Video Article

Synthesis of Wavelength-shifting DNA Hybridization Probes by Using Photostable Cyanine Dyes

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Abstract

In this protocol, we demonstrate a method for the synthesis of 2'-alkyne modified deoxyribonucleic acid (DNA) strands by automated solid phase synthesis using standard phosphoramidite chemistry. Oligonucleotides are post-synthetically labeled by two new photostable cyanine dyes using copper-catalyzed click-chemistry. The synthesis of both donor and acceptor dye is described and is performed in three consecutive steps. With the DNA as the surrounding architecture, these two dyes undergo an energy transfer when they are brought into close proximity by hybridization. Therefore, annealing of two single stranded DNA strands is visualized by a change of fluorescence color. This color change is characterized by fluorescence spectroscopy but can also be directly observed by using a handheld ultraviolet (UV) lamp. The concept of a dual fluorescence color readout makes these oligonucleotide probes excellent tools for molecular imaging especially when the described photostable dyes are used. Thereby, photobleaching of the imaging probes is prevented, and biological processes can be observed in real time for a longer time period.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54121/>

Introduction

Molecular imaging represents a fundamental technique for understanding biological processes within living cells.¹⁻³ The development of fluorescent nucleic acid based probes for such chemical-biological applications has become an expanding research field. These fluorescent probes need to meet a few requirements to become suitable tools for cell imaging. Firstly, the applied dyes should exhibit fluorescence with high quantum yields, large Stokes' shifts and, most importantly, high photostabilities to allow long-term *in vivo* imaging. And secondly, they should show a reliable fluorescence readout. Conventional chromophore-quencher-systems are based on the readout of a single fluorescence color by simple changes in fluorescence intensities.⁴ This approach bears the risk of false positive or false negative results due to autofluorescence of intracellular components or low signal-to-noise ratios due to undesired quenching by other components.⁴

We recently reported on the concept of "DNA traffic lights" that show dual fluorescence color readouts by using two different chromophores.⁵⁻⁶ The concept is based on the energy transfer (ET) from the donor dye to the acceptor dye which changes the fluorescence color (see **Figure 1**). This allows a more reliable readout and thereby provides a powerful tool for fluorescent imaging probes. Labelling of oligonucleotides with fluorescent dyes can be achieved by two different approaches. Dyes can be incorporated during the chemical DNA synthesis on a solid phase by using correspondingly modified phosphoramidite building blocks.⁷ This method is limited to dyes that are stable under standard phosphoramidite and deprotection conditions. As an alternative, post-synthetic modification methodologies were established in oligonucleotide chemistry. Here, we demonstrate the synthesis of one of our new photostable energy transfer pairs^{8,9} and the post-synthetic labelling of DNA by using copper-catalyzed 1,3-cycloaddition between azides and alkynes (CuAAC).¹⁰

Protocol

Caution: Please consult all relevant material safety data sheets (MSDS) before use. Several of the chemicals used in these syntheses are toxic and carcinogenic. Please use all appropriate safety practices that are typically required in organic chemistry laboratories, such as wearing a laboratory coat, safety glasses and gloves.

1. Synthesis of the Dyes

Note: Both dyes can be synthesized by the same types of reaction. **Figure 2** shows an overview of these reactions.

