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tors (7). The inability of intracellular LPS molecules to enhance *c-fos* expression in HeLa cells was unexpected.

Two principal mechanisms may account for this result. First, intracellular LPS may remain complexed to polycationic lipids or may be unable to escape from the endosomal compartment of HeLa cells. Second, LPS shows marked structural similarity to the second messenger ceramide and mimics ceramide in stimulating the ceramide-activated protein kinase (CAPK)/kinase suppressor of Ras (9). This enzyme blocks *c-fos* expression by uncoupling Elk-1 phosphorylation from ERK activation (13). It is possible that activation of CAPK led to the suppression of *c-fos* expression in LPS-loaded cells. Further studies carried out by applying the present methods to a variety of primary and immortalized cell types should resolve this issue.

In summary, the loading of LPS into HeLa cells by means of polycationic lipids results in relatively low acute toxicity, as judged from cell viability, morphology and *c-fos* expression, the latter being commonly activated by many stress pathways (14). Therefore, our method appears well suited to the study of acute actions of LPS in the intracellular compartment of mammalian cells.

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Simple Method for Preparation of Fluor/Hapten-Labeled dUTP

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ABSTRACT

Many projects, such as multiplex-fluorescence in situ hybridization (M-FISH) karyotyping, require the use of relatively large amounts of multiple fluor- or hapten-labeled nucleotides for the preparation of DNA probes. Such a requirement makes these experimental approaches prohibitively expensive for many researchers. The cost of such nucleotides can be reduced approximately 99% by purchasing the chemical precursors, fluor or hapten succinimidyl esters and 5-(3-aminoallyl)-2-deoxyuridine 5-triphosphate (AA-dUTP), and performing the simple coupling/purification described here. It is possible to finish four to ten different fluor/hapten dUTP preparations of 2.5 mM scale within a 24 h period. The reagent cost for each preparation ranges from \$33-\$237 per mM, depending on the fluor/hapten. This laboratory uses such nucleotide preparations to prepare FISH probes by nick translation or PCR amplification.

INTRODUCTION

Before the mid-1980s, nucleic acid probes were labeled isotopically and detected after hybridization by autoradiography. The use of non-isotopically labeled probes has, for numerous reasons beyond the safety issue (2), replaced isotopically labeled probes for hybridization. In particular, the ability of fluor-labeled probes to differentiate a number of different hybridization targets simultaneously has proven to be extremely powerful because each fluor can be differentiated by its spectral signature. Using multiple fluors, karyotypes of human metaphase chromosomes can be obtained with a single hybridization (3,4).

The choice of fluors/haptens for labeling depends on the following factors. They must be available as succinimidyl esters or their homologues. Each fluor used in a given experiment must have excitation and emission spectrum

with minimal overlap with the excitation and emission of the other fluors so that each fluor can be individually detected with the appropriate single-bandpass optical filters. Any fluor or hapten used must be capable of being incorporated as a dUTP analog during PCR amplification or nick translation. Alternatively, fluors that cannot be incorporated as fluor-dUTPs can still be used for fluorescence detection if they are coupled to avidin or an antibody directed against hapten-labeled probes.

Fluors also should be reasonably photostable. In recent years, the number of choices of fluors available as fluor-nucleotides or succinimidyl esters has increased so that the detection of as many as seven different fluors in a single hybridization is feasible. However, the cost of fluor nucleotides can still be a deterrent for some experimental approaches. Multiplex-fluorescence in situ

hybridization (M-FISH) or spectral karyotyping (SKY) requires large amounts of labeled nucleotides. It has prompted a simplification of the original method for nucleotide coupling that has been used to produce fluor/hapten dUTP in this laboratory without modification for many years (1). These modest changes produce relatively large amounts of labeled nucleotide for a minimum amount of time, effort and cost.

MATERIALS AND METHODS

Reagents

The reagents required are: DEAE 52 resin (Whatman, Maidstone, England, UK), 1 M triethylammonium bicarbonate buffer (TEAB), pH 7.5 (triethylamine; Sigma, St. Louis, MO, USA, and ammonium bicarbonate; JT Baker, Phil-

lipsburg, NJ, USA), 5-(3-aminoallyl)-2'-deoxyuridine 5' triphosphate (AA-dUTP; Sigma, St. Louis, MO, USA), 0.1 M sodium borate, pH 8.0 (Mallinkrodt, St. Louis, MO, USA), fluor-succinimidyl esters (Amersham Pharmacia Biotech; Molecular Probes; Roche Molecular Biochemicals) and dimethylsulfoxide (DMSO) (JT Baker).

