Multicolor Fluorescent Differential Display

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Y.-j. Cho, J.D. Meade[1], J.C. Walden[1], X. Chen[2], Z. Guo[1], and P. Liang
Vanderbilt University, Nashville, TN, [1]GenHunter Corporation, Nashville, TN, and [2]Medical College of Georgia, Augusta, GA, USA

ABSTRACT

Differential display and DNA microarray have emerged as the two most popular methods for gene expression profiling. Here, we developed a multicolor fluorescent differential display (FDD) method that combines the virtues of both differential display in signal amplification and DNA microarray in signal analysis. As in DNA microarray, RNA samples being compared can be labeled with either a red or green fluorescent dye and displayed in a single lane, allowing convenient scoring and quantification of the differentially expressed messages. In addition, the multicolor FDD has a built-in signal proofreading capability that is achieved by labeling each RNA sample from a comparative study with both red and green fluorescent dyes followed by their reciprocal mixings in color. Thus, the multicolor FDD provides a platform upon which a sensitive and accurate gene expression profiling by differential display can be automated and digitally analyzed. It is envisioned that cDNAs generated by the multicolor FDD may also be used directly as probes for DNA microarray, allowing an integration of the two most widely used technologies for comprehensive analysis of gene expression.

INTRODUCTION

The first method for the analysis of alteration in gene expression, which was developed in late 1970s, was two-dimensional protein gel electrophoresis, which allows comparative studies of the gene products and cellular protein species (11). Due to the problems of sensitivity and difficulty in recovery of protein species of interest for further molecular analysis, this method gradually gave way to the newer methodologies developed in the early 1980s. These methods included differential screening and subtractive hybridization, which focused on the analysis of the gene message, mRNA, as it can be copied into cDNA and then cloned into plasmids or bacterial phage vectors for propagation and analysis (10).

With the advent of PCR, differential display (DD) methodology was invented in 1992 (5). Since then, the method has greatly accelerated the rate at which differentially expressed genes are identified because of its simplicity, sensitivity, and ability to simultaneously compare multiple mRNA samples for the identification of both up- and down-regulated genes without prior knowledge of their sequences (8). In essence, DD involves a systematic amplification of the mRNA 3′ termini from a cell using a series of specially designed primer pairs (5,6). The amplified cDNAs from RNA samples are labeled with isotope and separated on a gel matrix to allow a side-by-side comparison of mRNA expression profiles. Although DD has become the most widely used method for cloning differentially expressed genes, nearly all of these studies focused on the isolation and characterization of a single gene from a comparative system using a limited number of primer combinations (9). There is a need to streamline and automate DD to increase its accuracy, comprehensiveness, and throughput. Although progress has been made in rational primer designs for DD, which provides a theoretical foundation for a comprehensive gene expression profiling (6), the dependence on radioactive detection has been a serious impediment for the method automation. Fluorescent labeling of DD-amplified cDNAs has been demonstrated more recently, but these studies were carried out with only single fluorophore-labeled primers with little knowledge of its sensitivity in a side-by-side comparison with traditional DD by isotopic labeling (3,4).

To further improve and streamline DD by incorporating the concept DNA microarray in signal detection and analysis, we have developed the multicolor fluorescent differential display (FDD) using dual color-coded fluorophores to label and detect each RNA sample being compared. We demonstrate that the dual color labeling method allows proofreading of positive signals to be automatically built in, which should greatly cut down the rate of false positives. The digital FDD image obtained can be automatically analyzed, quantified, and used to locate and retrieve differentially expressed genes of interest, whose nature can be rapidly identified by direct sequencing, without subcloning of the cDNAs.

MATERIALS AND METHODS

Cell Culture

Normal and ras-transformed rat embryo fibroblasts were cultured as described previously (7). The inducible p53 H1299 lung cancer cell line was

cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, G418 (250 µg/mL), puromycin (1 µg/mL), and tetracycline (1 µg/mL) as described previously (1). For the induction of p53 expression, cells were grown to 80% confluence and then incubated with DMEM in the absence of tetracycline for 4, 8, 12, and 24 h.

Immunoblotting

Cellular protein extraction and western blot analysis were carried out as described previously (15). Antibody against actin was from Sigma (St. Louis, MO, USA), and polyclonal antibody to p21 (C-19) was obtained from Santa Cruz Biochemicals (Santa Cruz, CA, USA).

RNA Isolation

After incubating for 4, 8, 12, and 24 h with DMEM without tetracycline to induce p53, total RNA was isolated with one-step acid-phenol extraction method using RNApure reagent (GenHunter, Nashville, TN, USA) according to the manufacturer's instructions. To remove all chromosomal DNA contamination, total RNA was treated with DNase I using the MessageClean® kit (GenHunter) as instructed.

Reverse Transcription of mRNA

The first-strand cDNA synthesis was performed with unlabeled anchored oligo-dT primers using the RNAimage® kit (GenHunter) essentially as described (6), except the dNTP concentration was increased from 20 to 200 µM.

Conventional DD Using ^{33}P

The conventional DD was performed using the RNAimage® kit in the presence of α-[^{33}P]dATP (NEN® Life Science Products, Boston, MA, USA) according to the manufacturer's instructions.

