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Professor Rainer Glaser
University of Missouri-Columbia
Associate Editor, *The Journal of Organic Chemistry*

RE: REVISED
YOYO-1 Green Fluorescent Dye: The Advanced Dye of the Future
By Kasey Royer and Michelle Lukosi

Dear Dr. Glaser:

Thank you very much for your letter of 20 April regarding the above cited paper. We value the constructive comments made by Reviewers 09, 05, and 07, and we have now prepared a revision and the changes made are listed below.

Major Changes

[M.1] Spectra have been moved to the Supporting Information.

[M.2] Our title has been modified to better fit the subject of our paper.

[M.3] Lots of formatting issues have been addressed.

Response to Reviewer 09

[09.1] Clarification of the location of the methane bridge has been established, with the addition of "located between the two aromatic rings at the ends of the molecule).

[09.2] We now have readers referring to Figure 1 to visualize how YOYO-1 intercalates with DNA (we didn't feel a new scheme or figure was appropriate for the Results and Discussion section).

[09.3] We changed the previous sentence to now read, "We developed a simple synthesis for YOYO-1, beginning with two parts benzoxazolicmonomethine cyanine, adding one part…ending with a reflux."

[09.4] The caption for Table 1 has been centered.

[09.5] "In inhomogeneous" was changed to "in homogeneous" and the apostrophe was removed from "spectra's."

[09.6] Figure 3's caption has been centered.

[09.7] A bibliography for the spectra has been added to the appendix.

Response to Reviewer 05

[05.1] We clarified what FISH is, by adding the sentence, "FISH (fluorescent in situ hybridization) is a technique used to diagnose chromosome abnormalities." Also clarified is the meaning of "decay of the fluorescence polarization anisotropy" fluorescence polarization anisotropy, which is difference between the fluorescence intensities, detected with a polarization parallel and perpendicular to the excitation polarization (simply put, the fluorescence differences between the emission and excitation states).

[05.2]We understand that more references in the introduction may give more validity, but we feel the general ideas of molecular fluorescent probes and nuclear probes were described quite well and our two sited references were extremely good sources and do justice to the topic. Any more references that we found simply repeated ideas that we had already found, so found it unnecessary to continue adding repetitive sources simply to increase our number of references.

[05.3] Additional sources have been added to the Results and Discussion section.

[05.4] The spectra in the Appendix have been centered. Results for DAPI are listed instead of those for YOYO-1 because YOYO-1 spectra have yet to be published, but DAPI is suspected to have very similar spectra.

[05.5] Grammatical errors have been addressed.

[05.6] A header was added with last names and email addresses have been unlinked.

Response to Reviewer 07

Title

[07.1] The title has been changed to "YOYO-1 Green Fluorescent Dye: The Advanced Dye of the Future"

Abstract

[07.2] Sentence 4 has been changed to "Luminescent properties were measured and recorded," and in sentence 5, "with results discussed later in the paper" has been removed.

[07.5] Sentences 8 and 9 have been condensed to one sentence, reading, "Overall, we believe that with the development of YOYO-1 we will now be able to learn even more about chromosome development, and breakthroughs may come in learning where development goes wrong in certain chromosomal mutations."

Introduction

[07.1] "Now" has been eliminated.

[07.2] Figure 1 has been taken from a source, and that source was sighted. It is a figure, not a scheme, and we have given create to the creator. We do not have the capabilities to recreate this image.

[07.3] The sentence has been modified to read, "as well as in chromosome division."

[07.4] Again, yes this figure was taken from a source, and again that source was sited. Again, it is a figure, not a scheme. We do not have the means to recreate it. We feel it gives the reader an idea of the variety of nuclear fluorescent probes that exist, so it will remain in our paper.

[07.5] “Luminescence spectra” has been changed to “UV/Vis spectra.”

[07.6] We corrected the reference numbering from reading “Figure 2” to the correct reference of “Figure 3.”

[07.7] The sentence has been changed to read, “TOTO-1 differs by only two sulfurs in place of the two oxygens in the YOYO-1 molecule.”

[07.8] Requested grammatical changes have been made.

[07.9] We left Scheme 1 as is because we feel that it is an important enough scheme to take up a large portion of the paper. Changing the orientation will only take up more space and we feel would be harder for the reader to follow.