1. Synthesis of 1-(3-azidopropyl)-4-(2-(1-methyl-1H-indol-3-yl)vinyl)pyridin-1-ium iodide (dye 1)

1. **Synthesis of 1-(3-iodopropyl)-4-methylpyridin-1-ium iodide (Figure 2A, step a)**
 1. Dissolve 466 mg 4-picoline and 5.91 g 1,3-diiodopropane in 10 ml of acetonitrile in a 20 ml headspace vial and seal tightly by a septum cap.
 2. Heat to 85 °C for 16 hr.
 3. Allow to cool to RT, then remove the solvent under reduced pressure using a rotatory evaporator.
 4. Add 20 ml of ethyl acetate to the residual oil and treat the mixture in an 120 W ultrasonic bath for 3 min.
 5. Collect the formed precipitate by filtration and wash five times with ethyl acetate. Dry the solid product under vacuum O/N.
 2. **Synthesis of 1-(3-azidopropyl)-4-methylpyridin-1-ium (Figure 2A, step b)**
 1. Dissolve 900 mg 1-(3-iodopropyl)-4-methylpyridin-1-ium iodide in 12 ml of acetonitrile in a 20 ml headspace vial, add 376 mg of sodium azide, and seal tightly by a septum cap.
 2. Heat to 85 °C for 16 hr.
 3. Allow to cool to RT, then remove the solvent under reduced pressure using a rotary evaporator.
 4. Add 15 ml of dichloromethane to the residue.
 5. Filter off and discard the resulting precipitate.
 6. Remove the solvent under reduced pressure using a rotary evaporator to obtain the product as brown oil.
 3. **Synthesis of 1-methyl-1H-indole-3-carbaldehyde (Figure 2A, step c)**
 1. Under inert gas (argon), dissolve 1.45 g indole-3-carbaldehyde, 1.52 g potassium carbonate and 2.70 g dimethyl carbonate in 10 ml absolute dimethylformamide in a 50 ml round-bottomed flask equipped with a reflux condenser.
 2. Stir the mixture at 130 °C for 19 hr.
 3. Allow to cool to RT, then pour the mixture on ice.
 4. Extract the aqueous layer three times with 150 ml ethyl acetate.
 5. Combine the organic layers, wash them with water, dry them over sodium sulfate and remove the solvent at 50 °C under reduced pressure using a rotary evaporator.
 4. **Coupling to 1-(3-azidopropyl)-4-(2-(1-methyl-1H-indol-3-yl)vinyl)pyridin-1-ium iodide (dye 1) (Figure 2A, step d)**
 1. Work under argon and under exclusion of moisture. Dissolve 90 mg 1-(3-azidopropyl)-4-methylpyridin-1-ium and 48 mg 1-methyl-1H-indole-3-carbaldehyde in 4 ml ethanol in a 20 ml round-bottomed flask equipped with a reflux condenser.
 2. Add 0.07 ml piperidine and heat to 80 °C for 4 hr.
 3. Allow to cool to RT.
 4. Collect the resulting precipitate by filtration and wash three times with diethylether.
 5. Add diethylether to the supernatant and collect the resulting precipitate by filtration. Wash three times with diethylether.
 6. Combine the precipitates.
2. **Synthesis of 1-(3-azidopropyl)-4-(2-(1-methyl-2-phenyl-1H-indol-3-yl)vinyl)quinolin-1-ium iodide (dye 2)**
 1. **Synthesis of 1-(3-iodopropyl)-4-methylquinolin-1-ium iodide (Figure 2B, step a)**
 1. Dissolve 715 mg 4-methylquinoline and 5.91 g 1,3-diiodopropane in 10 ml of acetonitrile in a 20 ml headspace vial and seal tightly by a septum cap.
 2. Heat to 85 °C for 16 hr.
 3. Allow to cool to RT, then remove the solvent under reduced pressure using a rotary evaporator.
 4. Add 20 ml of ethyl acetate to the remaining oil and treat the mixture in an 120 W ultrasonic bath for 3 min.
 5. Collect the formed precipitate by filtration and wash five times with ethyl acetate. Dry the solid product under vacuum O/N.
 2. **Synthesis of 1-(3-azidopropyl)-4-methylquinolin-1-ium (Figure 2B, step b)**
 1. Dissolve 900 mg 1-(3-iodopropyl)-4-methylquinolin-1-ium iodide in 12 ml of acetonitrile in a 20 ml headspace vial, add 333 mg of sodium azide, and seal tightly by a septum cap.
 2. Heat to 85 °C for 16 hr.
 3. Allow to cool to RT, then remove the solvent under reduced pressure using a rotary evaporator.
 4. Add 15 ml of dichloromethane to the residue.
 5. Filter off and discard the resulting precipitate.
 6. Remove the solvent under reduced pressure using a rotary evaporator to obtain the product as brown oil.
 3. **Synthesis of 1-methyl-2-phenyl-1H-indole-3-carbaldehyde (Figure 2B, step c)**
 1. Under inert gas (argon), dissolve 1.45 g 2-phenyl-1H-indole-3-carbaldehyde, 0.996 g potassium carbonate and 1.77 g dimethyl carbonate in 10 ml absolute dimethylformamide in a 50 ml round-bottomed flask equipped with a reflux condenser.
 2. Stir the mixture at 130 °C for 19 hr.
 3. Allow to cool to RT, then pour the mixture on ice.
 4. Extract the aqueous layer three times with 150 ml ethyl acetate.
 5. Combine the organic layers, wash them with water, dry them over sodium sulfate and remove the solvent under reduced pressure using rotary evaporator.
 4. **Coupling to 1-(3-azidopropyl)-4-(2-(1-methyl-2-phenyl-1H-indol-3-yl)vinyl)quinolin-1-ium iodide (Figure 2B, step d)**
 1. Work under argon and under exclusion of moisture. Dissolve 90 mg 1-(3-azidopropyl)-4-methylquinolin-1-ium and 59.7 mg 1-methyl-2-phenyl-1H-indole-3-carbaldehyde in 4 ml ethanol in a 20 ml round-bottomed flask equipped with reflux condenser.
 2. Add 0.06 ml piperidine and heat to 80 °C for 4 hr.
 3. Allow to cool to RT.
 4. Collect the resulting precipitate by filtration and wash with diethylether three times.
 5. Add diethylether to the supernatant and collect the resulting precipitate by filtration. Wash three times with diethylether.

