table 1. On average, while using 5-fold less input material, about 50% more genes were detected using the RLS detection approach than with fluorescence. This difference in number of positive features detected by RLS vs. fluorescence is consistent with data from previous experiments across a variety of microarray systems (data not shown). Slide-to-slide variation as indicated by median %CV, was equivalent or better than fluorescence, indicating comparable reproducibility and precision.

To compare feature intensities between slides, a linear normalization approach was used with both RLS and fluorescence. This linear normalization technique applies a transform algorithm to the data set of one of the arrays to normalize for assay variability. This algorithm is generated from the slope and y-intercept of a best-fit linear regression line that is generated when the bulk of the features (presumed to be non-differentially expressed features) are plotted against one another. In the one-color RLS assay, the normal lung array with the highest overall intensity was used as the control for normalization of the other RLS detected arrays. For the two-color fluorescence assays, the Cy5 data set was normalized to the Cy3 data set.

Label and Tissue Type	Median %CV Positive Features	Positivity Rate (S/B > 2.5)
RLS Normal Lung	29%	38%
Cy3 Normal Lung	33%	24%
Cy5 Normal Lung	30%	24%
RLS Lung Tumor	26%	31%
Cy3 Lung Tumor	29%	17%
Cy5 Lung Tumor	33%	25%

Table 1: Slide-to-slide variation and positivity rates for a 2 μ g input total RNA for RLS and 10 μ g input RNA for fluorescence detection on University Health Network 1.7k human oncology microarrays

A minimum of a 2-fold change in measured expression level was used as a threshold for establishing differential gene expression. Statistical significance was verified by performing a t-test to confirm a greater than 95% confidence level for those genes scored as differentially expressed. 20 genes were uniformly identified as differentially expressed and exhibited similar fold changes in expression patterns as measured by both RLS and fluorescence detection technologies (Figure 1). In addition, 26 genes were detected to be down-regulated by RLS Technology, but fell below the limits of detection for one or both fluorescence data sets (Table 2). Only a single gene was inconsistently determined as differentially expressed by the different methods, where RLS detection showed an increase in expression level in the lung tumor sample, while the fluorescence data showed no change. While the RLS result for this particular gene has not yet been confirmed by quantitative RT-PCR, it is interesting to note that the amplified material comprising this array feature was originally isolated from a human lung large cell carcinoma (Genbank accession number BE877666). RNA samples have been retained for confirmation of this gene's differential gene expression using outside quantitative RT-PCR techniques. In summary, the number of differentially expressed genes more than doubles (47 vs. 20) when using one-color RLS detection technology when compared with fluorescence, despite using 5-fold less input material per sample.

Of the 26 genes that were detected by RLS to be differentially expressed and were below the limits of detection for fluorescence, a subset of 5 randomly selected genes, were evaluated by manual quantitative RT-PCR techniques. Though the total RNA samples were treated with DNase I prior to purchase, a second DNase I treatment was performed as an additional precaution to remove any remaining genomic DNA contamination. This total RNA was then converted to cDNA using a poly d(T) primer and standard protocols. PCR reactions using gene specific primers and cDNA as a template were stopped every 5 cycles up to 40 cycles. The PCR products were separated on gels and quantitated by SYBR green staining. All 5 genes that were detected by RLS and undetected by fluorescence produced measurable products between 30 and 35 cycles for normal lung tissue and ranged from detectable at 35 cycles to undetectable at 40 cycles in human lung tumor tissue. Both a no RT enzyme and a template-free reaction were run in parallel as negative controls. These reactions were uniformly negative for the presence of PCR amplicons, indicating that the amplicons generated from the cDNA samples were derived from mRNA and not from any DNA contamination. The results of these RT-PCR experiments indicate that the mRNAs detected utilizing RLS were indeed present in the normal lung RNA sample. These RT-PCR results provide further evidence that RLS Technology is capable of reliably detecting lower abundance transcripts than are typically detected using fluorescence technology.