[07.10-11] The final two sentences have been changed to, “YOYO-1 allows one to follow a cell through mitosis (six stages thus far), so we expect the probe to help with breakthroughs in learning even more about a cell’s development at the chromosomal level and any mutations that may occur. Hopefully, this research will ultimately apply in humans.”

Materials and Methods

[07.1] Sentence 1 now reads, “YOYO-1 synthesis begins with…”

[07.2]“BisquaRternization” and “quaRternization”

[07.3] Synthesis details are in the Supporting Information.

[07.4] Supporting Information is referenced instead of the appendix.

[07.5] We feel that Scheme 2 is well explained by the caption. The caption says “two parts benzoxazolicmonomethine cyanine”, meaning the benzoxazolicmonomethine cyanine is clearly the molecule with the brackets inclosing it with a 2 outside of the brackets, and the N,N,N',N'-tetramethyl-1,3-propanediamine is the other reactant, with YOYO-1 as the product.

[07.6] “Once” was changed to “only after” and the end of the sentence was eliminated.

[07.7] The introduction has been changed to include “a few other nuclear probes…”

[07.8] Yes, this information is stated correctly. The methanol/HCl is an aqueous solution.

[07.9] “Spectra graphs” has been changed to “spectra”, but seeing as there was a debate in class where these graphs should be located, we chose to put them in our Results and Discussion section, as the spectrum of YOYO-1 are “results” of our experiment.

[07.10] Changed to “spectra” and “Supporting Information.”

[07.11] The Table 1 title has been moved to the top of the table.

[07.12] The table has been reorganized, with YOYO-1 followed by TOTO-1 and then DAPI.

Results and Discussion

[07.1] Saying it is nonfluorescent in aqueous media means that it doesn't fluoresce when it is simply in the cell; it must be bound to a nucleic acid to fluoresce.

[07.2] "Caught in the act" has been changed to "going through…"

[07.3] "YOYO-1" has been added to beginning of sentence 5.

[07.4] Noted.

[07.5] "dsDNA" has been changed to "double-stranded DNA."

[07.6] All grammatical errors of "spectra's" have been changed to "spectra."

[07.7] Excited-state dynamics refers to the fluorescence in the excited-state. This explanation has been added.

[07.8] Citations have been added. These references were already cited, and all information in this section came from the sources we have reported.

[07.9] We don't feel that this charge/pH information is relevant in our discussion, so we have chosen to not make any changes to this section.

[07.10] "H-dimer" has been changed to "homodimer."

[07.11] This sentence has been explained with the addition of the explanation of fluorescence polarization anisotropy.

[07.12] The sentence now reads, "In one case, we have observed a marked change of the depolarization dynamics upon increasing the dye concentration and this increase is explained by different binding modes." We feel that this change in phrasing reinforces the idea that this is "our" research.

[07.13-14] We've decided to keep both of these sentences in the Results and Discussion section, as we feel these are crucial RESULTS of our study.

[07.15] Figure 3 has been moved to the Supporting Information section.

Conclusion

[07.1] "Has been" has been changed to "is."

[07.2] A space, as well as others found, have been inserted where necessary.

[07.3] In sentence 2, "allowed" has been changed to "will allow."

[07.4] In sentence 4, "is" has been changed to "can be."

[07.5] "Also" was changed in this sentence.

[07.6] Comma inserted and "also" removed.

[07.7] RNase is the proper term. A comma after cases is not necessary.

[07.8] Noted.

[07.9] Your suggestions are noted, however we are going to keep our original sentence.

Supplemental Materials

[07.1] It is now titled “Supplemental Material Available.”

[07.2] The YOYO-1 UV spectrum is the first one found in the table of UV spectra.

Supporting Information

[07.1] The title of the paper has been changed to “YOYO-1 Green Fluorescent Dye: The Advanced Dye of the Future” from “YOYO-1 Green Fluorescent Dye to Follow Chromosomes Through Mitosis”

[07.2] The reason that we have spectra for DAPI and not for YOYO-1 is because spectra of this nature was not available for YOYO-1, but was obtained for DAPI, which is a compound of a similar nature, as was explained in our paper, page 6, at the end of the Materials and Methods section.

[07.3] The resolution for the DAPI spectra is quite fuzzy. However, we were unable to make these pictures become any more defined than they already are sadly and did not have anything else to provide.