Multicolor FDD

The anchored oligo-dT primers were synthesized and labeled at the 5′ end with either fluorescein (green) or Cy3 (red) on an ABI 392 DNA synthesizer (Applied Biosystems, Foster City, CA, USA). Rhodamine-labeled anchored primers were from Integrated DNA Technologies (Coralville, IA, USA). The RNAimage kit was used for the two-color-based FDD PCRs. The cDNA was amplified according to the manufacturer's instructions, except the final concentration of dNTP was at 200 µM and fluorescent primer was at 0.2 µM. DD PCR amplifications were carried out on a Mastercycler® gradient (Eppendorf Scientific, Westbury, NY, USA) under the conditions of conventional DD (6). The amplified cDNAs were separated on a 6% denaturing polyacrylamide gel in 1× TBE buffer. For ^{33}P-labeled PCR products, the gel was dried to a 3M paper and exposed to Biomax® film (Eastman Kodak, Rochester, NY, USA) for 16–48 h. For fluorescently labeled PCR products, gels were scanned without drying using the FMBIO® II fluorescent laser scanner with FMBIO ReadImage version 1.5 software (both from Hitachi Genetic Systems, Alameda, CA, USA). The Cy3- (or rhodamine) and fluorescein-labeled DD PCR products were detect-

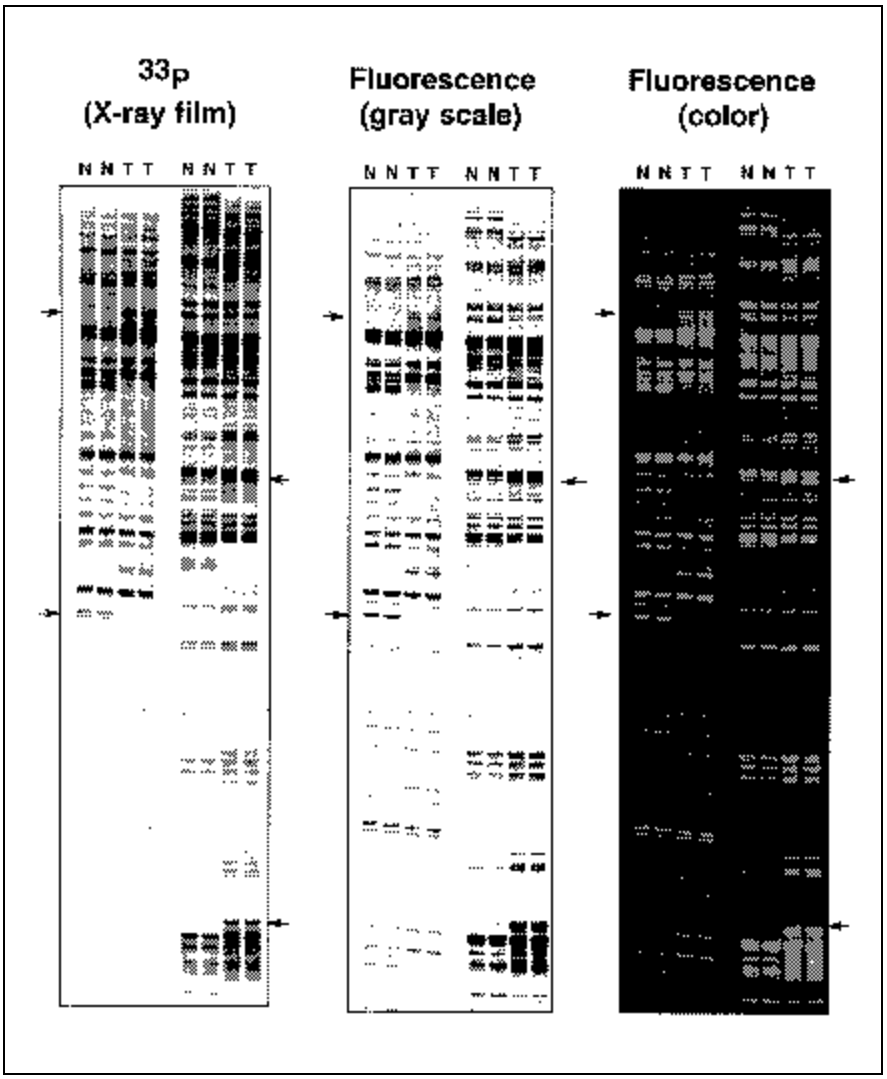

Figure 1. Comparison of radioactive and fluorescent DD. DNA-free total RNA from normal (N) and ras oncogene transformed (T) rat embryo fibroblasts were compared in duplicate by either conventional differential display with ^{33}P-labeled [α-]dATP or FDD with fluoroscein-labeled anchored primer under identical PCR conditions. The autoradiogram and fluorescent images in gray scale or color were compared in sensitivity and reproducibility as indicated. Reproducible differences were marked by arrows. The anchored primer, H-T$_{11}$G, was used in combination with two arbitrary primers, H-AP6 and H-AP29.

ed with the 585- and 505-nm cutoff filters, respectively. The digital FDD images were analyzed using FMBIO data analysis software (version 8.0) according to the manufacturer's instructions. For the scatter plots and quantification of bands from FDD, the measurement of peak height of each band in arbitrary units was used.

