



Introduction

Problems with current microarray and genomic analysis techniques, including hybridization perturbation, slow enzymatic labeling methods using expensive labeled nucleotides and sequence dependence, can be solved using a direct labeling approach. We have developed a series of new ultrasensitive fluorescent labeling reagents as well as allied protocols for directly labeling DNA or RNA samples isolated from live cells or tissues or prepared by PCR. These reagents efficiently and sensitively label oligonucleotides, oligoribonucleotides, DNA, RNA, mRNA, siRNA and microRNA samples for use in High-Throughput microarray analysis, Fluorescence In-Situ Hybridization (FISH), as well as for monitoring the levels of labeled DNA or RNA administered to cells in culture or *in vivo*. In addition to fluorescent dye labels (TAMRA, Fluorescein, NBD, Cy3, Cy5, Dansyl, BODIPY and R123) quencher and biotin labels were also prepared.

The effects of this direct labeling on hybridization efficiency, telomerase gene knock-down in SK-BR-3 (which overexpresses the HER2/c-erb-2 gene product) and T-47-D (having a mutant tumor suppressor protein p53 protein) human breast tumor cell lines as well as by comparison with existing techniques were examined. These assays also exhibited the ability to monitor changes in gene expression by microarray analysis (using the Affymetrix Mouse 430A and Mouse Gene 1.0 ST arrays) upon application of the anticancer drugs doxorubicin or resveratrol, in a dose-response and cell-specific manner. Uses of these methods to determine the pattern of gene expression in disease or upon therapeutic treatment as well as improve pharmacokinetic analysis of DNA or RNA based drug therapies were developed. This work is supported by grant IIP-0923953 from the National Science Foundation.

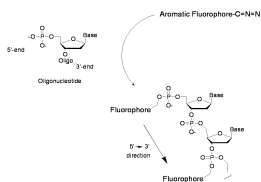


Figure 1: Labeling of DNA or RNA is through both terminal and internal phosphates on the backbone.

Methods

- ### Labeling Systems.
- The labeling compounds were prepared from standard fluorescent dyes and D-biotin using an aminoethylbenzaldehyde linking arm.
 - Stable tri-isopropylsulfonium hydrazide or toluenesulfonylhydrazide intermediates were prepared from the aldehyde precursors.
 - The hydrazide intermediates could be efficiently converted to the active diazo labeling agents using either strong basic conditions or with convenient polymer based reagents.
 - The active diazo labeling reagents were stable for several weeks at 20°C.
 - Labeling of dsDNA, ssDNA, oligonucleotides or RNA was accomplished in aqueous solution using an excess of the active diazo dye or labeling reagents for 1 hour at 37°C.
 - The labeled DNA or RNA could be purified by ethanol precipitation, silica gel spin column (Qiagen) or using gel electrophoresis.
 - The labeled DNA or RNA was stable for several weeks when stored at -80°C in H₂O or appropriate buffer.

- ### DNA and RNA Assays
- Analysis of the labeled DNA or RNA by gel electrophoresis exhibited expected gel shifts due to charge changes upon labeling.
 - Hybridization assays using two fluorophores and fluorescence resonance energy transfer (FRET) or by gel shifts indicated efficient hybridization.
 - The degree of substitution was dependent upon fluorophore structure, varied from 12-120 dyes / 1000 bp.
 - Labeling was found to be quantitative and could be used to quantitate the amount of DNA present. In addition, dye incorporation into nucleic acids is proportional to dye/DNA(RNA) ratio. The higher the ratio, the higher the incorporation.
 - Photostability of labeled DNA: measure of fluorescence over time indicated minimal photobleaching.
 - Chromosome spread Fluorescence In Situ Hybridization (FISH) analysis using Cy3 and TAMRA labeled satellite sequence probes toward centromere regions of chromosomes gave specific and bright labeling.
 - siRNA (amplified or anti-sense RNA) microarray analyses with TAMRA dye (M0776) or with biotin-X labeling gave expected labeling efficiency and excellent photostability.
 - mRNA analysis using both oligonucleotide and DNA probes indicated efficient mRNA labeling.