[07.4] The assignment did not specify as to where the UV spectra for our probe, and those to which we were comparing it to, should go. They can go in either the Results and Discussion or in the Supporting Information. However, being that there were so many I do agree that they would do better in the Appendix and have so moved them.

YOYO-1 Green Fluorescent Dye: The Advanced Dye of the Future

Kasey Royer and Michelle Lukosi

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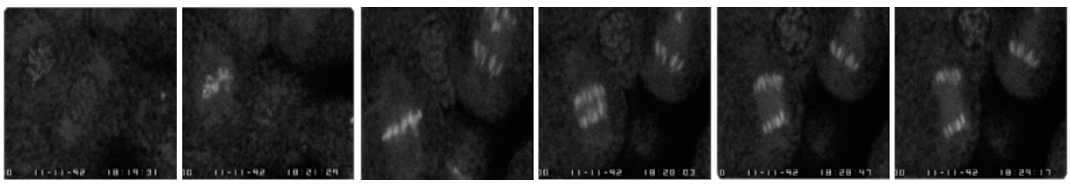
Abstract:

Recently synthesized is a new molecular fluorescent probe, YOYO-1. More specifically, this is a green nuclear fluorescent probe that binds to double stranded DNA, allowing one to follow a chromosome through at least six phases of mitosis and chromosome development. We developed a simple synthesis for YOYO-1, beginning with two parts benzoxazolicmonomethine cyanine, adding one part N,N,N',N'-tetramethyl-1,3-propanediamine, and ending with a reflux. Luminescent properties were measured and recorded. We also compared our nuclear probe to six other nuclear probes' luminescence, as well as two other nuclear probes' structures. YOYO-1 has strong emission once bound to nucleic acids, and although any nuclear probe can have carcinogenic effects, our probe is very luminescent at low concentrations and has very little to no interference with other organelles, minimizing toxicity. Overall, we believe that with the development of YOYO-1 we will now be able to learn even more about chromosome development, and breakthroughs may come in learning where development goes wrong in certain chromosomal mutations.



The six stages of mitosis tracked by YOYO-1 thus far

Introduction

Molecular fluorescent probes have illuminated cellular function and have thus enabled cellular exploration for years. Fluorescence has become an extremely powerful tool for investigating the structure and dynamics of matter or living systems at a molecular or supramolecular level. Polarity, fluidity, order, molecular mobility, and electrical potential are just a few of the characteristics that can be measured by fluorescent probes in things such as biological membranes, proteins, and nucleic acids in living cells[1]. A good, useful probe is often one that fluoresces only once bound to the organelle of choice.

Here, we will focus on nuclear fluorescent probes. These are probes that bind to either DNA or RNA (nucleic acids), and once bound, fluoresce, allowing one to follow a cell's developmental process at the nuclear level. There are three classes of classic dyes that target and bind to different locations on nucleic acids, including intercalating dyes, minor groove binders, and other nucleic acid stains[2]. A sample strand of DNA and possible target sites is shown below in Figure 1.

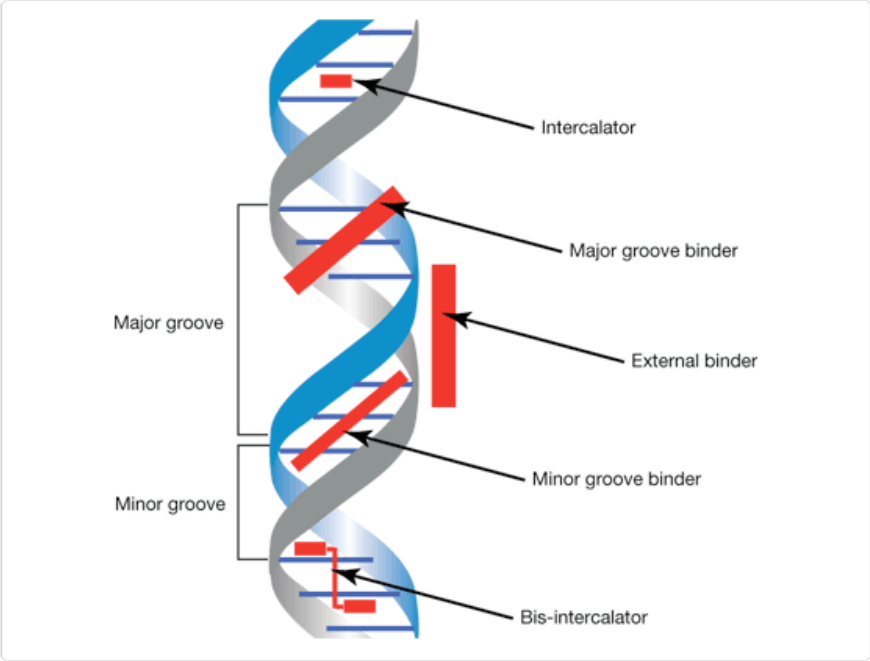


Figure 1. DNA with possible target sites of different nuclear probes[2]