6. Combine the precipitates.

2. Synthesis of the DNA Strands

Note: The synthesis of the DNA strands is carried out using the phosphoramidite method on a solid phase, as described by M. Caruthers¹¹ on a DNA synthesizer. The functioning of the synthesizer is tested before the synthetic procedure, and reagents are renewed if necessary.

1. Prearrangements

1. Dissolve the commercially available 2'-O-propargyl-deoxyuridinephosphoramidite (cU) in 1.2 ml of amidite diluent (extra dry acetonitrile). Move solution in the vial to a synthesizer vial and screw into synthesizer.
2. Perform a "leak test" (according to manufacturer's instructions) to make sure that no argon gas leak exists. If the "leak test" fails, screw in the vial more tightly.
3. Prime the cU solution by filling the tube that connects to the solid phase synthesis chamber.
4. Wash all lines with acetonitrile.

2. Synthesis of DNA strands

1. Use the connected computer to enter the DNA sequence and coupling method, following the prompts from the manufacturer's protocol. The coupling step of the cU building block takes 168 sec with 7 pulses (each pulse are 16 μ l) compared to 40 sec with 7 pulses for a conventional phosphoramidite as building blocks.
2. Mount the column containing the CPG (controlled pore glass) as solid phase that is modified with 1 μ mol of the first base (DNA synthesis is performed from 3' to 5') into the synthesizer.
3. Start the synthesis on the DNA synthesizer¹¹.

3. Workup of synthesized DNA strands

1. Dry the columns with the synthesized DNA strands under vacuum O/N.
2. Use a pincer to open the column and release the CPG into a reaction vial. Add 700 μ l of concentrated aqueous ammonia solution to deprotect the oligonucleotide and release the DNA strand from the CPG.
3. Close the vial and apply a security lid onto the reaction vessel to prevent it from bursting, and heat to 50 °C for 18 hr.
4. Remove ammonia by centrifugation under reduced pressure (100 mbar) at 30 °C for 30 min.
5. Start filtration from CPG: Centrifuge vessel at 11,000 x g for 3 min. Take supernatant and transfer it into a centrifugal device with a pore size of 0.45 μ m. Centrifuge at 1,000 x g for 4 min.
6. Meanwhile add 300 μ l of double distilled water to the CPG, vortex for 20 sec and centrifuge at 11,000 x g for 3 min. Transfer the supernatant into the centrifugal device and centrifuge at 1,000 x g for 4 min. Repeat this washing procedure twice.
7. Remove the water from the combined aqueous solutions by centrifuging at 0.1 mbar and 25 °C O/N.