Reamplification, Sequencing, and Subcloning of cDNA Bands from FDD

The FDD images were obtained using the FMBIO II laser scanner with fluorescent markers. The real-size printout of the multicolor FDD images were used to align the gels, and the bands of interest were excised directly from the gel. After the gel slice was boiled for 10 min in 100 µL water, the cDNA was ethanol precipitated in the presence of glycogen (GenHunter) and resuspended in water. Reamplification was carried out using the same primer set and PCR conditons in a 40-µL reaction. For DNA sequence analysis, the reamplified cDNAs after gel purification with QIAex® (Qiagen, Valencia, CA, USA) were analyzed by cycle sequencing with the corresponding arbitrary primers using an automated DNA sequencer (Applied Biosystems). For cloning the differentially expressed cDNAs, PCR-TRAP® kit (GenHunter) was used according to the manufacturer's instructions.

RESULTS

Fluorescent DD with Equal Sensitivity to Traditional DD

As a first step in the development of a multicolor DD, it is crucial that the FDD has a similar sensitivity in signal detection to the traditional DD with isotope labeling. However, DD with fluorescent dye-labeled one-base anchored oligo-dT primers carried out under the same conditions for conventional DD but gave poor fluorescence signals, especially for cDNAs of higher molecular weight (data not shown). It is known that the dNTP concentration used in the original DD for both the reverse transcription and PCR steps was intentionally kept low for the efficient incorporation of radioactively labeled nucleotide into amplified cDNAs (5). Thus, increasing the dNTP concentration should enhance the signal for FDD, which relies on the use of fluorescently labeled anchored primers for signal detection. Such optimized FDD was shown to be essentially identical in both sensitivity and reproducibility to that of radioactive DD (Figure 1). The FDD image was obtained with a laser-activated fluorescence scanner, FMBIO II, and stored digitally for data analysis. The FDD image can be displayed in either pseudocolor in gray scale or in real colors from the corresponding fluorophores (Figure 1).

