

Cyanine dye dUTP analogs for enzymatic labeling of DNA probes

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ABSTRACT

Fluorescence *in situ* hybridization (FISH) has become an indispensable tool in a variety of areas of research and clinical diagnostics. Many applications demand an approach for simultaneous detection of multiple target sequences that is rapid and simple, yet sensitive. In this work, we describe the synthesis of two new cyanine dye-labeled dUTP analogs, Cy3-dUTP and Cy5-dUTP. They are efficient substrates for DNA polymerases and can be incorporated into DNA probes by standard nick translation, random priming and polymerase chain reactions. Optimal labeling conditions have been identified which yield probes with 20–40 dyes per kilobase. The directly labeled DNA probes obtained with these analogs offer a simple approach for multicolor multisequence analysis that requires no secondary detection reagents and steps.

INTRODUCTION

Since the introduction of biotinylated nucleic acid probes in early 1980's (1), fluorescence *in situ* hybridization (FISH) has found increasing application in many areas of research and clinical diagnosis. Examples include molecular cytogenetics (2, 3), nuclear organization (4–6), gene mapping (7–12) and gene amplification (13), prenatal (14, 15), tumor diagnosis (16, 17) and pathogen detection (18–21). Fluorescence techniques have advantages over isotopic techniques in safety, spatial resolution, speed of detection and simultaneous detection of multiple sequences in single cells using different fluorescent labels. The simultaneous use of FISH probes with different fluorophores is especially useful for detection of chromosomal translocations, deletions and amplifications, for gene mapping on chromosomes and for chromosome enumeration (22–25).

It has been demonstrated that up to seven different DNA targets can be simultaneously visualized by using combinatorial labeling of DNA probes with three fluorochromes (24). This technique is based on labeling each individual probe with a different combination of up to three markers including two indirect labels (biotin and digoxigenin). The availability of new colors of

deoxynucleoside triphosphates would extend the power of combinatorial labeling. Furthermore, new fluorescent dNTPs will be valuable when combinatorial labeling is not optimal such as possible mis-identification of the sequence due to fluorescence signals that are spatially overlapping. Instead of combinatorial labeling, each nucleic acid probe can be labeled with a fluorophore that is spectrally distinct. This approach would provide probes of high sensitivity with simplified labeling and detection of multiple sequences.

In this study, we introduce two cyanine dye-conjugated dUTP analogs for direct enzymatic labeling of nucleic acid probes. Direct labeling significantly simplifies the hybridization procedure by eliminating the detection steps with affinity binding proteins. This time- and cost-efficiency is magnified in multicolor multisequence hybridization assays. A directly labeled probe can be used in combination with probes labeled with other fluorochromes to obtain spectrally distinct signals. As many as five target sequences plus DAPI counter-staining of the chromosomes can be detected with currently available fluorophores and microscope systems.

Nick translation, random priming and polymerase chain reactions (PCR) are the most commonly used enzymatic DNA labeling methods. Biotin-dUTP, digoxigenin-dUTP, FITC-dUTP and other chemically modified deoxynucleoside triphosphates have been successfully incorporated into DNA probes by these enzymatic reactions (24, 26–32). We have studied the incorporation of the cyanine dye dUTP analogs by nick translation, random priming and PCR labeling reactions and have optimized the labeling conditions so that the maximal number of dyes can be incorporated into probes and the brightest hybridization signals can be obtained. Multicolor FISH with three probes directly tagged with FITC, Cy3 and Cy5 is demonstrated.

MATERIALS AND METHODS

Synthesis of cyanine dye conjugated dUTPs

The synthesis of 5-(3-amino)allyl deoxyuridine 5' triphosphate (AA-dUTP) was based on a previously published procedure (1). The characterization of AA-dUTP was performed by ninhydrin

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assay (33), UV spectrum (1) and comparison to proton NMR spectrum provided by Dr David Ward. AA-dUTP is also commercially available.

