Method Article

Modified synthesis of 6-carboxyfluorescein (6-FAM): application to probe labeling for conventional cytogenetics

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ABSTRACT

Aims: Fluorescent in situ hybridization (FISH), the routine technique of molecular cytogenetics, is widely used to detect and localize the presence of specific nucleic acids sequences in chromosomes, cell nucleus space, cells and tissue samples through the use of highly complementary probes to targets sequence. Expansion of FISH method application for research purposes and medical diagnostics requires efficient and low-cost production of labeled nucleic acid probes.
Methodology and Results: We developed an effective method of fluorescein hydroxyalkylcarboxamides synthesis. This modification of the basic protocol of 6-carboxyfluorescein (6-FAM) synthesis enabled the production of highly reactive conjugate perfectly suitable for terminal labeling of newly generated oligonucleotides. Efficiency of 6-FAM labeled oligonucleotides obtained by the use of modified protocol has been proved for conventional cytogenetics.
Conclusion: The suggested procedure of 6-FAM labeled oligonucleotides synthesis allows obtaining the high yield of directly labeled FISH probes. The introduction of this method into practice of cytogenetic studies will improve their efficiency and reduce the cost of an examination.

Keywords: 6-carboxyfluorescein phosphoramidite, p-nitrophenyl ether of carboxyfluorescein, fluorescent in situ hybridization, fluorochrome, oligonucleotide synthesis, FISH probe

1. INTRODUCTION

The main technique of molecular cytogenetics is fluorescent in situ hybridization (FISH) requiring labeled nucleic acid probes to detect their position on mitotic or interphase chromosomes in cell nucleus space [1]. DNA or RNA probes (or their analogs) should be conjugated directly with fluorophores or capable of indirect binding fluorescent molecules via non-fluorescent linkers [2]. If the target of interest is of appropriate size (≥ 70 kb), direct attaching of fluorophores to nucleic acid probes is more preferable as it allows to avoid additional antibody-incubation steps and related nonspecific background [3]. For direct labeling both enzymatic and chemical reactions are used. The most popular lab protocols of direct FISH-probe labeling involve different kinds of polymerases to incorporate enzymatically fluorophore-conjugated nucleoside triphosphates by polymerase chain, random priming or nick translation reactions [4]. To visualize loci carrying repeat sequences, oligonucleotides covalently labeled on the 5'-terminus with different fluorescent dyes are used [5].

6-Carboxyfluorescein (6-FAM) is one of the most common and the simplest fluorescent reagents used in oligonucleotide synthesis. 6-FAM is highly reactive, water-soluble single isomer of fluorescein, with a relatively large molar absorptivity (75,000 M−1 cm−1) and a high quantum yield (0.9). Its absorbance/emission maxima lie in the visible region of the spectrum (492/517 nm respectively), so 6-FAM is compatible with the most commonly used fluorescence detection instruments. According to all these reasons, 6-FAM-labeled primers are used widespread in real-time PCR, sequencing and fragment analysis. Specific 6-FAM-labeled oligonucleotides can be used per se for a direct visualization of bacteria in human and animal clinical samples by FISH with rDNA sequences [6, 7]. The use of 6-FAM-labeled oligonucleotides for needs of conventional cytogenetics, i.e. for localization of corresponding nucleotide sequence on mitotic or interphase chromosomes, is rather rare [3, 8].