3. "Clicking" Procedure

1. Add 50 μ l doubly distilled water, 25 μ l of a sodium ascorbate solution (0.4 M in water), 34 μ l tris-[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (0.1 M in DMSO/tBuOH 3:1), 114 μ l of the azide (0.01 M in DMSO/tBuOH 3:1) and 17 μ l of a tetrakis(acetonitrile) copper(I)hexafluorophosphate solution (0.1 M in DMSO/tBuOH 3:1) to the lyophilized alkyne-modified DNA sample.
2. Incubate the sample at 60 °C for 1.5 hr.
3. Cool to RT.
4. Add 150 μ l Na₂EDTA (0.05 M in water) and 450 μ l sodium acetate (0.3 M in water) to the DNA sample and transfer to a 10 ml tube.
5. Add 10 ml ethanol (100%) and keep at -32 °C for 16 hr.
6. Centrifuge vessel for 15 min at 1,000 x g and remove supernatant.
7. Wash DNA-pellet with 2 ml cold ethanol (80%).
8. Dry pellet under reduced pressure.

4. HPLC Purification

Note: Before separating the DNA strands make sure that the HPLC is working properly, enough solvent is available and the column is clean. Rinse column with the starting concentration of buffer/acetonitrile.

1. Dissolve the crude DNA-pellet in 250 μ l of doubly distilled water and transfer to an HPLC vial.
2. Start HPLC run of 45 min with a gradient of 0% acetonitrile to 15% of acetonitrile for dye 1 and a gradient of 0% acetonitrile to 17% of acetonitrile for dye 2. Monitor the run by checking 260 nm (DNA absorption) and 459 nm (dye 1) or 542 nm (dye 2). Collect those fractions that show absorption in both wavelength channels.
3. Check the collected fractions for the right mass by matrix assisted laser desorption ionization (MALDI) mass spectrometry using a matrix consisting of 3-hydroxypicolinic acid (3HPA) and diammoniumhydrogencitrate.
 1. Prepare the matrix by mixing 900 μ l of a saturated 3-HPA solution in 1:1-mixture of acetonitrile and water with 100 μ l of a diammoniumhydrogencitrate solution (100 g/L) in water.
 2. Pipette 1-2 μ l of the DNA probe on the target and let it dry on air.
 3. Add 0.2 μ l of the 3-HPA/diammoniumhydrogencitrate matrix to the sample and mix until it crystallizes.
 4. Perform MALDI mass spectrometry.

- Combine those HPLC fractions that exhibit the right mass.

5. Determination of Concentration

Note: The concentration is determined by measuring the absorption at 260 nm using a UV/Vis spectrophotometer, based on the extinction coefficients (ϵ_{260}) of the DNA bases and the dye.

- Dissolve the DNA in 100 - 200 μ l doubly distilled water.
- Apply 1 μ l of the DNA solution on the UV/Vis spectrophotometer.
- Measure the absorption at 260 nm. Repeat three times.
- Take the average value to calculate the concentration of the solution. For the calculation of the extinction coefficients, use $\epsilon_{260\text{nm}}$ of the four natural bases¹² and the $\epsilon_{260\text{nm}}$ of the purchased building block cU (considered as natural uridine)¹² to obtain the $\epsilon_{260\text{nm}}$ of the whole DNA strand. For dye 1, use $\epsilon_{260\text{nm}} = 10,200 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ and for dye 2, use $\epsilon_{260\text{nm}} = 13,100 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$. Calculate the concentration of the solution following Lambert-Beers' law based on the extinction coefficients and measured absorbances.¹³

6. Sample Preparation and Spectroscopy

1. Preparation of single strand samples

- Prepare separately 1 ml of 2.5 μ M solutions of each of the modified DNA1 and DNA2 in 200 mM NaCl, 50 mM NaP_i buffer, pH = 7.