Nuclear probes are extremely significant, as they allow for visualizing nuclei and chromosomes, and they also are very useful in the analysis of chromosome banding patterns, as well as in chromosome division. Different probes will fluoresce different colors, and a few of these variations are shown below in Figure 2. A few characteristics are key for a useful nuclear probe, including high molar absorptivity, very low intrinsic fluorescence (little to no fluorescent properties when not bound to nucleic acids), very large fluorescence upon binding to nucleic acids, and moderate to high affinity for nucleic acids with little or no staining of any other organelles or biopolymers[2].

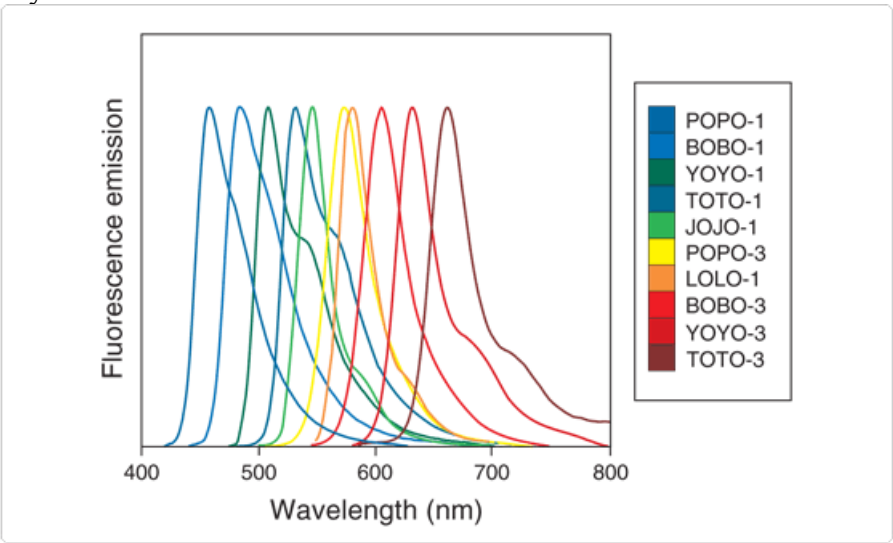
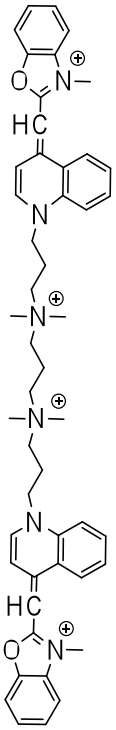


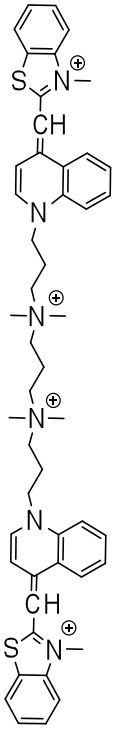
Figure 2. Fluorescence Emission Spectra of DNA-bound nuclear probes[2]

Here we report the results of YOYO-1, a green fluorescent dye that we have synthesized to follow chromosomes through cell division. We have microinjected YOYO-1 into cells in order to follow mitotic chromosomes through at least six cell cycles in fertilized sea urchin eggs[2]. We have measured the UV/Vis spectra of YOYO-1, which can be seen in the Results and Discussion section (Figure 3). We compared YOYO-1 to TOTO-1 and DAPI, as well as a few other nuclear probes, which all fluoresce different colors when bound to nucleic acids. As shown in Scheme 1, YOYO-1 and TOTO-1 have extremely similar structures. TOTO-1 differs only by two sulfurs in

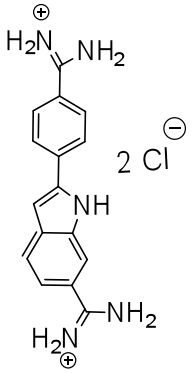
place of the two oxygens in the YOYO-1 molecule. DAPI is much smaller and its differing structure may explain its different fluorescent properties (as listed in Table 1).