Multicolor FDD Analysis of p53-Regulated Gene Expression

To demonstrate the feasibility of the multicolor FDD, RNA samples from a

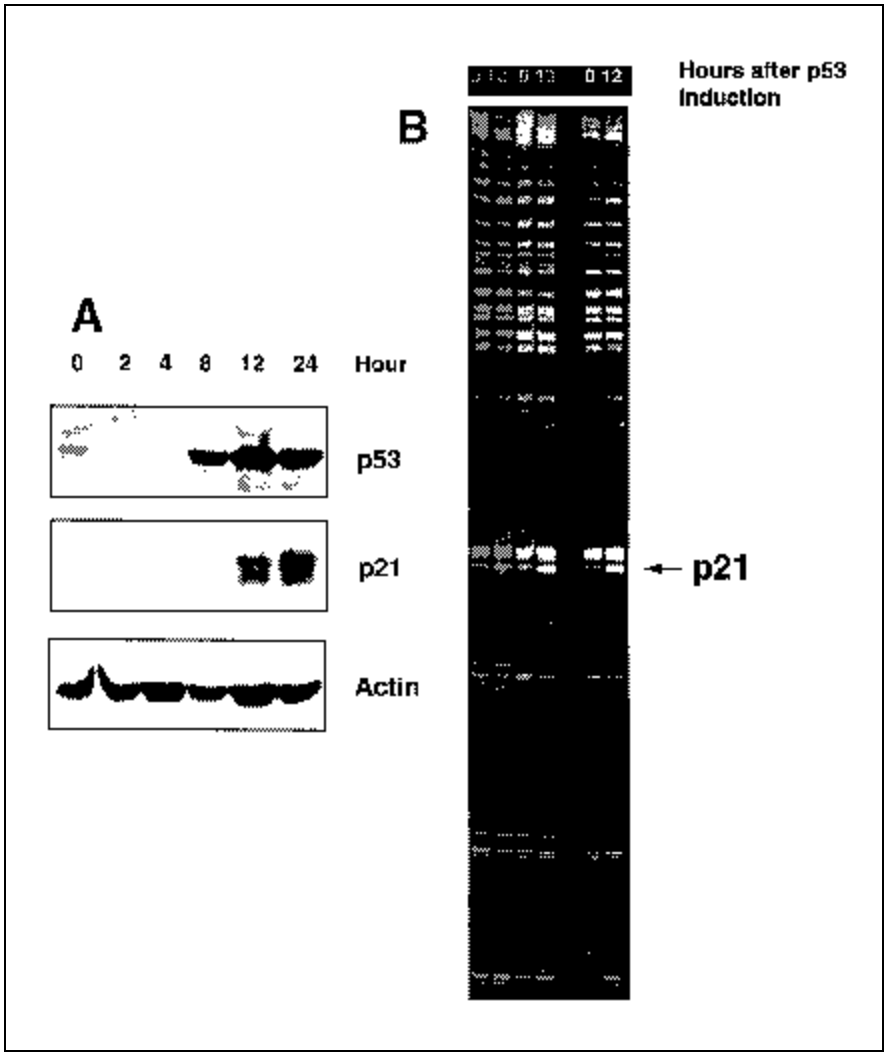

Figure 2. Multicolor fluorescent DD analysis of p53 tumor suppressor-gene induced gene expression. (A) Tetracycline-regulated wild-type p53 gene expression in H1299 cells. p53 was induced by the removal of tetracycline from the culture media for the time indicated and, the induction of p53 was analyzed by western blot using a polyclonal antibody against p53. The same samples were also analyzed by western blot for the p53-dependent induction of p21 and equal sample loading using antibody against human p21 and β-actin, respectively. (B) FDD analysis of p53-dependent p21 gene expression (indicted by the arrow) using either Cy3- (red) or fluoroscein-labeled anchored primer, HT11A, in combination with the arbitrary primer, H-AP-p21 (5′-AAGCTTAGTGTAC-3′). RNA samples before (0 h) and 12 h after p53 induction were compared. To determine the priming efficiency of the anchored primers labeled with the red and green fluorophores, equal amounts of both primers were included in the same DD PCRs (in yellow color).

human lung carcinoma cell line, H1299, before and after induction of the wild-type p53 tumor suppressor gene (1) (Figure 2A) were reverse transcribed and amplified by DD. Both fluorescein (green) and Cy3 (red)-labeled one-base anchored oligo-dT primers were used in combination with rationally designed arbitrary 13-mers (6). For the positive control, an arbitrary 13-mer, H-AP-p21, was designed to target a p53 target gene p21 (2) (Figure 2A). The DD PCR samples labeled in either red or green fluorescence were displayed by denaturing polyacrylamide gel electrophoresis, which showed excellent reproducibility (Figure 2B). The cDNA species displayed by red and green fluorescent anchored primers were very comparable, and both revealed the p53-dependent induction of p21 mRNA (Figure 2B). To determine if different fluorescent labels would affect the priming efficiency of the anchored primers, the same DD PCRs were carried out in the presence of an equal amount of red and green fluorescent anchored primers. As one would predict for equal priming efficiency, all cDNA bands displayed were in yellow color and were comparable to the cDNA pattern displayed by either anchored primer alone (Figure 2B). The induction of p21 was also confirmed by DNA sequence analysis of the band after reamplification and northern blot analysis (data not shown).

For the multicolor FDD, the RNA samples from H1299 cells before and after 12 h of induction of p53 were reverse transcribed and amplified by DD with both red and green fluorescent anchored primers in combination with the H-AP-p21, as described above. Fluorescently labeled cDNAs were then displayed (*i*) without mixing (in either red

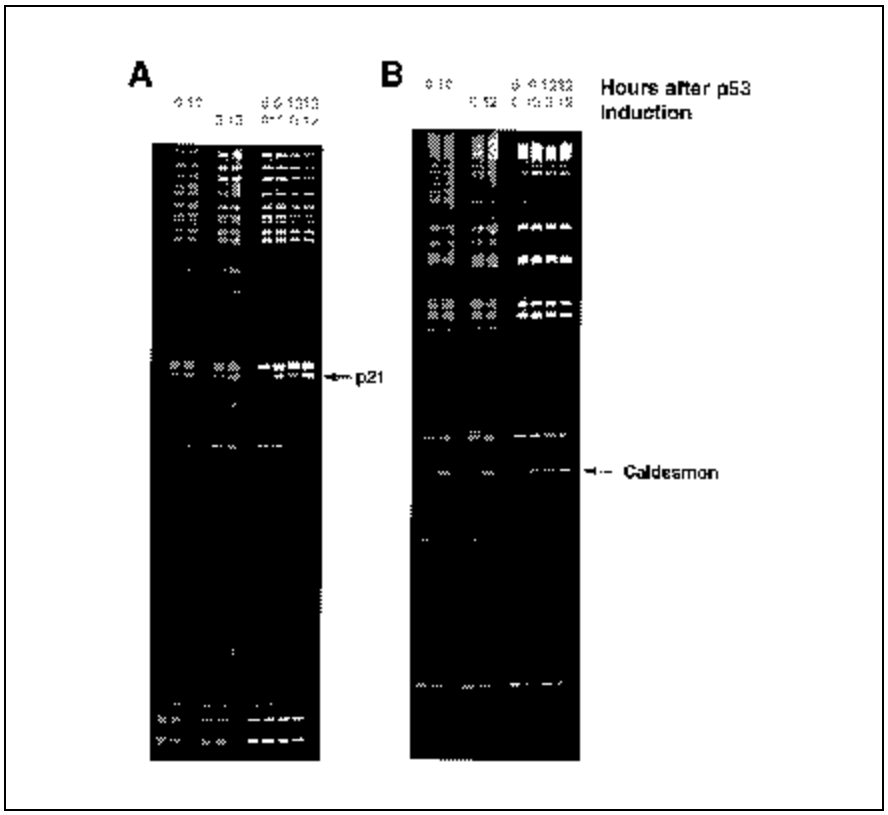

Figure 3. The multicolor FDD analysis of p53-regulated gene expression. DD was carried out with either a green or red fluorescent anchored primer, HT11A, in combination with either H-AP-p21 (A) as in Figure 2B, or H-AP7 (B). The fluorescence-labeled PCR products were then either self-mixed (0 h red/0 h green; 12 h red/12h green) or reciprocally cross mixed (0 h red/12 h green; 12 h red/0 h green) as indicated and compared to the unmixed PCR samples on the same gel. The arrows indicate the successful proofreading of truly differentially expressed genes by the multicolor FDD in complementary colors (green/red) for the reciprocal cross mixings. Most of the non-differentially expressed genes appeared either yellow or do not change in color.