Detection Method for Differentially Expressed Genes	Number of Genes
Differentially expressed by both RLS and fluorescence	20
Differentially expressed by RLS, below detection threshold for fluorescence	26
Differentially expressed by RLS, not by fluorescence	1
Total number of differentially expressed genes detected by fluorescence	20
Total number of differentially expressed genes detected by RLS	47

Table 2: Comparison of differentially expressed genes detected by RLS and fluorescence.

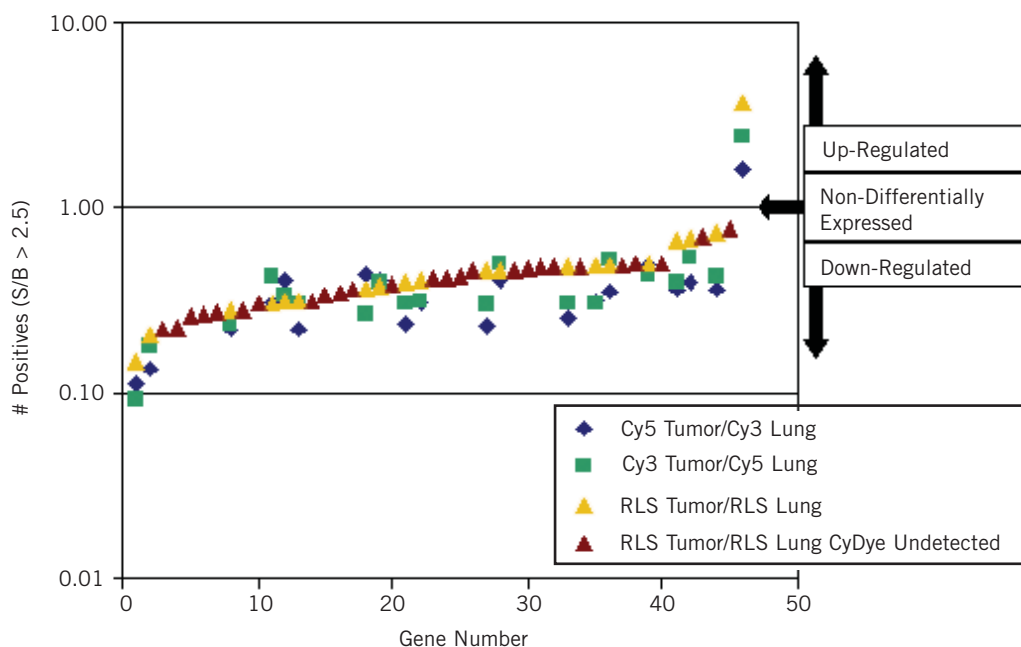


Figure 1: Differential gene expression using a one-color RLS detection and a fluorescent two-color reciprocal labeling detection approach. Red triangles represent the 26 array features which were scored as differentially expressed by RLS detection, but fell below the threshold for detection with fluorescence.

Conclusion

RLS Technology is an effective detection method for one-color differential gene expression, providing sensitivity that enables more genes to be accurately scored as differentially expressed. When coupled with a linear normalization strategy, a one-color RLS detection approach enables detection of differentially expressed genes as accurately as a two-color fluorescent reciprocal method, eliminating inter-label variability and providing a solution to address inter-slide variation. The fold-differences in gene expression measured by RLS with 2 μg of starting material were consistent with those measured by fluorescence detection using 10 μg of starting material. The increased sensitivity of RLS Technology enables the detection of lower abundance transcripts that were not detected by fluorescence, even when using 5-fold more starting material. A total of 26 additional differentially expressed genes were detected using RLS Technology. Confirmatory RT-PCR studies were performed on a subset of the RLS positive genes, assuring the validity of the RLS data.

Finally, the increased sensitivity of RLS Technology lowers the amount of starting material required for microarray analysis. Reduced sample consumption enables either more experimental replicates to be performed to increase statistical accuracy, or allows other assay types to be performed with the same sample. Thus, RLS Technology offers an effective and significant improvement to standard fluorescence detection methods of gene expression using microarrays.