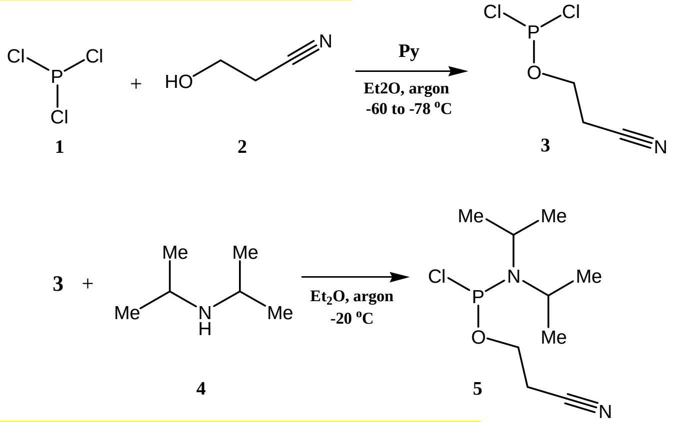
2. METHODOLOGY

Synthesis of 6-FAM-phosphoramidite (**14**) is shown in Fig. 1 (schemes 1-3). In the first step β-cyanoethylphosphorodichloridite (**3**) was obtained [9, 10]. Cyanoethanol (**2**) (17.3 g in diethyl ether) was added slowly to a cooled solution (–78℃) of pyridine (1 equiv.) in diethyl ether containing PCl_3 (1 equiv.). After 1 hour exposure at –78℃ the solution was filtered from pyridinium hydrochloride and solvent was removed under reduced pressure at room temperature to isolate light-yellow liquid of compound **3**. Then the solution of N,N-diisopropylamine (**4**) (2 equiv.) in diethyl ether was added to a solution of compound **3** in diethyl ether during 60 min at –20 ℃ under argon atmosphere. After 20 hours of stirring at room temperature, the amine hydrochloride was removed and the solution was concentrated under vacuum. The concentrated residue was carefully distilled (103-106℃/0.08 mm) to obtain a 29% total yield of the phosphitylating agent the N,N-diisopropylamino-2-cyanoethoxychlorophosphine (**5**) (Scheme 1).

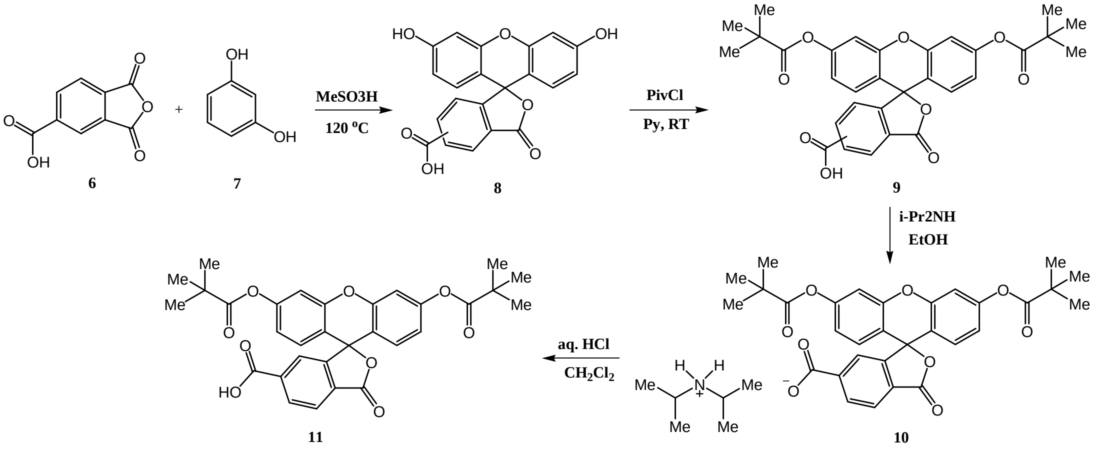
In the next step synthesis of 5(6)-carboxyfluorescein (**8**) was realized [11]. Synthesis was carried out as follows: 1,2,4-benzencarboxylic anhydride (**6**) (2.50 g) and resorcinol (**7**) (2 equiv.) were heated in concentrated methanesulfonic acid (20.0 mL) during 2 h at 120℃. The mixture was added to 250 mL of rapidly stirred ice-water. The obtained brown solid was filtered and washed with water. The resulting precipitate was dissolved in 4M NaOH (25 mL) and acidifed with conc. H_2SO_4 until the light brown precipitate of 5(6)-carboxyfluorescein (**8**) was formed (89% yield). Then the 5(6)-carboxyfluorescein (**8**) was treated by pivaloyl chloride to obtain the nonfluorescent lactone **9** [12]. During the reaction, pivaloyl chloride (12.8 mL) was added drop wised to a cooled and stirred solution of compound **8** (8 g) in dry pyridine (80 mL) with further stirring of the mixture at room temperature during 12 h. The pure 6-isomer of carboxyfluorescein dipivalate **11** was isolated as its diisopropylamine salt **10** from crude mixture of 5- and 6-dipivalates **9**. To obtain a pure isomer **11** the mixture of dipivalates **9** was initially dissolved in absolute ethanol, then it was treated by diisopropylamine with further cooling of the solution to –20℃. The resulting solid **10** was removed by filtration and washed with cold ethanol. The diisopropylamine salt **10** was converted to the carboxylic acid **11** by treatment with hydrochloric acid (Scheme 2).