or green color), (*ii*) with self-mixing (0 h red/0 h green; 12 h red/12 h green), or (*iii*) with reciprocal cross mixing (0 h red/12 h green; 12 h red/0 h green) (Figure 3A). As shown in Figure 2B, the cDNA patterns displayed were very comparable when either the red or green fluorescent anchored primer was used. However, importantly, both self-mixing (in yellow color) and reciprocal cross mixing (in either green or red color) confirmed nicely the induction of p21 by p53 tumor suppressor gene based on the principle of multicolor FDD outlined above. Although the majority of cDNA species displayed here were in yellow, as one would predict for equally expressed messages, a few bands appeared to be preferentially amplified with either the red or green anchored primers. These cDNA bands also representing equally expressed mRNAs did not exhibit complementary color (green and red) during reciprocal mixings and thus could be easily differentiated from truly differentially expressed genes. Using a different primer pair, another candidate p53 target gene, Caldesmon, which we identified by an earlier conventional DD (data now shown), was confirmed by multicolor FDD (Figure 3B). Thus, the proofreading of a reproducible difference is achieved by a dual color output, with green-red representing genes down-regulated, red-green representing genes up-regulated, and yellow-yellow, green-green, and red-red representing equally expressed genes for the comparison of a pair of mRNA samples.