Materials and Methods

Arrays

Human cDNA microarrays containing 3,840 elements printed on Corning GAPS II slides (Corning Inc., Corning, NY) were purchased from the University Health Network Microarray Centre (Toronto, Canada). The 3,840 elements consist of 1,728 unique human cDNA clones spotted in duplicate, 256 elements derived from an *Arabidopsis thaliana* cDNA clone for use as negative controls and

128 elements of 3X SSC spotting buffer. Slides were post-processed by UV crosslinking and assayed using the One-Color Microarray Toolkit Protocol (Genicon Sciences, San Diego, CA).

cDNA Target Labeling and Purification:

Reagents:

The following reagents and materials were used to generate labeled target cDNAs: human lung total RNA, (Ambion, Austin, TX); human lung tumor total RNA, (Invitrogen, Carlsbad, CA); Superscript II reverse transcriptase, RNaseOUT, 5X first-strand buffer, 0.1 M dithiothreitol, oligo d(T)₍₁₂₋₁₈₎ primers (Invitrogen, Carlsbad, CA); Cy3 and Cy5 dUTP (Amersham Pharmacia Biotech, Piscataway, NJ); Biotin-11-dUTP (Enzo Diagnostics, Farmingdale, NY); sodium hydroxide, Tris-HCl (Sigma, St. Louis, MO); QIAquick PCR Purification Kit, (QIAGEN, Valencia, CA).

Protocol:

cDNA targets were prepared for each RNA and label type by direct incorporation using (per reaction) 7.5 μg total RNA, 0.75 μg oligo d(T)₍₁₂₋₁₈₎ and nuclease free water to 15 μl . The mix was incubated at 70°C for 10 minutes, chilled to 42°C for 2 minutes before adding reverse transcriptase mix (6 μl , 5X first-strand buffer; 3 μl , 0.1 M dithiothreitol; 0.9 μl RNaseOUT; 0.6 μl , 50XdNTPs (25 mM dATP, dGTP, dCTP, 10 mM dTTP); 3 μl , 1mM Biotin-11-, Cy3 or Cy5 dUTP; 1.5 μl SuperScript II) and then incubated for 60 minutes at 42°C. The reaction was terminated by addition of 5 μl 1N NaOH and incubating for 10 minutes at 70°C, chilled and neutralized with 5 μl 1M Tris-HCl, pH 7.4. cDNA targets were purified using the QIAquick PCR Purification Kit using the recommended protocol and eluted with 50 μl buffer EB.

Pre-Hybridization, Hybridization, and Wash Conditions

40 glass microarrays from a single printing lot were pre-hybridized according to the Genicon Sciences One-Color Microarray Toolkit's Pre-Hybridization Protocol (Genicon Sciences, San Diego, CA). The

slides were rinsed in DI water and dried under a stream of clean, filtered air. Labeled cDNA from human lung or human lung tumor total RNA was prepared and hybridized according to the Genicon Sciences One-Color Microarray Toolkit's Hybridization Protocol. Briefly, 10 slides at 2 µg total RNA input for human lung, 10 slides at 2 µg total RNA input for human lung tumor, 10 slides at 10 µg total RNA input Cy3 lung || 10 µg Cy5 lung tumor, and 10 slides at 10 µg total RNA input for Cy5 human lung || 10 µg Cy3 lung tumor had their respective hybridization mixtures prepared, denatured at 95°C for 5 minutes, and applied utilizing LifterSlips (Erie Scientific, Portsmouth, NH). All slides were incubated at 42°C for 16 hours in a hybridization chamber, and subsequently washed according to the Genicon Sciences One-Color Microarray Toolkit's Post-Hybridization Wash Protocol. The fluorescent-labeled slides were rinsed in DI water and dried under a stream of clean, filtered air.

RLS Particle Binding

RLS Particle Binding was performed according to the Genicon Sciences One-Color Microarray Toolkit Protocol (Genicon Sciences, San Diego, CA). Briefly, slides were blocked with RLS Blocking Solution, and then 80 nm gold RLS Particles functionalized with anti-biotin antibodies were added for a 60 minute binding step. After a brief wash to remove non-specifically bound RLS Particles, a final rinse in DI water and drying under a stream of clean, filtered air, the slides were archived by dipping the slides into Archiving Solution.