To synthesize Cy3-dUTP, AA-dUTP was dissolved in 0.1M sodium borate buffer pH 8.7. An equal molar amount of Cy3.29OSu (a succinimidyl ester, available from Biological Detection Systems, 955 William Pitt Way, Pittsburgh, PA 15238) dissolved in small amount of neutral water was added to the AA-dUTP in aliquots within half an hour. Total incubation was 1 to 2 hours at room temperature. The labeled product was purified by reversed-phase chromatography. The column was first washed with H₂O and the product was eluted with increasing MeOH:H₂O gradient. Fractions were checked on reversed-phase thin layer chromatography (TLC) and those containing cyanine-dUTP were pulled. The TLC plate was first run in distilled water and let dry. Then, 30:70 (v/v) MeOH:H₂O was used to separate the dUTP analog from free dye and other impurities. R_f of the free dye was 0.8 and that of Cy3-dUTP was 0.58. In case of Cy5-dUTP, 37:63 (v/v) MeOH:H₂O was used. R_f of the free dye was 0.70 and that of the Cy5-dUTP was 0.51. The product was concentrated by rotary evaporation to a small volume and further purified by reversed-phase HPLC on a 250×4.6mm C18 column (Alltech). A linear gradient of 0.05M phosphate buffer, pH6.0 and MeOH was used. The HPLC fractions were collected and rotary evaporated to remove MeOH. They were then desalted on disposable C18 guard columns (Millipore) with H₂O and eluted with 50:50 (v/v) MeOH:H₂O. The final product was concentrated to a small volume and stored in sterile water at -20°C.

Labeling probes by nick-translation

Nick translation was performed following standard procedures (34). The DNase I and the *E.coli* DNA polymerase I reactions were carried out simultaneously and sometimes sequentially. In a simultaneous reaction, the template DNA was incubated in a total of 50 µl of nick translation buffer (10× from Boehringer Mannheim), with 20 µM of each of the dNTPs, cyanine-dUTP, and 5 units of DNA polymerase I and the appropriate amount of DNase I. Several reactions with a series of dilutions of DNase I (Boehringer Mannheim, 10 units/µl) were tested. The reaction mixtures were incubated at 16°C for 90 min and stopped by adding 2 µl of 0.5M EDTA and 1.25 µl of 5% SDS. When sequential reactions were used, the DNA template was incubated with about 1/500 unit (template size- and amount-dependent) of DNase I (Boehringer Mannheim, 10 units/µl stock) in nick translation buffer at 37°C for 10 min. The fragment length was checked on 1% agarose gel while the reaction was sitting on ice. If the fragments were not short enough, the sample tube was returned to the 37°C water bath and further incubated. When the desired fragment length was reached, the nicked DNA was ethanol precipitated and resuspended in sterile H₂O. The fragments were then incubated in a total of 50 µl of nick translation buffer with 20 µM of each of the dNTPs, cyanine-dUTP, 5 units of DNA polymerase I (Boehringer Mannheim) for a minimum of 2 hours. Typically, in order to obtain reasonably accurate UV/VIS spectral measurement, 2 µg of the template was nick translated in each sample. Unincorporated Cy3-dUTP was removed by double ethanol precipitation, spin columns or Ultrafree filters (Millipore). The purified probes were in 200 µl sterile water for spectroscopic measurements.

Labeling probes by random priming

For random priming reactions, 2 µg of linearized plasmid probe was denatured at 95°C for 5 minutes and immediately cooled on ice. The template was incubated with random priming buffer (10× from Boehringer Mannheim), hexanucleotides (Boehringer Mannheim, 10× hexanucleotide solution), 100 µM of each of the dNTPs, cyanine-dUTP, and 5 units of Klenow DNA polymerase (Boehringer Mannheim). The reaction mixture was incubated at 37°C for 30 minutes to 2 hours. The labeled probes were purified by ethanol precipitation and brought up in 200 µl sterile water for spectroscopic measurement.