Scatter plots with values of either fluorescein- or Cy3-labeled cDNA sig-

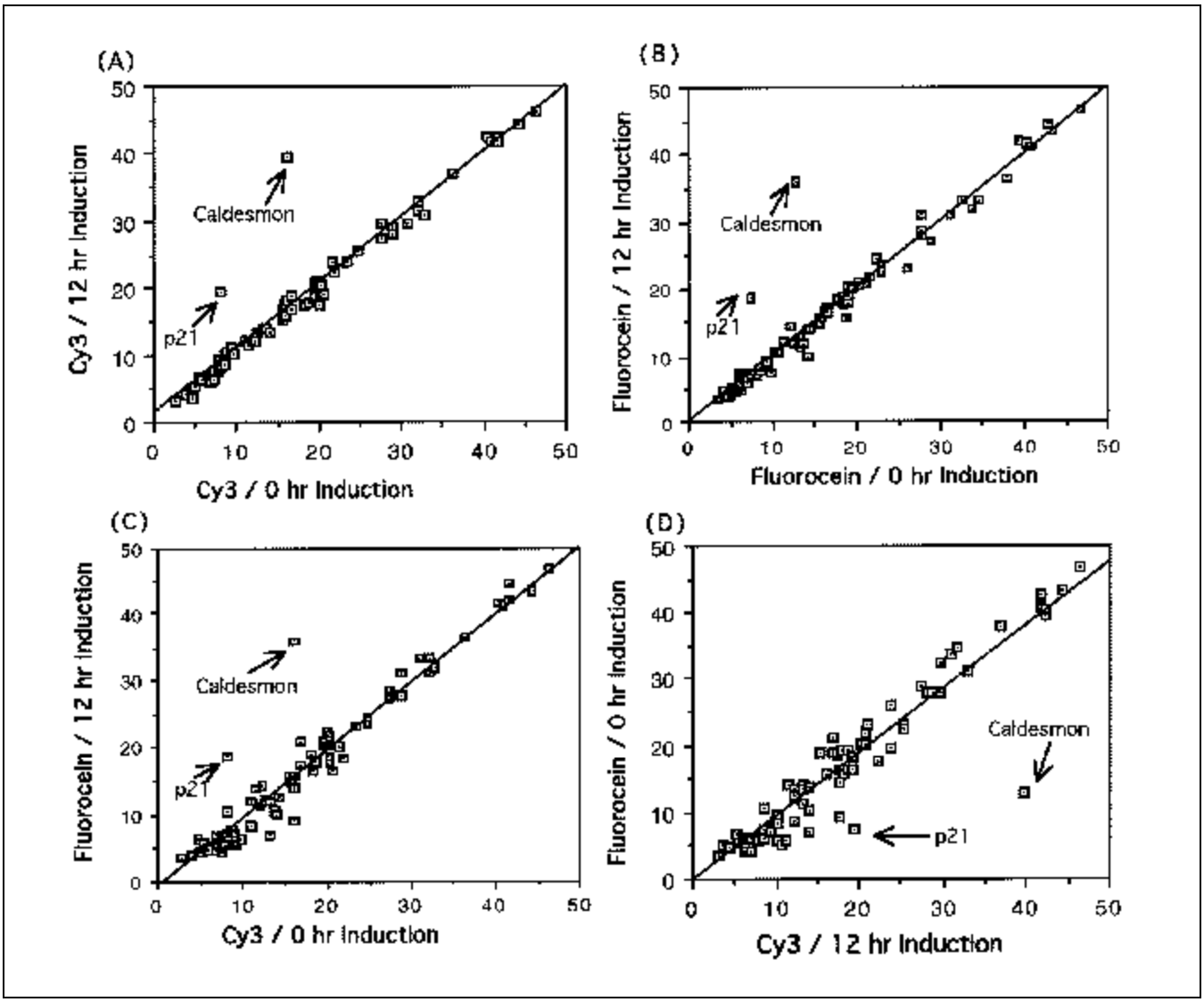

Figure 4. Scatter plots for single-color FDD and the multicolor FDD of p53 tumor suppressor gene-induced gene expression. RNA samples before (0 h) and 12 h after p53 induction were compared. The scatter blots for one-color fluorophore from FDD are shown in A and B. In this case, the ratio of the fluorescent signal (1/100 of peak height in arbitrary units) for each band from non-induced and induced condition shown in Figure 3, A and B, were plotted. For scatter plots of two-color labeling, the ratio of normalized Cy3 and fluorescein signals of each band from the reciprocal mixing of the multicolor FDD experiments shown in A and B were plotted (C and D). The arrows indicate the successful proofreading of truly differentially expressed genes in complementary colors by both single- and dual-color FDD.

nals from Figure 3, A and B, clearly revealed the excellent reproducibility of FDD (Figure 4, A and B) with a very tight distribution pattern centered in an straight diagonal line as expected, except for p21 and Caldesmon. Scatter plots with ratio of fluorecein and Cy3 signals for the reciprocal mixing of the multicolor FDD also confirmed that both p21 and Caldesmon were induced by p53 (Figure 4, C and D). It is worth noting that the distribution of the scatter pattern appeared to be less tight with mixed colors compared to single-color fluorescence, which supports the observation that a certain degree of dye-specific amplification of cDNAs occurred. Nonetheless, only p21 and Caldesmon exhibited greater than twofold signal difference in both plots from the reciprocal mixing experiments.

Multicolor FDD Analysis of Time-Dependent p53-Regulated Gene Expression

One of the advantages of DD over DNA microarray is its ability to simultaneously compare more than two RNA samples, which helps to quickly narrow down the genes of interest to be identified. With dual-colored fluorescent labels, FDD can also retain this advantage, so more subtle changes in gene expression in a cell upon exposing to a chemical or induction of gene can be readily detected. This was demonstrated again in the study of p53 tumor suppressor gene-regulated gene expression. Exponentially growing H1299 cells with a tetracycline-repressible wild-type p53 gene was allowed to turn on the p53 expression by removing tetracycline from the culture medium. The RNAs were harvested at 0, 4, 8, and 12 h after tetracycline removal and compared by multicolor FDD with either a green or red fluorescence-labeled anchored oligo-dT primer in combination with H-AP-p21. As expected, a cDNA fragment corresponding to the size of p21 PCR product was reproducibly induced after 4 h of induction when either fluorescent anchored primer was used (Figure 5). The induction of p21 was better visualized by mixing the 0-h DD PCR sample labeled with one fluorophore equally with those of 0, 4, 8, and 12 h labeled with the other fluorophore (Figure 5). Thus, the multicolor FDD reveals changes in gene expression precisely as horizontal changes (or time course dependent) in fluorescence color or intensity of a cDNA fragment. The equal mixing of the cDNAs labeled with red and green fluorescence can be easily verified by fluorescence scanning using the corresponding cutoff filters, which showed excellent color separation (Figure 5).