Preparation of DEAE52 Resin

The DEAE 52 anion exchange resin is prepared by first removing fines and then washing successively with 5–10 volumes of 0.1 M NaOH, double-distilled water, 0.1 M HCl, double-distilled water and 0.1 M ammonium bicarbonate, pH 7.5–8.0. The resin can be stored in 0.1 M ammonium bicarbonate buffer at 4°C for months. One gram of dry resin yields about enough material for 10 columns of 0.5 mL each.

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Analysis of the Fluor/Hapten Coupled Nucleotides

We tested the nucleotides in PCR labelings (chromosome painting libraries) and by nick translation (YAC clones) followed by in situ hybridization. Dilutions of the nucleotide mixture (1:2 with TE buffer [10 mM Tris-HCl, 1 mM EDTA, pH 8.0]) usually gives excellent results in the protocol described here. This dilution should give a fluor/hapten concentration equal to or less than 20 μM with 140 μM dTTP also added. Five pools of human chromosome libraries (kindly provided by J. Trent, P. Meltzer and M. Bittner at the National Center for Human Genome Research, Bethesda, MD, USA) were PCR-labeled by the incorporation of fluor or hapten dUTPs prepared as described above. PCRs included 4 μL of DNA template (approximately 500 ng), 20 μM fluor-dUTP, 140 μM dTTP, 200 μM d(AGC)TP, 3 mM MgCl_2 , 2

Table 1. Nucleotide Synthesis Protocol

Coupling Reactions

1. Add 8 mL of 0.1 M sodium borate, pH 8.0, to 10 mg of AA-dUTP to obtain a 2 mM solution. This solution is sufficient for six 2.5 μM coupling reactions. Unused AA-dUTP solution is stable at -20°C .
2. Add 200 μL of DMSO to 5–10 μM of fluor-succinimidyl ester (2.5–10 mg, depending on the molecular weight of the fluor-succinimidyl ester and the fluor:AA-dUTP ratio used). Mix and rapidly transfer to 1.25 mL of 2 mM AA-dUTP (2.5 μM). A fluor:AA-dUTP ratio of 4:1 yields nucleotides that generate strong hybridization signals; equally strong signals have been obtained when AA-dUTP was coupled with a 2.5 molar excess of fluor-ester.
3. Incubate the reaction mixture at room temperature for 4 h. While the mixture is incubating, prepare the DEAE52 column. After 4 h, cool the sample on ice, then run on a 0.5 mL DEAE52 column.

Column Preparation

Equilibrate the resin and the buffers to 4°C . Pour 0.5 mL of resin into a suitable column for each fluor coupling. Wash with 10 mL of 1 M TEAB, pH 7.5. Then wash with 10 mL of 50 mM TEAB, pH 7.5.

Column Run

1. Apply the sample to the top of the resin, but do not allow it to completely sink in or air bubbles will form in the column. When the sample has almost sunk in, gently apply a small amount of 50 mM TEAB to the liquid remaining on top of the resin. Repeat several times until the liquid above the resin is colorless.
2. Wash with 5 mL of 50 mM TEAB.
3. Wash with 5 mL of 100 mM TEAB. Most of the free fluor should be eluted in this wash.
4. Elute fluor nucleotides with 2 mL of 0.6 M TEAB.
5. Dry the 0.6 M TEAB eluate by vacuum centrifugation to remove the ammonium ions. Resuspend the dried nucleotide in 2 mL of 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.0. Each reaction contains a maximum of 2.5 μM of fluor-dUTP. For convenience, we assume the yield is 2 mL of 1 mM fluor/hapten dUTP.