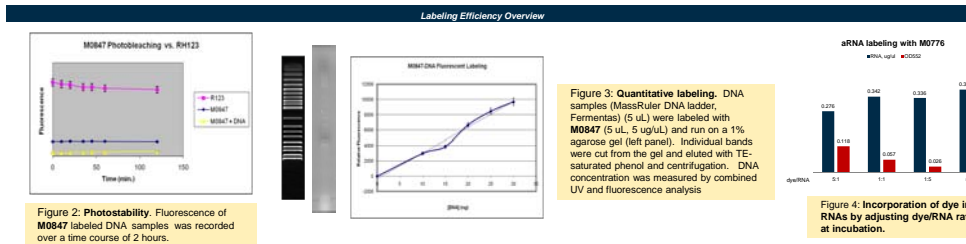


Figure 2: Photostability. Fluorescence of M0847 labeled DNA samples was recorded over a time course of 2 hours.

Figure 3: Quantitative labeling. DNA samples (MassRuler DNA ladder, Fermentas) (5 μ L) were labeled with M0847 (5 μ L, 5 μ g/ μ L) and run on a 1% agarose gel (left panel). Individual bands were cut from the gel and eluted with TE-saturated phenol and centrifugation. DNA concentration was measured by combined UV and fluorescence analysis

Figure 4: Incorporation of dye in RNAs by adjusting dye:RNA ratio at incubation.

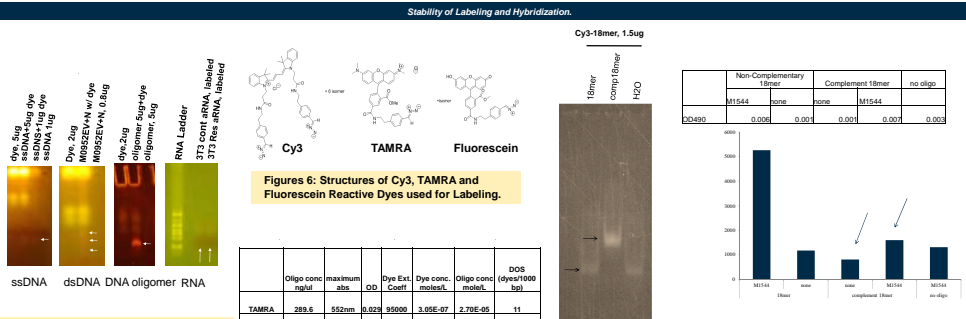


Figure 5: TAMRA labeling reagent M0766 is able to label ssDNA, dsDNA, oligonucleotides and amplified RNA; with minimal shifts in mobility on agarose gel electrophoresis chromatography.

	Oligo conc	oligo conc	maximum abs	Dye Ext. Coeff.	Dye conc. mole/L	Dye conc. mole/L	Oligo conc. mole/L	DOS (dyes/1000 bp)
TAMRA	289.6	552nm	0.02	85000	3.05E-07	2.70E-05	11	
Fluorescein	266.7	499nm	0.015	93000	1.61E-07	2.49E-05	6	
Cy3	235.6	546nm	0.31	130000	2.45E-06	2.39E-05	112	

Table: Incorporation of dye in DNA oligomers: TAMRA, Fluorescein and Cy3.

	Non-Complementary 18mer	Complement 18mer	no oligo
M1544	none	none	M1544
20490	0.008	0.001	0.007

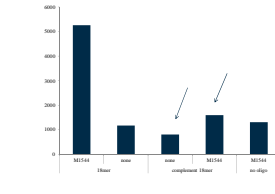


Figure 7: Gel shift analysis upon hybridization of single-strand DNA oligomers. Figure 8: Hybridization of TAMRA (M0766) and Fluorescein (M1544) labeled 18-mer probes and Fluorescence resonance energy transfer (FRET) analysis: FRET Excitation at 490 nm / Emission at 565 nm.