Labeling probes by PCR

PCR reactions were carried out in a total of 100 µl mix containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 1.5mM MgCl₂, 1% Triton X-100, 40 ng of the template DNA, 0.25µM of 5'-end labeled primers, 5 units of Tli or Taq DNA polymerase (Promega), and 60 µM of each of the dNTPs. In the control reaction, 60 µM of all the four normal dNTPs and no Cy3-dUTP was used. In others, 50% of dTTP was substituted with different amounts of Cy3-dUTP. The template used was a chromosome 1 plasmid probe containing 900 bp insert (ATCC cat. no. 59863). The primer sequences are: GCTGCAGGTCGACTCTAG (65-Reverse) and GAATTCGAGCTCGCCCGG (65-Forward), which direct the amplification of the insert. The primers were labeled at 5'-end with Cy5.18OSu and purified to homogeneity by HPLC as previously described (35). PCR was performed in DNA Thermal Cycler (Perkin Elmer Cetus). After an initial denaturation at 94°C for 4 min, 25 cycles of PCR were carried out with denaturation at 94°C for 1 min, annealing at 52°C for 2 min and extension at 72°C for 3 min. The labeled probes were purified using Prep-A-Gene (BioRad) to remove unreacted Cy3-dUTP and excess Cy5 labeled primers. The probes were in 150 µl sterile water for spectroscopic measurement.

PCRs were carried out in 100 µl to provide sufficient amount of labeled probes for spectroscopic measurement, but it can be scaled down to 10 µl in practical labeling reactions in order to save material.

Characterization of fluorescently labeled probes

UV/VIS spectra were obtained with a HP 8452 diode array spectrophotometer (Hewlett-Packard) equipped with a deuterium lamp. Fluorescence emission and excitation spectra were taken on Spex Fluorolog 2 spectrofluorometer (Spex Industries, Inc.) equipped with a Xenon lamp. Excitation and emission slits were 1 and 3 mm respectively, corresponding to bandpasses of 1.8 and 5.4 nm.

For characterizing nick translated probes, UV/VIS absorption spectra of the purified probes were obtained. The labeling density of the labeled probe measured as dye-per-kilobase ratio (d/kb) were calculated using Beer's law. The extinction coefficient of Cy3 is 150,000 L/mol.cm (36) and the average extinction coefficient of a base was taken as 10,000 L/mol.cm (37). For random priming labeled probes, the d/kb were not calculated. In addition to the bases in the labeled probe, the template and excess primers contribute significantly to 260 nm absorption. Although the exact d/kb could not be calculated for random priming labeled probes, the ratio of dye absorbance to 260 nm absorbance still reflected the relative amount of dye molecules incorporated and could be used to monitor the incorporation.

For characterizing PCR labeled probes, the probes were purified with Prep-A-Gene. Cy3 and Cy5 emission spectra were measured for each sample and Cy5 emission spectra of the control samples were taken. Cy3/Cy5 molar ratios were converted from Cy3/Cy5 fluorescence intensity ratios using calibration standards of known Cy3 and Cy5 molar ratio. The amplification efficiency is relative to the control sample.

In situ hybridization

The hybridization protocol for interphase nuclei and metaphase chromosome spreads is based on a previously published procedure (26). When using Cy3- and Cy5-dUTP analogs, no secondary reagent is needed for detection. After post-hybridization wash, the slides were stained with 25 μ g/ml Hoechst in Hank's Balanced Salt Solution (HBSS) for 10 minutes at room temperature and washed twice in HBSS for 5 minutes. The slides were mounted in crystal mount (Biomed Corp.) and sealed with nail polish.

Microscope imaging and spot analysis

Images were obtained with a Zeiss fluorescence microscope equipped with a 100 \times oil immersion objective, a Macintosh Quadra 950, a mercury arc lamp and a cooled CCD camera (Photometrics). The imaged fields were 512 \times 512 pixels. A software package based on BDS Image (Biological Detection Systems, Inc. Pittsburgh, PA) was used for image collection and processing, including registration, uniform field correction and signal intensity measurement. A subroutine, 'spot analysis' was written to quantify hybridization signals. The 'FISH signal intensity' is defined as the sum of all pixel intensities within the spot minus the overall background. It is related to the brightness of the probe, the efficiency of hybridization, and the efficiency of the detection system. The overall background intensity was derived by multiplying the average background pixel intensity by spot area. The average background pixel intensity was determined at the periphery of the observed spot. The peripheral region for each spot was determined by dilating the spot until its intensity began to increase or no longer decreased by a pre-determined threshold intensity. The program also calculated signal-to-background, which was defined as maximum pixel intensity within the spot divided by the background pixel intensity. For each slide sample, about 30 signal dots were analyzed and results averaged. The standard deviation was in the range of 10% to 30%.