YOYO-1 – N,N,N',N'-tetramethyl-N,N'-bis{3-[4-[(3-methyl-2-(3H)-benzoxazolilydene)-methyl]-quinolinium-1-yl]-1,3-propanediyl}-1,3-propanediammonium ion



TOTO-1 - N,N,N',N'-tetramethyl-N,N'-bis{[4-[(3-methyl-2-(3H)-benzothiazolilydene)-methyl]-quinolinium-1-yl]-1,3-propanediyl}-1,3-propanediammonium ion



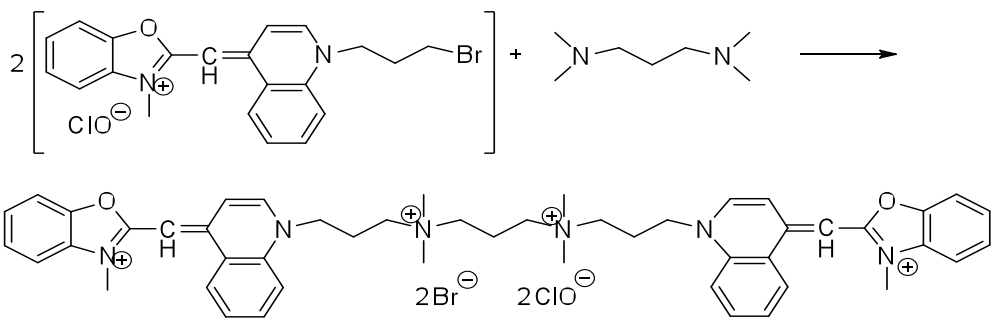
DAPI - 1H-Indole-6-carboximidamide, 2-[4-(aminoiminomethyl)phenyl]-, dihydrochloride

Scheme 1. YOYO-1 nucleic dye shown with two comparable dyes, TOTO-1 and DAPI.

Our new YOYO-1 probe is significant in that it does not fluoresce until bound to double-stranded DNA, and once bound, it emits a bright green fluorescence at low concentrations. YOYO-1 allows one to follow a cell through mitosis (six stages thus far), so we expect the probe to help with breakthroughs in learning even more about a cell's development at the chromosomal level and any mutations that may occur. Hopefully this research will ultimately apply in humans.

Materials and Methods

YOYO-1 synthesis begins with the reaction of 1-(3-iodopropyl)-4-[(3-methyl-2-(3H)-benzothiazolilydene)methyl]quinolinium iodide or 1-(3-iodopropyl)-4-[(3-methyl-2-(3H)-benzoxazolilydene)methyl]quinolinium iodide with N, N, N`, N`-tetramethyl-1,3-propanediamine. The starting materials of 1-(3-iodopropyl)-4-[(3-methyl-2-(3H)-benzothiazolilydene)methyl]quinolinium iodide and 1-(3-iodopropyl)-4-[(3-methyl-2-(3H)-benzoxazolilydene)methyl]quinolinium iodide are known as benzoxalicmonomethinecyanines, and these are bisquarternized with four different compounds. After quarternization, reflux, and isolation, the synthesis is complete[1]. Scheme 2 outlines the synthesis, and a more detailed description is provided in the Supporting Information.



Scheme 2. Synthesis reaction of YOYO-1: two parts benzoxazolicmonomethine cyanine with one part N,N,N',N'-tetramethyl-1,3-propanediamine.

Once YOYO-1 was synthesized, its luminescent properties were measured. On its own, the probe does not fluoresce. The green fluorescence is seen only after YOYO-1 binds to double-stranded DNA. The luminescent properties of YOYO-1 and of comparable nuclear probes (which are listed in Table 1 below) were all measured in aqueous solutions with dyes bound to

nucleic acids (DNA or RNA), and all spectra are measured in methanol acidified with a trace of HCl[2]. Actual spectra of YOYO-1 and of the comparable dyes are located below, in the results section. Other spectra, including NMR and IR, for the comparable probe DAPI, are located in the Supporting Information.