We developed an effective method of fluorescein hydroxyalkylcarboxamides synthesis based on the reaction of hydroxyalkylamines with fluorescein carboxylic acid p-nitrophenyl ether. The p-nitrophenyl ether **12** was obtained *in situ* from substituted carboxyfluorescein **11** and 4-nitrophenol in the presence of dicyclohexylcarbodiimide (DCC). Compound **12** readily reacts with aminohexanol in methylene chloride giving fluorescein dipivaloyl-6-(N-(6'-hydroxyhexyl))-carboxamide (**13**). Formation of compound **13** proceeds smoothly and it is not accompanied by side reactions, such as removing of the pivaloyl substituents from phenolic groups. As usual in such reactions the activation of the carboxyl group of fluorescein is carried out by means of the formation of N-succinimidyl [12, 13] or pentafluorophenyl [13, 14] ethers. The following phosphitylation of the carboxamide **13** with *N,N*-diisopropylamino-2-cyanoethoxychlorophosphine (**5**) in the presence of diisopropylethylamine (DIPEA) furnishes the desired phosphoramidite **14** (Scheme 3). The resulting 6-FAM-phosphoramidite **14** was used in automated DNA synthesizers for the preparation of fluorochrome-labelled oligonucleotide.

Scheme 1. Synthesis of phosphitylating agent **5**



Scheme 2. Synthesis of 6-carboxyfluorescein dipivalate **11**



Scheme 3. Synthesis of 6-FAM-phosphoramidite **14**

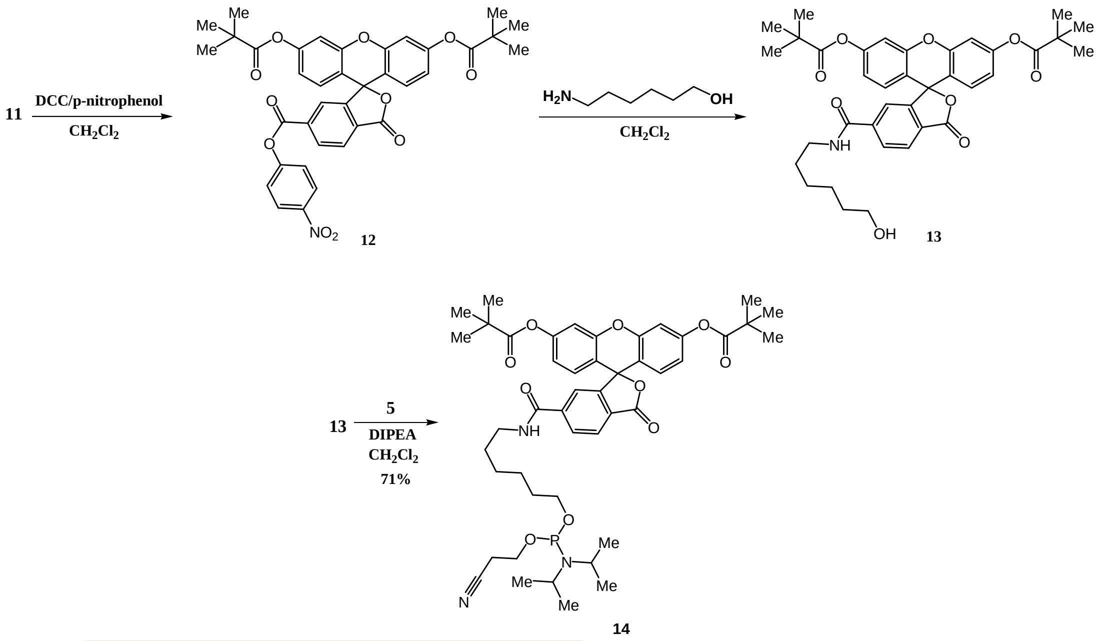


Fig. 1. **Preparation of 6-FAM-phosphoramidite 14**

Oligonucleotide synthesis was performed using ASM-800 DNA/RNA synthesizer (Biosset, Russia) on a 0.1 μM scale according to standard program for long sequences. 6-FAM-labelled oligonucleotides were cleaved from solid support, deprotected with 30% water solution of ammonium hydroxide in 24 hours at 22°C and lyophilized. Then oligonucleotides were resuspended in 50% formamide and purified on a denaturing polyacrylamide gel. 5’-FAM labeled oligonucleotide derivatives were analyzed using 7% polyacrylamide gel electrophoresis in 0,5x TBE-buffer [15] at 250V, stained with Coumassie Blue and visualized in blue light (440-485 nm).