RESULTS AND DISCUSSION

Synthesis and purification of Cy3-dUTP and Cy5-dUTP analogs

Like many other succinimidyl ester dyes, the cyanine dyes react with primary amino groups. Therefore, an amino-group-containing linker was attached to dUTP in order to obtain a cyanine-dUTP analog. The common modification site on dUTP is the C-5 position of the pyrimidine ring, which does not participate in the base-pair hydrogen bonding. We used 5-(3-amino)allyl-dUTP (AA-dUTP) for the synthesis of cyanine dye-dUTP analogs. In order to avoid cross-linking, Cy3.29OSu, a mono-reactive form of Cy3.18 (36) was used to label AA-dUTP. Figure 1 illustrates the chemical structure of cyanine-dUTP analogs. The space between the dye moiety and the dUTP

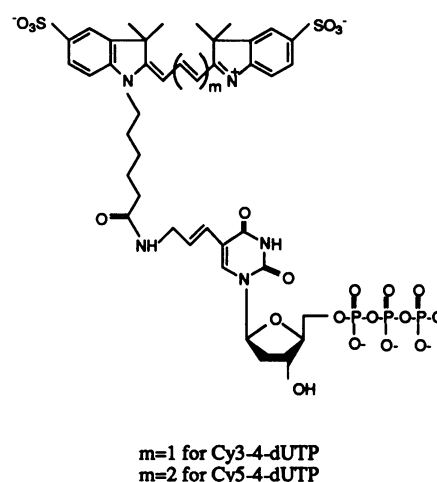


Figure 1. Chemical structure of cyanine-4-dUTP analogs.

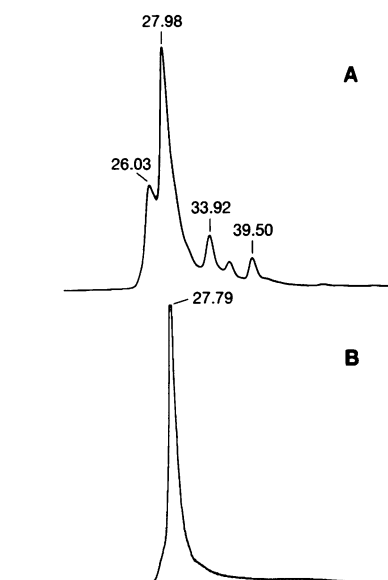


Figure 2. HPLC profile of Cy3-dUTP monitored at 550 nm with visible detector. A: Cy3-dUTP pre-purified by TLC (Materials and Methods) B: The second peak in A was collected and re-injected on HPLC using the same gradient. The numbers shown are time (minutes) after injection.

is 4 atoms and therefore the conjugates are termed Cy3-4-dUTP and Cy5-4-dUTP according to previously used nomenclature (28). For simplicity, Cy3-dUTP and Cy5-dUTP are abbreviations used in the remainder of this manuscript. It should be noted that there is a 6 atom space between the fluorochrome and its reactive group, giving a total of 10 atom space between the fluorochrome and the base.

The Cy3-dUTP conjugate was purified by reversed-phase column as described in materials and methods. The yield of the coupling step was about 50%. The product was further purified on a C18 reversed phase HPLC column. As shown in Figure 2A, five components were resolved which were collected individually and lyophilized. Each of the HPLC components of

Table 1. Characterization of probes generated by nick translation

[dTTP] μ M	15	10	0	0	0	0
[Cy3-4-dUTP] μ M	5	10	20	30	40	50
labeling density (d/kb)	—	8	30	19	25	24
FISH signal intensity	NC	3500	29,800	38,500	34,000	28,000
signal-to-background	—	1.87	6.29	5.60	5.81	5.41

The template was nicked with DNase I to obtain fragments with average length of 150–250bp. In the subsequent labeling reactions, dTTP was substituted with increasing amounts of Cy3-dUTP. Labeling density (d/kb) of purified probes were obtained from UV/VIS absorption spectra. Hybridization signals were quantified by microscope imaging (Materials and Methods). NC=not consistent.