Table 1. Luminescent properties of nuclear probes[2]

Nuclear Probe	Abs. Max. (nm)	Em. Max. (nm)	ε (cm^{-1})	Color of Fluorscence
YOYO-1	491	509	99,000	Green
TOTO-1	514	533	117,000	Green
DAPI	358	461	24,000	Blue
Nuclear Yellow	355	495	36,000	Yellow
Hoechst 33342	350	461	45,000	Blue
BOBO-3	570	604	148,000	Orange
YOYO-3	612	631	167,000	Orange

Results and Discussion

YOYO-1 nucleic acid stain is a useful green-fluorescent nuclear counterstain because of its bright nuclear signal and low cytoplasmic background staining. The dye shows intense green fluorescence upon binding to nucleic acids, and a wash step is not required because the dye is essentially nonfluorescent in an aqueous medium. We have found that the YOYO-1 dye provides simple and reliable green-fluorescent counterstains for FISH analysis. FISH (fluorescent in situ hybridization) is a technique used to diagnose chromosome abnormalities[4]. It has been used to observe chromosomes going through cell division in fixed cells and tissues. YOYO-1, a dimeric cyanine dye, has been used to observe mitotic chromosome movement in live cells. The fluorescent dye has been microinjected into cells in order to follow mitotic chromosomes

through at least six cell cycles in fertilized sea urchin eggs. Incorporation of the fluorescent tracer does not interfere with subsequent progress through the cell cycle, and fluorescent strands of DNA can be followed as they assemble into chromosomes and segregate into daughters and granddaughters[1].

The extraordinary stability of the nucleic acid complexes formed with our dimeric cyanine dyes ensures that the dye–DNA association remains stable during electrophoresis. Electrophoresis is the motion of dispersed particles relative to a fluid under the influence of a spatially uniform electric field; it is ultimately caused by the presence of a charged interface between the particle surface and the surrounding fluid. Binding of the YOYO-1 dye to DNA initially results in homogeneous binding that yields double bands in DNA gel electrophoresis. These double bands can be avoided by incubating complexes for times long enough to allow binding to come to equilibrium or by heating samples to 50°C for at least two hours. Binding of our other dimeric nucleic acid stains to DNA does not seem to give this problem. The YOYO-1 dye has no fluorescence on its own, but becomes strongly fluorescent after binding to double-stranded DNA[1]. The stain preferentially binds to double-stranded DNA, but will stain single-stranded DNA with lower performance.

The molecule of the YOYO-1 dye was compared to TOTO-1 and DAPI, two other nuclear probes. We then collected data of the YOYO-1 dye such as its absorption spectra and compared it to TOTO-1 and DAPI, as well as a few other nuclear probes. Luminescent properties are listed in Table 1 and spectra are shown in Figure 3. The excited-state dynamics (fluorescence when in the excited-state) of the DNA intercalator YOYO-1 and of three derivatives has been investigated in water and in DNA using ultrafast fluorescence spectroscopy. In the free form, the

singly charged dyes exist both as monomers and as homodimers, while the doubly charged dyes exist predominantly as monomers. Both forms are very weakly fluorescent. The early fluorescence dynamics of these dyes in DNA exhibits substantial differences compared with that measured with their homodimeric YOYO analogues, which are ascribed to dissimilarities in their local environment. Finally, the decay of the fluorescence polarization anisotropy (which is simply the difference in fluorescence between the exciting states of a photon[4]) reveals ultrafast hopping of the excitation energy between the intercalated dyes. In one case, we have observed a marked change of the depolarization dynamics upon increasing the dye concentration and this increase is explained by different binding modes. Indeed, the major fluorescence enhancement mechanism of the monomeric dyes upon intercalation (refer to Figure 1 for a visual of the intercalation location) is the inhibition of the large amplitude motion around the methane bridge (located between the two aromatic rings at the ends of the molecule) that leads to an ultrafast nonradiative deactivation. This study also reveals that time-resolved fluorescence anisotropy combined with numerical simulations can be a very powerful tool for obtaining direct information on the binding mechanism of these dyes to DNA.

YOYO-1 has been shown to be successful in the lab, as well as in the market, where it has been promoted as safer to work with and free from the complex waste disposal than other fluorescing dyes, such as ethidium. However anything capable of binding DNA with high affinity is a possible carcinogen.