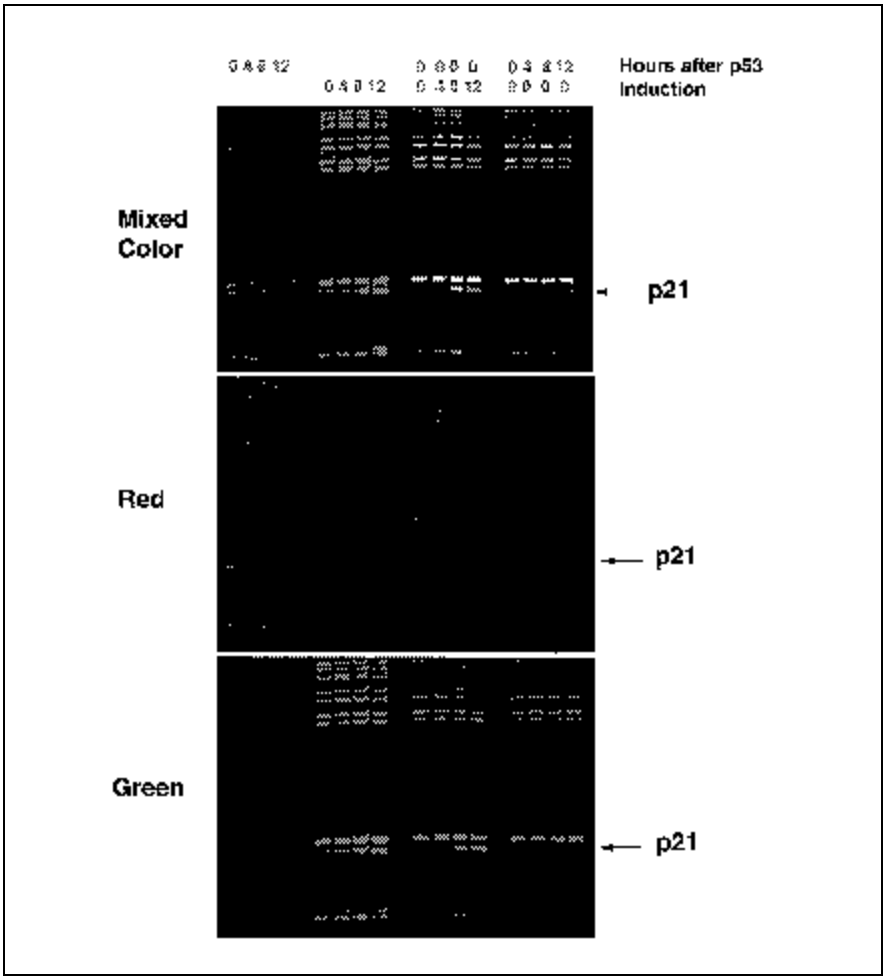

Figure 5. The multicolor FDD analysis of time-dependent p53-regulated gene expression. RNA samples from H1299 cells before (0 h) and after 4, 8, and 12 h of p53 induction were compared by DD with either red or green fluorescence. To better reveal p53-regulated genes such as p21 by multicolor FDD, the PCR samples before p53 induction (0 h) labeled in either red or green fluorescence were mixed equally with all the PCRs labeled in the complementary color (green or red). As indicated by the arrow, p21 expression proofread by the complementary color was induced by p53. Color separation in either red or green fluorescence by the corresponding emission cut-off filters indicated that there was no bleeding of either fluorescence during image scanning.

Quantification of Change in the Level of Gene Expression

The fluorescent image of the multicolor FDD could also be analyzed using the FMBIO Data Analysis software for the accurate quantification for the difference in the level of a gene expression of interest (Figure 6). The fluorescent intensity measured in digital form could be used to determine precisely the fold of induction of a gene. For the p53-dependent expression of p21, the maximum induction was measured to be 3.5 and 3.7 fold for the green and red fluorescent labels, respectively.

DISCUSSION

Despite the fact that more differentially expressed genes have been identi-

fied by DD than by all the other methods combined, there has been a lack of effort to significantly improve the throughput and accuracy of the method by automation in data acquisition and analysis. This partly had to do with the use of isotope in cDNA labeling, which is hazardous and difficult to automate. In contrast, DNA microarray has attracted a great deal of attention because of its potential for a complete automation in data acquisition and analysis. Although DNA microarray, which is based on the principle of a reverse northern blot, offers the potential to obtain a simple snapshot of all the expressed genes in a cell, the method at the moment is imperfect. First, the method is not foolproof, as revealed by a recent and more carefully carried out DNA microarray experiment, which revealed that not all differences in gene expression detected were reproducible from experiment to experiment (12). Although the most ingenious aspect of DNA microarray has been the use of dual-colored fluorophores for quantification and scoring for differentially expressed genes, few published works have used the same principle to check for data reproducibility.

In this study, we have successfully developed the multicolor FDD that has a similar sensitivity to traditional DD with isotopic labeling. The concept of dual color fluorescent labeling of cDNA from DNA microarray has been integrated into DD to give rise to multicolor FDD. Unlike DNA microarray, the multicolor FDD can easily verify the efficiency and uniformity in cDNA labeling with each fluorophore by the pattern of displayed cDNA bands. By labeling each of the RNA being compared with both red and green fluorescence, followed by either self-mixing or reciprocal cross mixing in color of labels, the multicolor FDD has the signal proofreading capability built in. Essentially, the reproducible difference in gene expression should be scored by the complementary colors of a cDNA band during reciprocal cross mixings of the labeled cDNAs compared. For example, p21, a p53 target gene, was confirmed by multicolor FDD (shown in green-red) to be a p53-inducible gene (Figures 3, 4, and 5). By the same token, a cDNA detected by multicolor FDD in red-green color during reciprocal cross mixings of the labeled cDNAs would indicate a p53-repressible gene.

Although little difference in FDD cDNA patterns was observed for the anchored primer labeled by either the red or green fluorescent dye, as shown in Figure 2B, it should be noted that not all cDNAs displayed by multicolor FDD when self-mixed in color of fluorescence appeared completely yellow. This was also reflected as a less tight and straight pattern in two-color scatter plots (Figure 4, C and D) compared to those of one color (Figure 4, A and B). The subtle variation with two-color labels appeared to be unlikely caused by the difference in fluorescent labels attached to the primers since nearly all cDNA bands exhibited perfectly yellow color when equal amounts of the red and green primers were present in the same PCRs (Figure 2B). Thus, the minor difference in cDNA labeling when two-colored primers were separately used could be due to the tube or positional difference during the PCR. Future effort will be directed towards sorting out the exact cause for this subtle discrepancy. Nonetheless, the bands shown in red-red or green-green in lanes with reciprocal mixings could be easily differentiated from those that are truly differentially expressed, which