Table 2. Characterization of probes generated by random priming

[dTTP] μ M	50	0	0	0
[Cy3-4-dUTP] μ M	50	100	150	200
OD _{dye} /OD ₂₆₀	1.9%	4.1%	4.2%	4.2%
FISH signal intensity	1,650	10,400	15,600	8,400
signal-to-background	1.74	2.04	2.94	2.24

Linearized template DNA was labeled as described in Materials and Method. dTTP was partially or completely substituted by various amounts of Cy3-dUTP. The order of OD_{dye}/OD₂₆₀ indicates relative incorporation since base absorption resulted from the template, primer and the labeled fragments is constant for all experiments. Hybridization signals were quantified by microscope imaging.

Cy3-dUTP was tested in nick translation reaction. All the components incorporated into DNA probe equally well. Furthermore, when the probes labeled with each of the fractions were used in interphase nuclei hybridization, spot analysis of the signals showed similar intensity (data not shown). Because of the small quantities of these material, further analysis was not performed. For the consistency of the data, only the major HPLC component (Figure 2B) was used in all the studies described in this work.

Enzymatic preparation of Cy3-labeled DNA probes by nick translation

Nick translation utilizes three different enzymatic activities, DNase activity of DNase I, 5' to 3' exonuclease activity and 5' to 3' DNA polymerase activity of *E. coli* DNA polymerase I. The procedure generates a pool of partially labeled DNA fragments.

It was in our interest not only to label DNA probes with cyanine-dUTP but also to provide quantitative measurement of the incorporation and the brightness of the labeled probes. Unlike non-fluorescent markers, the incorporation of the fluorescent dyes into DNA probes can be conveniently and quantitatively monitored by measuring the absorbance or the fluorescence emission of the labeled probes. For example, the absorption of the dye and bases can be obtained from UV/VIS absorption spectrum of a labeled probe. Labeling density measured as dye-per-kilobase ratios (d/kb) can then be calculated using Beer's law knowing the extinction coefficient of the dye and the average extinction coefficient of the bases. This approach was used to monitor the extent of cyanine-dUTP incorporation under various labeling conditions. While it is important to incorporate a high number of dyes into a DNA probe, it is also important not to over-label DNA because dye-dye contacts can lead to

fluorescence quenching (36). Overlabeling may also reduce hybridization efficiency of the probe. Thus, it is essential to quantify actual FISH signals in order to identify the optimal labeling density. Therefore the probes labeled at various d/kb were hybridized with interphase nuclei on slides and images were obtained with an imaging microscope. Relative signal intensities and the signal-to-background of the hybridization signals were measured using 'spot analysis' program (Materials and Methods).

Optimization of the labeling conditions was performed using *SsrI* plasmid probe, a moderately repetitive sequence of 2.5 kilobase appearing in tandem arrays on human chromosome 19 and less frequently on chromosome 4 (38). The pUC12 plasmid containing 2.5 kb *SsrI* insert (total of 5 kb) was used in the labeling reactions.

One important variable in the enzymatic labeling reactions is the relative substitution of dTTP by the dUTP analog. For instance, it has been reported that, when incorporating biotin- or digoxigenin-labeled nucleotides, complete substitution of dTTP by the modified analogs does not give the brightest signal (39,40). The situation is different for cyanine labeled dUTPs. Table 1 shows labeling density (d/kb) as well as hybridization signal intensities of nick translated probes using different Cy3-dUTP concentrations. It is obvious that the probe is significantly brighter when all the dTTP is completely replaced by an equal amount of Cy3-dUTP. Intensities of the hybridization signals parallel the d/kb measurement, indicating that fluorescence quenching is not significant at the higher labeling density. The results also reveal that higher Cy3-dUTP concentrations do not improve labeling density or hybridization signal-to-background. Lower percent substitution results in a much lower labeling density. The sample with 25% substitution did not result in measurable dye absorption of the labeled probe. Although the hybridization signals were observable, they were too weak for valid quantification.