For molecular cytogenetic analysis we tested 6-FAM-conjugated oligonucleotides complementary to telomere sequence (5'-FAM-CTAA{CCCTAA}6C-3’) and chicken tandem repeat PO41 (5′-FAM-TATGGGGCTCTATGGGGCTCTATGGGGCGGC-3′) (NCBI acc. # AF124926) [4, 16] as molecular probes for DNA/DNA FISH on chicken metaphase chromosome. Chromosome preparations were made from cells derived from 4-day-old chicken embryos according to standard protocol including treatment with colcemid (0.1 μg/ml for 1 h at 37℃), hypotonic swelling with 0.55% KCl for 20 min at 37℃ and methanol-glacial acetic acid (3:1) fixation. Chromosomes were pretreated with RNaseA (100 μg/ml), pepsin (0.0005% in 0.01N HCl) and formaldehyde (1% in PBS, 50mM MgCl2) according to routine procedures. The 6-FAM-conjugated oligonucleotides were dissolved to a final concentration of 20 ng/μl in hybridization buffer (50% formamide, 2xSSC, 10% dextran sulfate) with 50-fold excess of Escherichia coli tRNA (Roche). DNA of chromosomes and probes were denatured together on the slide covered with a coverslip and sealed with rubber cement at 82℃ for 5 min. After overnight hybridization in a humid chamber at room temperature, slides were washed in three changes of 2×SSC at 28-30℃, dehydrated in 70-96% ethanol series, air-dried and mounted in antifade solution containing 1% 4’,6-diamidino-2-phenylindole (DAPI). Preparations were examined using a Leica DM4000B fluorescence microscope equipped with a monochrome CCD camera DFC350FX and appropriate filters. QFISH (Leica) software was used to capture and process chromosome colour images.

3. RESULTS AND DISCUSSION

The most important step in production of fluorophore-conjugated probe is coupling of 6-FAM molecule to 5-terminus of an oligonucleotide chain. The method of fluorescein hydroxyalkylcarboxamides synthesis described here allows to produce highly reactive conjugate perfectly suitable for terminal labeling of newly generated oligonucleotides.

We tested 6-FAM-conjugated oligonucleotides as molecular probes for DNA/DNA FISH on metaphase chromosome sets. We report that due to the high brightness, using of 6-FAM labeled oligonucleotides as hybridization probes is perfectly suited for FISH to cytogenetic purposes (Fig. 2). Background fluorescence is absent, specific signals fluoresce brightly in green spectrum.

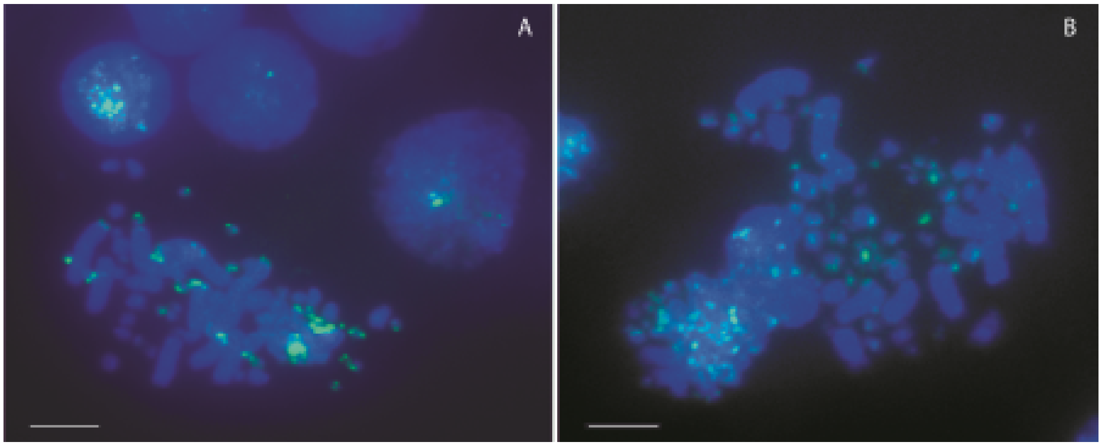


Fig. 2. FISH with 6-FAM labelled oligonucleotide probes (green signals) on chicken mitotic chromosomes: (A) localization of PO41 sequences; (B) localization of telomere repeats. Chromosomes were counterstained with DAPI (blue), scale bar – 5 mkm.

4. CONCLUSION

Here we described new modification of 6-FAM labeled oligonucleotides synthesis which allows to obtain the high yield of directly labeled nucleic acid probes. According to our protocol fabrication of FISH probes for conventional cytogenetics can become more efficient and cheap.

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COMPETING INTERESTS

The authors declare no competing interests.

AUTHORS' CONTRIBUTIONS

Alexander Stepakov carried out the chemical synthesis of 6-carboxyfluorescein. Svetlana Galkina carried out the cytogenetic validation. Denis Bogomaz generated oligonucleotide probes labeled with 6-FAM. Elena Gaginskaya participated in the design and coordination of the study. Alsu Saifitdinova initiated the study and drafted the manuscript. All authors read and approved the final manuscript.

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DEFINITIONS, ACRONYMS, ABBREVIATIONS

FISH - Fluorescent in situ hybridization
6-FAM - 6-carboxyfluorescein
DCC - dicyclohexylcarbodiimide
DIPEA - diisopropylethylamine