Conclusion

The synthesis of the YOYO dye family is a major breakthrough in the development of fluorescent DNA probes for molecular biology. They will allow, for the first time, DNA

detection with sensitivity comparable to that of radioactive probes[3]. YOYO-1 finds usage in several areas of biochemistry and molecular biology. It can be used as a dye for the quantification of double stranded DNA in some methods of real time PCR, as well as being used to visualize DNA in gel electrophoresis. In addition to labeling pure nucleic acids, YOYO-1 can be used for labeling of DNA within cells for flow cytometry and fluorescence microscopy. In these cases, RNase treatment may be required to reduce background from RNA in the cells. YOYO-1 is not only relatively safe to work with and free of the complex waste disposal associated with other fluorescing dyes, it has shown resilience and reliability in the laboratory.

Supplemental Material Available: The appendix contains a detailed description of the synthesis of YOYO-1. It also contains UV spectrum of YOYO-1 as well as a few other spectrums of the nuclear probes listed in Table 1. The appendix can be obtained by contacting the authors.

References

[1] Gadjev, N.; Deligeorgiev, T.; Timcheva, I.; Maximova, V. Synthesis and Properties of YOYO-T-type homodimericmonomethine cyanine dyes as noncovalent nucleic acid labels. *Elsevier Science Ltd.* **2003**, *57*, 161-164.

[2] Johnson, I.; Spence, M.Probes for the Nucleus.*Molecular Probe Handbook, A Guide to Fluorescent Properties and Labeling Technologies.***2011**, *11,* Chapters 8 & 12.

[3] Furstenberg, A.; Vauthey, E.Ultrafast Excited-State Dynamics of Oxazole Yellow DNA Intercalators.*J. Phys. Chem. B.* **2007**, *111,* 12610-12620.

[4] Fergus, K. FISH Analysis.*Biology Online.* **2009**.

[5] Diaspro, A. Fluorescence Polarization. *National Facility for Multi-Photon Excitation Fluorescence Spectroscopy on Biomolecules*.**2000**.

Supporting Information

YOYO-1 Green Fluorescent Dye: The Advanced Dye of the Future

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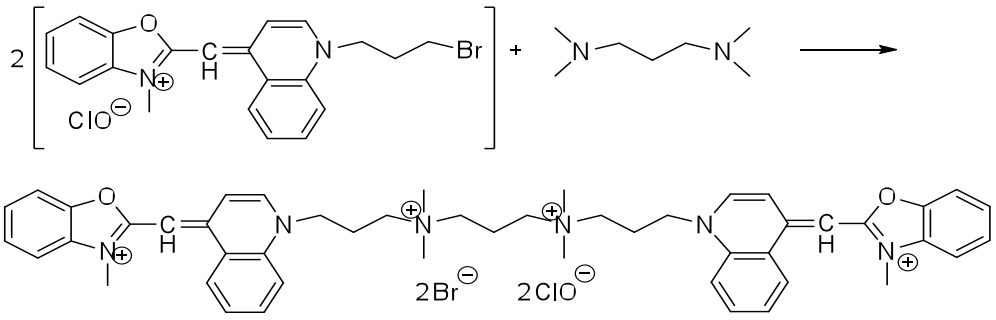
Table of Contents

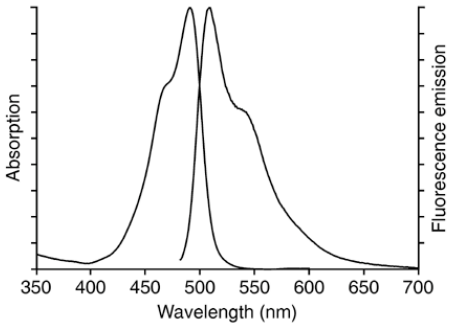
Synthesis of YOYO-1

The monomethine cyanine dyes (1-(3-iodopropyl)-4-[(3-methyl-2-(3H)-benzothiazolilydene)methyl]quinolinium iodide and 1-(3-iodopropyl)-4-[(3-methyl-2-(3H)-benzoxazolilydene)methyl]quinolinium iodide) were dissolved in 10-20 mLmethoxyethanol, while stirred and heated. The diamine (1 mM of N, N, N`, N`-tetramethyl-1,3-propanediamine) was then added (which is known as being quarternized) and this mixture was refluxed for 1-5 hours. TLC plates were used to observe the completion of the reaction. Once the reaction was complete the resultant precipitate was filtered off (by suction), washed with ethanol/ether in a 1:1 ration, and air-dried.