It is evident that 100% substitution of dTTP by Cy3-dUTP is necessary to obtain the brightest probes. When this condition is used, the labeling density is in the range of 20 to 40 d/kb in routine experiments. This number is somewhat lower than expected, since when 100% substitution is applied, approximately 25% of the bases (d/kb ~250) would be labeled if the complete template sequence is replicated. However, several studies have suggested that modified nucleoside triphosphate analogs are incorporated less efficiently than the natural nucleoside triphosphate (28, 39, 40). In the T7 RNA polymerase *in vitro* transcription system, truncated transcripts are generated and the yield of full length transcript is very low when all the ATP is replaced by aminoethyl-ATP, especially with AT-rich template (39). It is conceivable that heavy labeling may cause steric perturbation at the binding and/or catalytic site of the enzyme that would cause the polymerase to fall off the template and stop polymerization. For example, in nick translation with modified dUTP, nucleotide replacement may cease when several adenines are next to each other in the template. Thus, there would be regions in the template that are not labeled at all, while the regions that are labeled may have close to 25% labeling density. The method used to calculate the d/kb only provides an average labeling density throughout the complete length of the probe.

Another important variable in nick translation reaction is DNase I concentration or probe length. For most *in situ* hybridization experiments, an average probe length in the range of 150–250 was found optimal (26, 27). We found in our experiment that labeling density was a function of DNase I concentration. The

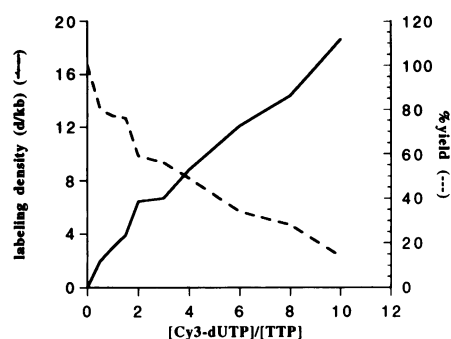


Figure 3. Labeling density and amplification yield as a function of Cy3-dUTP/dTTP molar ratio in PCR. PCRs were performed and the labeling densities as d/kb were measured as described in Materials and Methods.

labeling density increased inversely with the probe length. For example, 44 d/kb was obtained if the probe was nicked to an average length shorter than 100 bases, whereas 20 d/kb and 12 d/kb were obtained for probes with average lengths of 150 bases and 500 bases respectively. According to this result, shorter probes have higher average number of dyes per unit probe and therefore might be preferred on the basis of fluorescence signal intensity. However, heavily labeled short probes may form unstable hybrids due to possible steric perturbation with hydrogen bonding. Therefore the hybridization and wash stringency may have to be adjusted to obtain the best signal-to-background.

Labeling probes by random priming

In a random primed labeling reaction, degenerate oligonucleotides (6 mer or longer) are used as primers to drive the synthesis of uniformly labeled DNA probes by DNA polymerase. For random primed labeling, it was also found that complete substitution of dTTP with Cy3-dUTP provided the brightest probe (Table 2). In this case, only absorption ratio of the dye and the bases instead of d/kb was obtained, because the unlabeled template and excess oligonucleotide primers were not completely removed and contribute significantly to the 260 nm absorption reading. Again, spot analysis of the hybridization signals paralleled the absorption ratio measurement.

Labeling probes by polymerase chain reaction

Since the introduction of polymerase chain reaction (PCR) technologies, numerous applications have been devised including fluorescence labeling of DNA probes. Labeling by PCR offers the advantage of simultaneous amplification and labeling of a specific probe or a specific region of a probe.