2. Preparation of double strand samples

- Prepare 1 ml of a solution containing 2.5 μ M of DNA1 and 2.5 μ M of DNA2 in 200 mM NaCl, 50 mM NaP_i buffer, pH = 7 in a reaction vessel.
- Secure the lid with a safety cap and heat to 90 °C for 10 min. Then turn off heating and allow to cool down to RT O/N.

3. Absorption spectroscopy

Note: To determine the excitation wavelength for the fluorescence spectroscopy absorption spectra are recorded.

1. Single strand measurements

- Record a blank measurement containing only 200 mM NaCl, 50 mM NaP_i buffer, pH = 7.
- Transfer the prepared solution of DNA1 into a 1 cm quartz glass cuvette and record the absorption. Correct the measurement against the blank data. Determine the maximum value of dye absorption.
- Repeat for DNA2.

4. Fluorescence spectroscopy

1. Single strand measurements

- Record a blank measurement containing only 200 mM NaCl, 50 mM NaP_i buffer, pH = 7; using the excitation wavelength of dye 1.
- Transfer the DNA1 solution into a 1 cm quartz glass cuvette.
- Record the fluorescence spectrum.

2. Double strand measurement

- Transfer the double strand solution into a quartz glass cuvette.
- Record the fluorescence spectrum using the excitation wavelength of dye 1.

5. Visualization experiments

Caution: Appropriate eye protection should be worn to avoid UV damage to the eyes!

- To gain a better understanding of what the recorded spectra are showing, irradiate the cuvettes with handheld UV-lamps (**Figure 5**). Observe the change in fluorescence color from the single to the double stranded DNA.

Representative Results

Absorption and fluorescence spectra of the single and double stranded DNA are recorded as shown in **Figure 4**.

The recorded absorption spectra (**Figure 4** right) show absorption maxima λ_{max} at 465 nm for single-stranded DNA1 (dye 1) and 546 nm for single-stranded DNA2 (dye 2). The annealed DNA1_2 (dye 1 & dye 2) shows maxima at both 469 nm and 567 nm. Both absorption maxima show a bathochromic shift, 4 nm for dye 1 and 21 nm for dye 2. These small spectroscopic changes are the result of excitonic interactions between the dyes within the double-helical DNA architecture.

The corresponding fluorescence spectra (**Figure 4** left) exhibit maxima λ_{max} at 537 nm for single-stranded DNA1 (dye 1, excitation at 435 nm), at 607 nm for single-stranded DNA2 (dye 2, excitation at 535 nm) and at 615 nm for annealed DNA1_2 (dye 1 & dye 2, excitation at 435 nm). DNA1_2 shows solely the emission maximum of dye 2 although dye 1 is selectively excited which evidences that the energy transfer occurs efficiently from dye 1 to dye 2. This gives an emission contrast ratio of 1:57.

The difference between single and double strand is obvious by comparing the emission color in the cuvette during excitation with a standard UV-lamp as shown in **Figure 5**.

Figure 1. Basic concept of an energy transfer in DNA. The donor modified DNA strand (green) shows fluorescence at 535 nm upon irradiation at 435 nm. If it is hybridized with the acceptor-modified counter strand (red) the resulting double strand shows only the red fluorescence of the acceptor dye at 610 nm. [Please click here to view a larger version of this figure.](#)

Figure 2. Synthesis overview of dye 1 and dye 2. The synthesis of the two cyanine dyes 1 (A) and 2 (B) is carried out in three consecutive steps.⁹ (A) a) 1,3-diiodopropane, CH₃CN, 85 °C, 16 hr, 94%; b) NaN₃, CH₃CN, 85 °C, 16 hr, 87%; c) K₂CO₃, dimethyl carbonate, DMF, 130 °C, 19 hr, 89%; d) piperidine, EtOH, 80 °C, 4 hr, 80%. (B) a) 1,3-diiodopropane, CH₃CN, 85 °C, 16 hr, 49%; b) NaN₃, CH₃CN, 85 °C, 16 hr, 93%; c) K₂CO₃, dimethyl carbonate, DMF, 130 °C, 19 hr, 98%; d) piperidine, EtOH, 80 °C, 4 hr, 44%. [Please click here to view a larger version of this figure.](#)