μM 6MW primer, 20 mM Tris-HCl, pH 9.5 and 8 U of *Taq* DNA polymerase (Life Technologies, Rockville, MD, USA) per 100 μL reaction. The reaction conditions were 94°C for 45 s, 54°C for 45 s and 66°C for 4 min, for 30 cycles. After PCR labeling, DNA size was brought to below 500 bp by the addition of one-tenth volume of 10–20 ng/ μL DNaseI (Roche Molecular Biochemicals, Mannheim, Germany) and 20 mM MgCl_2 , followed by incubation for 6 min at room temperature. The reaction was stopped by the addition of one-tenth volume of 50 mM EDTA and 0.02% SDS with 2–3 min incubation at 95°C .

For M-FISH/SKY, 1–2 μg of each chromosome painting library (uniquely labeled with either DEAC, FITC or bi-

otin, Cy3 or Rhodamine-6G, Texas Red[®] or digoxigenin) were pooled and combined with 50 μg of human Cot1 DNA before ethanol precipitation. The probe pellet was resuspended partially in 6 μL of hybridization buffer by incubation at 37°C for 30 min with occasional vigorous mixing, followed by the addition of 6 μL of formamide and subsequent vigorous mixing. Human metaphase spreads were obtained by standard blood-culture and slide-preparation techniques. Slides were stored before use in 100% ethanol, from overnight to several days at -20°C . Before hybridization, the slides were pre-treated with 0.005% pepsin/10 mM HCl for 30–45 s at 37°C . The slides were placed immediately in $1\times$ PBS for 5 min and then dehydrated through an

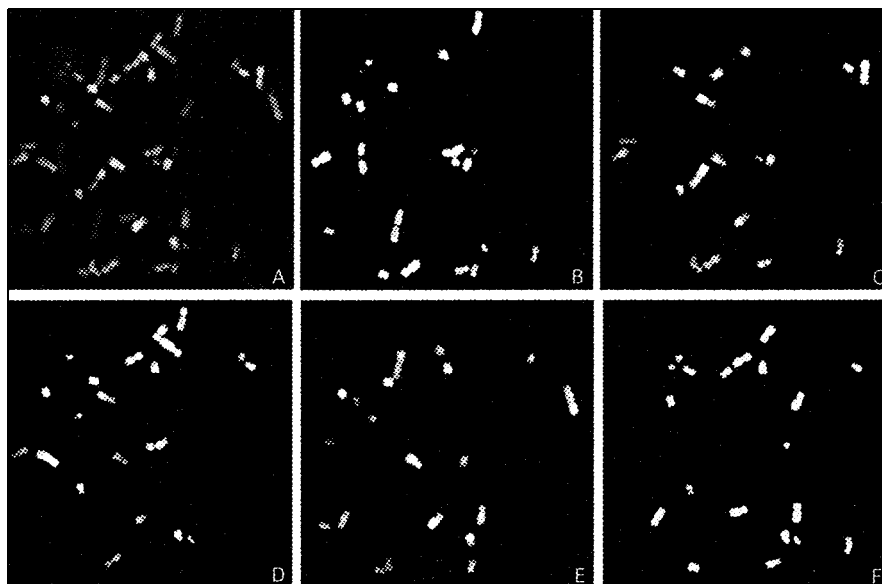


Figure 1. A normal human metaphase spread hybridized with the 24-chromosome probe cocktail labeled by PCR. Panel A shows the merged and pseudocolored image. Panels B–F show individual gray-scale images of hybridization with chromosome subsets labeled with FITC, Cy3, Texas Red, digoxigenin (Cy5.5) and DEAC.

ethanol series. The chromosomes were denatured in 70% formamide/2× standard saline citrate (SSC) for 90 s at 70°C, followed by dehydration through an ethanol series. Hybridization was done overnight (14–20 h) at 37°C.

After hybridization, slides were washed 3× for 5 min in 50% formamide, 2× in SSC at 42°C and 3× for 5 min in 0.5× SSC. After blocking with BSA, the digoxigenin-labeled probes were detected by anti-digoxigenin (Roche Molecular Biochemicals, Indianapolis, IN, USA) that had been coupled with Cy5.5 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using standard dye-protein coupling protocols. Anti-digoxigenin is stored as 1 mg/mL stock solutions and diluted 1:800 in 4× SSC/0.01%/Tween® 20/3% BSA before use. After 30 min incubation at 37°C, slides were rinsed 3 × 3 min in 4× SSC/0.1% Tween. The DNA was counterstained