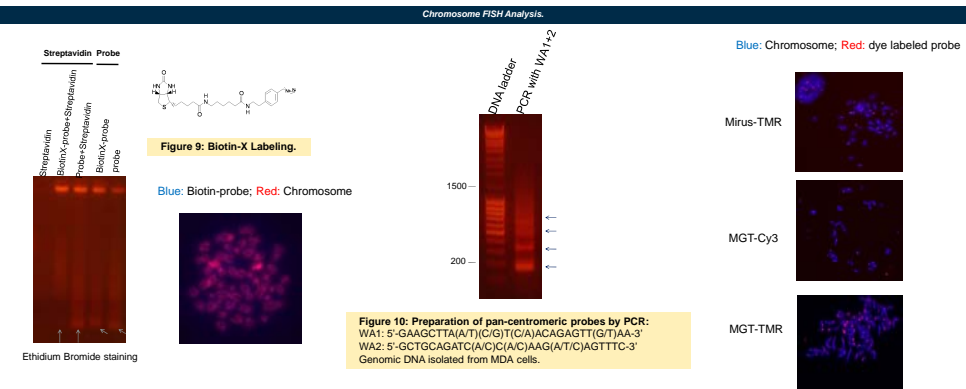


Figure 9: Biotin-X Labeling. Blue: Biotin-probe; Red: Chromosome

Figure 10: Preparation of pan-centromeric probes by PCR: WA1: 5'-GAAGCTTA(A/T)(C/G)T(C/A)ACAGAGTT(G/T)AA-3' WA2: 5'-GCTCGAGATC(A/C)(A/C)AAG(A/T)CAGTTTC-3' Genomic DNA isolated from MDA cells.

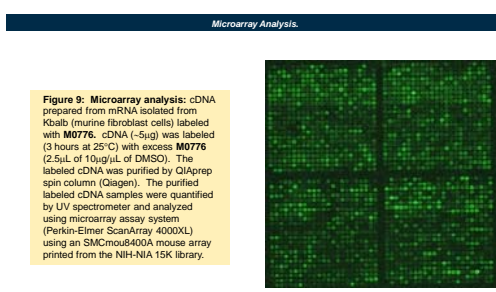
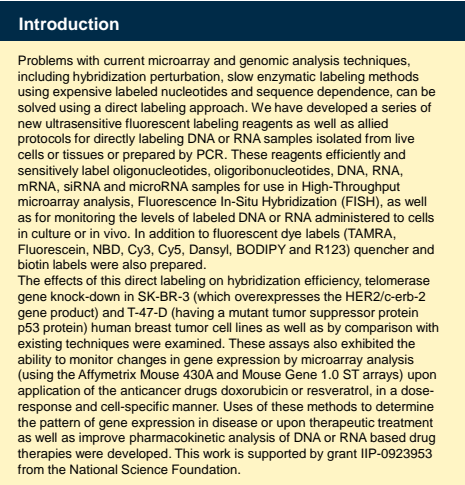


Figure 9: Microarray analysis: cDNA prepared from mRNA isolated from K562 (murine fibroblast cells) labeled with M0776. cDNA (-5 μ g) was labeled (3 hours at 25°C) with excess M0776 (2.5 μ L of 10 μ g/ μ L of DMSO). The labeled cDNA was purified by QIAprep spin column (Qiagen). The purified labeled cDNA samples were quantified by UV spectrometer and analyzed using microarray assay system (Perkin-Elmer ScanArray 4000XL) using an SMCmou4000 mouse array printed from the NIH-NIA 15K library.

Discussion and Conclusions

In order to produce a new fluorescent DNA and RNA labeling system, we developed a set of active fluorophore and D-biotin labeling compounds that could be used to directly labeling dsDNA, ssDNA RNA and oligonucleotide probes, using both standard and long-wavelength emission dyes and probes. The results of our *in vitro* experiments indicate that technical feasibility has been accomplished. For the 14 labeling reagents tested the data indicates:

- Up to four color (multiplexed) labeling of DNA samples can be accomplished.
- The DNA and RNA samples can be labeled quantitatively.
- The labeling reagents are stable under standard microarray and gel analysis assay conditions, and exhibit improved photostability compared with currently available methods.
- The labeling reagents can identify DNA samples isolated from biological sources without interference from protein or other contaminants present.
- The expression level for DNA and mRNA (cDNA) samples isolated from cell lines correlated to treatment with various antineoplastic reagents, indicated that the methods can be used to monitor changes in expression directly.
- Microarray analysis indicated that the labeling reagents perform as well or better than existing methods.
- The methods are amenable to high-throughput FISH assay formats.
- A new system for producing the diazomethane labeling compounds in situ, using a simple base activation step that provides high stability, improved purity and long-shelf life was developed.

The combined results of this work clearly indicate the potential of utilizing the direct labeling reagents for analysis of DNA and RNA expression levels from live cells and tissues *in vivo*, enabling the exciting commercial applications in numerous biotechnology areas.

References

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