Figure 3. Structure of cU-building block, dyes 1 and 2 and sequences of DNA1 and DNA2. The dyes 1 and 2 are linked to the 2' position of the ribose by a triazole linker within the given sequences of DNA1 and DNA2.⁹ [Please click here to view a larger version of this figure.](#)

Figure 4. Fluorescence of single and double stranded DNA strands (left) and absorption of single and double stranded DNA strands (right). Emission spectra of DNA1 (black) excited at 464 nm, DNA2 (red), excited at 535 nm and 435 nm (pink), and DNA1_DNA2 hybrid (blue), excited at 435 nm are shown. The absorption spectra of the single stranded DNA1 (black), DNA2 (red), and the hybridized double strand DNA1_DNA2 (blue) are shown on the right side show. [Please click here to view a larger version of this figure.](#)

Figure 5. Emission color of the single stranded DNA with donor dye (left) and double stranded DNA with donor and acceptor dye (right) during excitation by an UV-lamp. The image of the cuvette containing the single stranded DNA1 (left side) shows bright green fluorescence, and the image of double stranded DNA1_DNA2 (right side) shows red fluorescence during excitation by a handheld UV-lamp at 366 nm. [Please click here to view a larger version of this figure.](#)

Discussion

This protocol shows the complete procedure to label DNA post-synthetically via CuAAC by azide-modified fluorescent dyes. This includes the synthesis of the dyes and the alkyne-modified DNA as well as the labeling procedure.

The synthesis of the dyes follows four steps. All products can be obtained by a rather simple precipitation due to their positive charge and no time consuming column chromatography is needed. The introduction of the azide functionalities before the central coupling steps shortens the syntheses of the dyes to four instead of five consecutive reaction steps. The application of 1,3-diiodopropane instead of 3-iodopropanol for the alkylation step, the Appel reaction and the following Finkelstein reactions to convert the hydroxyl function into the iodo functionalities were avoided. These changes in comparison to the published procedures allow us to synthesize the dyes in larger scales and shorter periods of time.

The applied dyes exhibit an excellent photostability but not the necessary stability during solid-phase DNA synthesis and workup. Hence, the post-synthetic labelling strategy was chosen. Alternatively, labelling with such precious dyes during the DNA synthesis represents ultra-mild coupling and deprotection conditions (e.g., deprotection with potassium carbonate). This represents a significantly more expensive alternative and requires the synthesis of the corresponding dye-modified phosphoramidites as DNA building blocks or labelling on the solid support (on-bead).

DNA1 is labeled by dye 1, the donor dye, and DNA2 by dye 2, the acceptor dye. When DNA1 and DNA2 are annealed both dyes get into close proximity. An efficient energy transfer is observed when dye 1 is excited. In principal, excitonic interaction between both dyes may interfere with an efficient energy transfer since the latter process requires the uncoupled and excited dye 1 and uncoupled dye 2. Excitation of the coupled

dyes would initiate different photophysical pathways.¹⁴ Our previous studies^{9,15} revealed that the applied 3'-5'-diagonal arrangement of dyes provides the structural basis for only little excitonic interactions between the dyes and efficient energy transfer.

The high efficiency of the demonstrated energy transfer enables precise fluorescence readout in *in vitro* and *in vivo* experiments. This offers a wide range of applications, including the visualization of DNA hybridization in molecular beacons, binding of target molecules in nucleic acid based aptamers as well as the visualization of the integrity of small interfering RNA (siRNA) inside cells. The major limitation is the requirement to label both DNA strands. In the future we aim to focus our work on the development of doubly labeled oligonucleotide probes bearing both dyes in one strand.

Disclosures

The authors have nothing to disclose.

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