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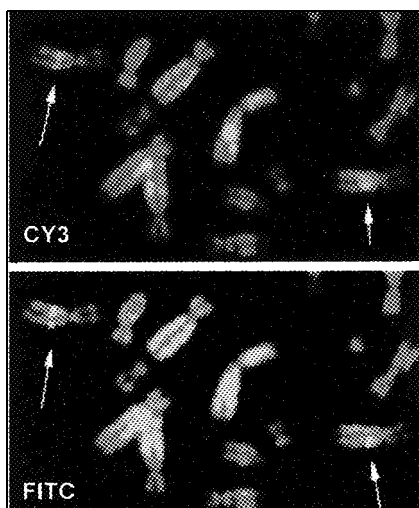


Figure 2. A partial metaphase showing signals from YAC clones labeled by nick translation with Cy3 and FITC.

with DAPI. An antifade medium, VECTASHIELD[®], (Vector Laboratory, Burlingame, CA, USA) and coverslips were applied. Images of the fluorescently-labeled probes were captured using M-FISH software (PSI) with a Sensys charge-coupled device (CCD) camera (Roper Scientific, Trenton, NJ, USA) and a Provis AX-70 microscope (Olympus America, Melville, NY, USA).

Probes were also prepared from YAC DNA by nick translation using standard methods. The fluor nucleotide concentration used was 40 μ M. The probes were pre-annealed with 20 μ g human Cot1 DNA and hybridized and detected as described above.

RESULTS AND DISCUSSION

We have described a simplified method for the preparation of fluor- or hapten-labeled nucleotide, which permits the simple preparation of these probes for hybridization at a low cost. In this procedure, the initial fluor/hapten coupling is performed essentially as originally described (1). However, separation of the free fluor from the other reaction products is accomplished by absorption onto a minimal volume of DEAE52 cellulose followed by step elution, rather than separating all the reaction components using gradient separation on a large column. Because the fluor/hapten dUTP is eluted in a much

smaller volume, the subsequent concentration of the end products is carried out in a SpeedVac[®] (Savant, Holbrook, NJ, USA) with a substantial saving of time and effort. The original protocol required three to five days for one or two fluor/hapten couplings. The modified protocol permits the preparation of 5–10 preparations, each producing 4 mL of 1 mM fluor/hapten dUTP within 24 h. Although the dUTP preparation contains both the precursor allylamine dUTP and the fluor- or hapten-labeled derivative, the use of this mixed preparation results in bright FISH signals.

Hybridization results of probes labeled with nucleotides prepared by this protocol are shown in Figures 1 and 2. Figure 1 shows the results of hybridization of the labeled chromosome painting libraries to normal human chromosomes. The probes were labeled by PCR as described above. The exposure times varied: 1.3 s for DEAC, 10 s for FITC, 0.5 s for Cy3, 0.2 s for Texas Red and 2 s for Cy5.5. Figure 1, panel A, shows the merged and pseudo-colored metaphase spread. Figure 1, panels B–F, show individual gray-scale images of individual fluor signals. Figure 2 shows the hybridization of two different human YAC clones labeled by nick translation.

The cost of nucleotide preparation by this protocol ranges from \$1.00–\$5.93 per equivalent of 25 μ L of 1 mM concentration. Differences in cost from one preparation to another depend on the choice of fluor or hapten because significant differences exist in the price of succinimidyl esters. In addition, fluors differ in the strength of their fluorescent signal; therefore, some fluor nucleotides can be used at lower concentrations in labeling mixtures. Among the fluor dUTPs, DEAC and Texas Red give the strongest signals. Virtually any fluor or hapten dUTP can be prepared using the protocol described here, providing the fluor/hapten-succinimidyl ester is available. The following fluors or haptens have been used with satisfactory results: DEAC, FITC, cy3, Rhodamine-6G, Texas Red, biotin and digoxigenin. The Cy3 and Rhodamine-6G have similar excitation/emission, but Rhodamine-6G is significantly less expensive. When avidin-Cy5 and anti-digoxigenin-Cy5.5 are used for detection, six of these fluors/haptens can be

used simultaneously with DAPI as a DNA counterstain. Thus, with appropriate filter sets, up to 63 probes can be hybridized simultaneously.

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