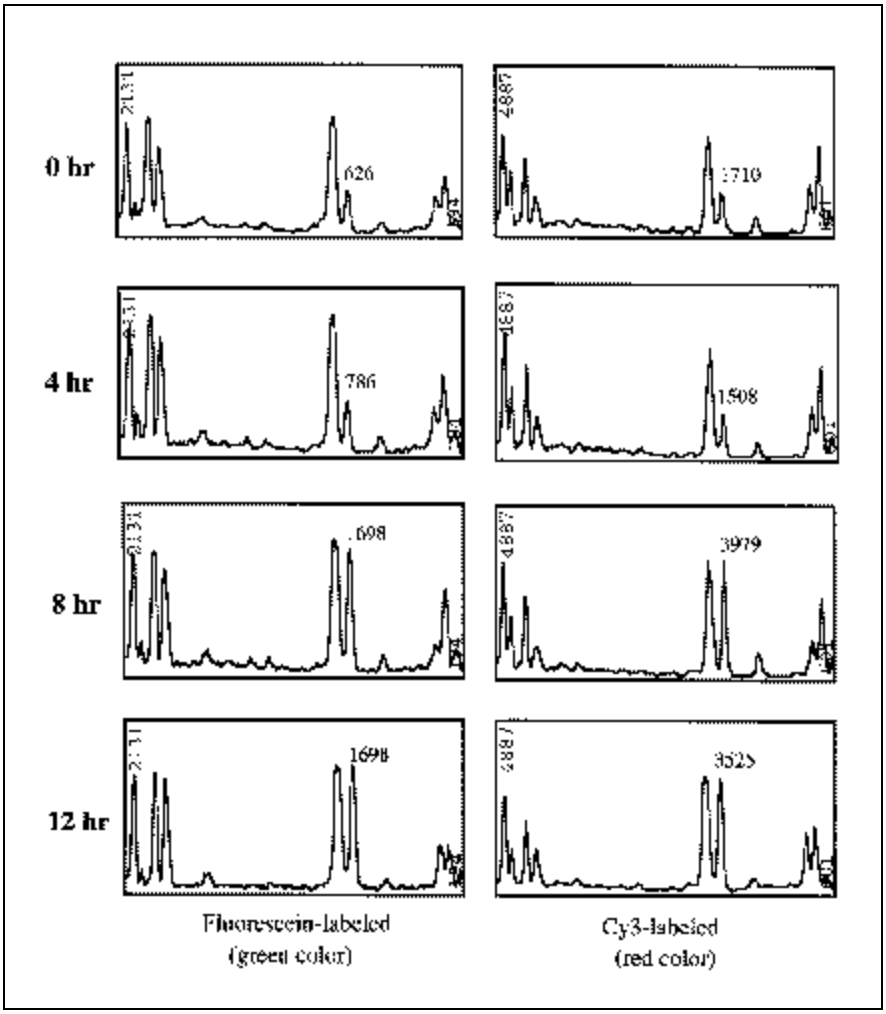

Figure 6. Quantification of differentially expressed genes by FDD. Region of the multicolor FDD image where p21 was located from Figure 4 was quantified in either green or red fluorescence using FMBIO Analysis software. The peak heights in fluorescent intensity (arbitrary unit) for p21 message before and after 4, 8, and 12 h of p53 induction were obtained. After subtracting the background signal (lower right figure of each window of analysis), the fold of induction of p21 could be derived. The upper left figure indicated the scale of the fluorescence intensity. The maximum induction of p21 was 3.5 and 3.7 fold measured by the green and red fluorescent labels, respectively.

have to be in complementary colors of fluorescence. In addition to the ability to proofread the signal, the multicolor FDD could also allow an accurate quantification of the difference in the level of gene expression based on the digital measurement of the fluorescence intensity of individual bands. As in DD, the differentially expressed cDNA from the multicolor FDD can be conveniently located by aligning the gel on top of the true-size print of a fluorescent multicolor FDD image. The cDNA then can be reamplified and directly sequenced with the arbitrary 13-mers.

Compared to DNA microarray, a few micrograms of total RNA are sufficient for multicolor FDD for a coverage of up to 95% of all the genes expressed in a cell (9), whereas several orders of magnitude more RNA are needed for a DNA microarray (13). In addition, the multicolor FDD detects genes with no prior knowledge of their sequence information, whereas DNA microarray can only detect whatever genes spotted on a chip. Furthermore, the multicolor FDD breaks down the complexity of the mRNA species to be analyzed by using numerous combinations of DD PCR primers, whereas DNA microarray uses a very complex probe made by reverse transcription of all the mRNAs. Such complex cDNA probes have been shown to cause problems of cross hybridization of homologous sequences, which greatly complicate the gene expression analysis for higher eukaryotes (12). Thus, the multicolor FDD not only provides a platform on which DD analysis of gene expression may be automated but also cDNAs labeled by the multicolor FDD may be directly used as probes for DNA microarray. Such probes made by FDD not only are more sensitive and less complex than those made by the reverse transcription reaction (14) but also they can be more accurately checked for uniformity and consistency in labeling by different fluorophores before being mixed for hybridization. As in multicolor FDD, reciprocal cross-mixed fluorescently labeled cDNAs when hybridized to duplicate arrays should allow proofreading of a differentially expressed gene in complementary colors. Thus, the multicolor FDD sets a foundation for a potential integration of the two most promising methodologies for the comprehensive analysis of gene expression.

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Address correspondence to:

Dr. Peng Liang
Vanderbilt-Ingram Cancer Center
Department of Cell Biology
School of Medicine
Vanderbilt University
Nashville, TN 37232, USA
e-mail: peng.liang@mcmail.vanderbilt.edu