Array Imaging

Cy3 and Cy5 labeled arrays were read with a GenePix 4000B scanner (Axon, Union City, CA) at 10 µm resolution, 100% Laser Power, and PMT 600 volts to obtain maximal signal intensities at high target input without signal saturation. Total read time for each slide was approximately 1.75 minutes. RLS labeled arrays were read with a white light/CCD-based GSD-501 RLS Detection and Imaging instrument, (Genicon Sciences, San Diego, CA) at 10 µm resolution and 0.4 second exposure time. The total read time for each slide was approximately 0.5 minutes. The resulting images were analyzed using ArrayVisionRLS image analysis software (Genicon Sciences, San Diego, CA, and Imaging Research Inc., St. Catherines, Canada).

Analysis

ArrayVisionRLS parameters used for analysis include: MTM density as the principal measure with a MAD threshold of 6, spot segmentation enabled, obvious outliers confirmed visually, flagged, and excluded from analysis. Background was defined as the median MTM density from the 256 A. thaliana features (camera bias of 100 subtracted from all MTM density measures for RLS calculations). A signal to background (S/B) ratio greater than 2.5 was defined as a detected feature. The average number of detected features at the specific input levels for the 10 replicate arrays was calculated as an output for comparison between labels.

Linear Normalization

Linear normalization of RLS detected microarrays: the array with hybridized normal lung targets that had the highest overall intensity was used as the control. Since these pre-spotted arrays were not printed with positive control features for normalization, linear normalization transform parameters (slope and intercept) were derived from pairs of presumably non-differentially expressed features on the differing slides. All replicate lung tumor features were linearly

normalized to the control array using the derived linear correlation transform parameters. For each pair of arrays being normalized, a scatter plot was generated using the features described. The normal lung features were placed on the independent x-axis and the lung tumor features were placed on the dependent y-axis. The slope and y-intercept of the best-fit linear regression line was determined. Subsequently, each feature on the lung tumor array was transformed by the equation $y'=(y-b)/m$ where: y' is the normalized, and y is the original lung tumor feature expression value; b is the y-intercept; and m is the slope of the best-fit linear regression line. For two-color fluorescence normalization, the Cy5 data set was normalized to the Cy3 data set using the same parameters as RLS linear normalization.

Quantitative RT-PCR

DNase I treated, RT-PCR certified total RNA from normal human lung (Ambion, Austin, TX) and human lung tumor (Invitrogen, Carlsbad, CA) was purchased. The samples of total RNA were treated a second time in-house with DNase I as an additional step to remove genomic DNA contamination in the samples by incubating 14 µg total RNA with 1.5 µl DNase I, 0.5 µl RNaseOUT and 4 µl 5X first-strand buffer for 15 minutes at 37°C. The reactions were terminated by heating at 95°C for 5 minutes. An aliquot of 1.4 µg was removed for use as a reverse transcriptase negative control (-RT) while the rest of the total RNA was converted to cDNA by adding 1.5 µg oligo d(T)₍₁₂₋₁₈₎ and water to 30 µl. The reaction was heated to 70°C for 10 minutes, then cooled to 42°C before the addition of reverse transcriptase mix (5X first-strand buffer, 8.4 µl; 0.1 M dithiothreitol, 6 µl; 50XdNTPs {25 mM dATP, dGTP, dCTP, dTTP}; SuperScript II, 3 µl) and incubated for 60 minutes at 42°C. The reaction was terminated by adding 10 µl 1N NaOH and incubating for 10 minutes at 70°C, chilled and neutralized with 10 µl 1M Tris-HCl, pH 7.4. Probes were purified using the QIAquick PCR Purification Kit using the recommended protocol and eluted with 50 µl EB buffer. PCR reactions contained 125 - 250 nM each gene specific primer, 10 - 50 ng cDNA, 25 µl 2X HotStarTaq Master Mix, up to 6 mM MgCl₂ and nuclease free water to 50 µl. Replicate reactions were stopped every 5 cycles up to 40 cycles with a no template reaction and a -RT reaction run to 40 cycles serving as negative controls for each primer pair. The PCR products were separated on 10% TBE Novex gels, stained with SYBR Green I stain, imaged and quantified with an Alphasampler 2200 (Alpha Innotech Corporation, San Leandro, CA).

References

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