We have characterized the incorporation of Cy3-dUTP into DNA probes via PCR. By using primers labeled at the 5' end with Cy5, a dye that absorbs and fluoresces at wavelengths distinct from Cy3, we were able to accurately measure the d/kb of PCR amplified probes. The Cy5 fluorescence (680nm) or absorbance (650nm) signal is proportional to the number of amplified sequences created (amplimers) and provides an accurate measurement of relative amplification efficiency. At the same time, the internal incorporation of Cy3-dUTP was monitored at a separate wavelength (550 nm for absorption and 575nm for fluorescence). After the purification of PCR labeled probes, the

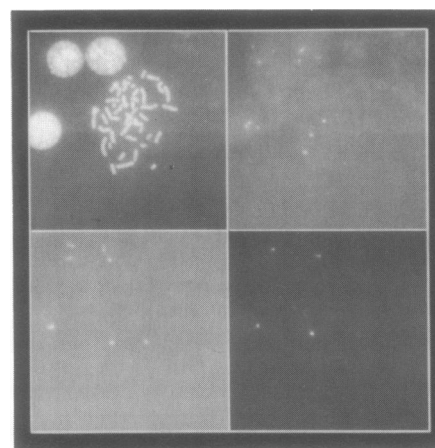


Figure 4. Multicolor FISH image. Images of four fluorescence signals from a human male lymphocyte chromosome spreads after hybridization. The cyanine dye labeled probes were prepared by nick translation. Upper-left: Hoechst fluorescence signal, indicating location of molecular DNA; Upper-right: hybridization signal from Cy3-labeled Chromosome 1 probe; Lower-left: hybridization signal from Cy5-labeled *SstI* probe hybridized to Chromosome 19; Lower-right: hybridization signal from FITC-labeled X chromosome probe (generous gift from Integrated Genetics).

relative Cy3 to Cy5 fluorescence intensity ratios were measured and were converted into Cy3 to Cy5 molar ratios of the labeled probe. These ratios together with the total length of the amplified probe (0.9kb) were used to calculate d/kb. Although the spectral overlap between Cy3 emission and Cy5 absorption is relatively small, energy transfer from Cy3 to Cy5 could occur when Cy3 molecules are inserted within approximately 5.5 nm of the 5' terminal Cy5 label (the distance for 50% energy transfer calculated by Dr Reddington, personal communication). A distance of 5.4 nm along a double helix corresponds to approximately 16 base pairs. Since 18 base primers are situated between the 5'- Cy5 energy acceptor and the closest place a Cy3 dUTP can be inserted, it is unlikely that energy transfer appreciably causes underestimation of d/kb.

As an amplification template, a Sp65 plasmid probe against a chromosome 1 repetitive sequence was used and the primers directed amplification of the 900 bp insert. The amplification was confirmed by loading a fraction of each sample on 1% agarose gel. The same volume of material before amplification was loaded and nothing was observed on the gel (gel not shown). Because of the net negative charges on the dye (Figure 1), labeled probes had only slightly retarded mobility on the gel. However, when longer linkers were used between dUTP and the fluorochrome, the mobility shift was significant (Zhu *et al.*, unpublished results).

Several thermal stable DNA polymerases were investigated for their ability to incorporate cyanine-labeled dUTP. The experiment indicated that the extent of label incorporation could increase by a factor of two or three when using polymerases other than the commonly used Tag DNA polymerase (data not shown). Tli (Promega), Pfu (Stratagene), Deep Vent and Vent Exo⁻ (New England Biolabs) were among those that incorporated most Cy3-dUTP per kb of synthesized DNA under conditions for each of the enzymes as recommended by the manufactures.

The effect of relative amount of Cy3-dUTP to dTTP on the PCR labeled product was tested using *Tli* DNA polymerase. In this experiment, dTTP concentration was kept constant, at 30 μ M in final reaction mixture and increasing amounts of Cy3-dUTP, ranging from 0 and 300 μ M final concentration were added. A control reaction was performed which contained 60 μ M of dTTP and no Cy3-dUTP. The relative yield of amplification in presence of Cy3-dUTP was compared with this control sample. Figure 3 shows that the labeling density (d/kb) increases almost linearly with the increase in Cy3-dUTP concentration, however, at the expense of lower amplification efficiency. As expected, it is the molar ratio of Cy3-dUTP to dTTP in the reaction mixture that governs the frequency with which Cy3-dUTP enters DNA. At higher dTTP concentration, higher Cy3-dUTP concentration is required for a specified labeling density. When the Cy3-dUTP to dTTP molar ratio is in the range of 0.5 to 10, the labeling density varies from 2 to 20 d/kb with relative yields decreasing from 80% to 15% correspondingly. This labeling density with Cy3-dUTP is at the lower end of what can be achieved by nick translation method (20–40 d/kb). While 100% substitution of dTTP with Cy3-dUTP in nick translation provided the best labeling density, complete substitution of dTTP in PCR resulted in no observable amplification by both gel electrophoresis and spectroscopic measurements.

When equal amounts of dTTP and Cy3-dUTP were used in the PCR reaction, the labeling density averaged only 3.3 d/kb. Assuming equivalent amount of all four bases in the sequence and no discrimination between modified and unmodified nucleoside triphosphates, the expected replacement would be 125 d/kb. Thus it is clear that the polymerase discriminates between dTTP and the modified analog with a preference for the dTTP. It is known that many DNA polymerases show little discrimination between dUTP and dTTP in DNA synthesis under most circumstances (41). Therefore the discrimination is mainly caused by the attachment of the linker molecule and/or the fluorophore on the base. This is not surprising given the precise binding site of nucleoside triphosphates on DNA polymerase (42). However, the labeling density increases almost linearly with Cy3-dUTP concentration, demonstrating that the analog is, nevertheless, a good substrate for *Tli* DNA polymerase and other DNA polymerases. When the same experiment was carried out with Taq DNA polymerase, similar result and plot were obtained (data not shown).

The fact that the amplification drops as the labeling density increases suggests that an increasing number of labels incorporated into the template inhibits DNA synthesis in subsequent cycles. Perhaps, heavily labeled DNA template strands do not provide the appropriate recognition sites required for the binding of DNA polymerase, especially where several modified nucleotides are next to each other. By contrast, nick translation and random priming, which translate from unmodified template, produce labeled probe efficiently even with 100% cyanine-labeled dUTP.

Our results demonstrate that there is a trade-off between the labeling density and the amplification yield in PCR labeling reactions. For producing probes for chromosome painting or repetitive sequences, relatively lower labeling density may be sufficient. However, for single copy gene detection where sensitivity is extremely important, higher labeling density may be necessary and the yield may have to be sacrificed.

Four color FISH with three directly labeled probes

Figure 4 demonstrates 4-color hybridization images obtained with human lymphocytes and probes that were directly labeled with Cy3-, Cy5- and FITC-dUTP.

CONCLUSION

Two cyanine-dUTP analogs, Cy3-dUTP and Cy5-dUTP have been synthesized and proven to be efficient substrates for DNA polymerases. An important parameter, cyanine-dUTP to dTTP molar ratio, has been optimized in standard nick translation and random primed labeling reactions. In both cases, complete substitution of dTTP with cyanine-dUTP yields the brightest probes and hybridization signals. In nick translation, labeling density of about 20–40 d/kb can be routinely obtained under the optimal labeling conditions. In polymerase chain reaction, however, complete substitution of dTTP does not result in detectable amount of full length DNA. The labeling density increases as the Cy3-dUTP concentration increases relative to dTTP concentration. However, amplification efficiency drops for more heavily labeled probes. Typically 20 d/kb can be reached at a molar ratio of 10:1 (Cy3-dUTP/dTTP) with amplification efficiency of 15% of that when dTTP is used without labeled dUTP.

Probes labeled with Cy3-dUTP and Cy5-dUTP by nick translation, random priming and PCR have been successfully used in fluorescence *in situ* hybridization experiments. We believe that this development will extend the current ability of FISH and make multisequence analysis a simple, powerful and practical tool in many areas of research and clinical diagnostics.

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