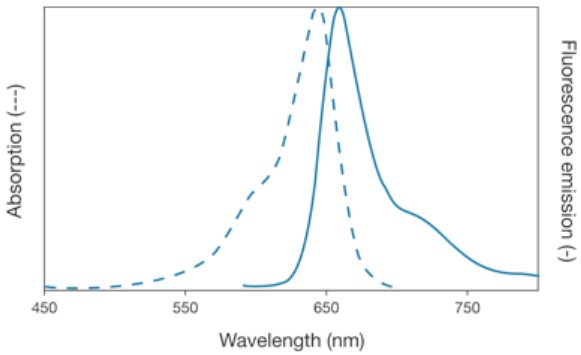


Scheme 2. Synthesis reaction of YOYO-1: two parts benzoxazolicmonomethine cyanine with one part N,N,N',N'-tetramethyl-1,3-propanediamine.

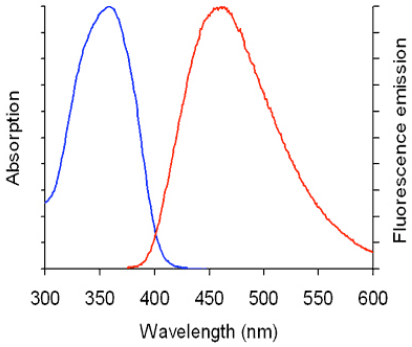
YOYO-1 UV Spectrum



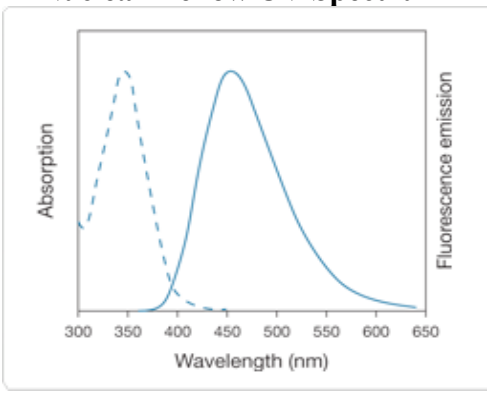
YOYO-3 UV Spectrum



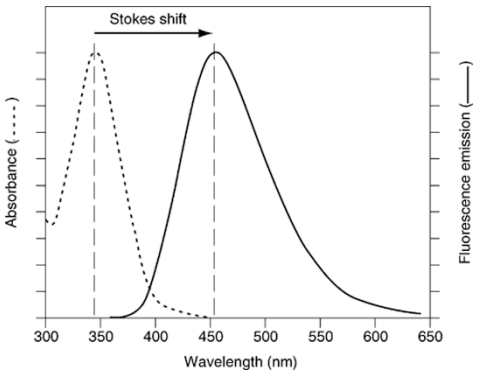
DAPI UV Spectrum



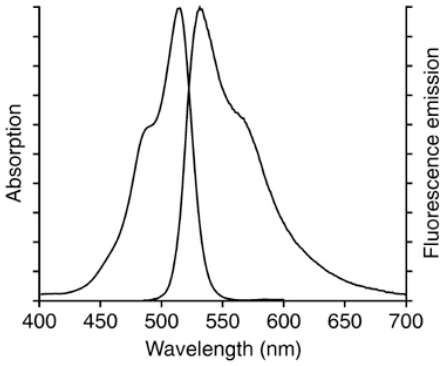
Nuclear Yellow UV Spectrum



Hoechst 33342 UV Spectrum



BOBO-3 UV Spectrum



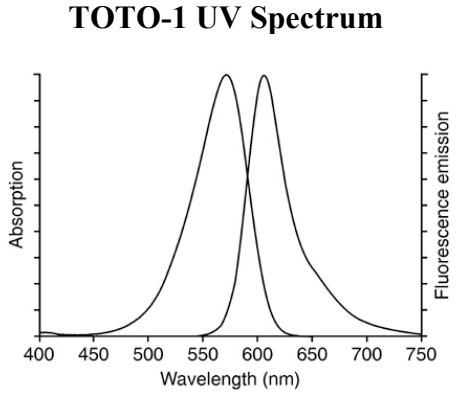
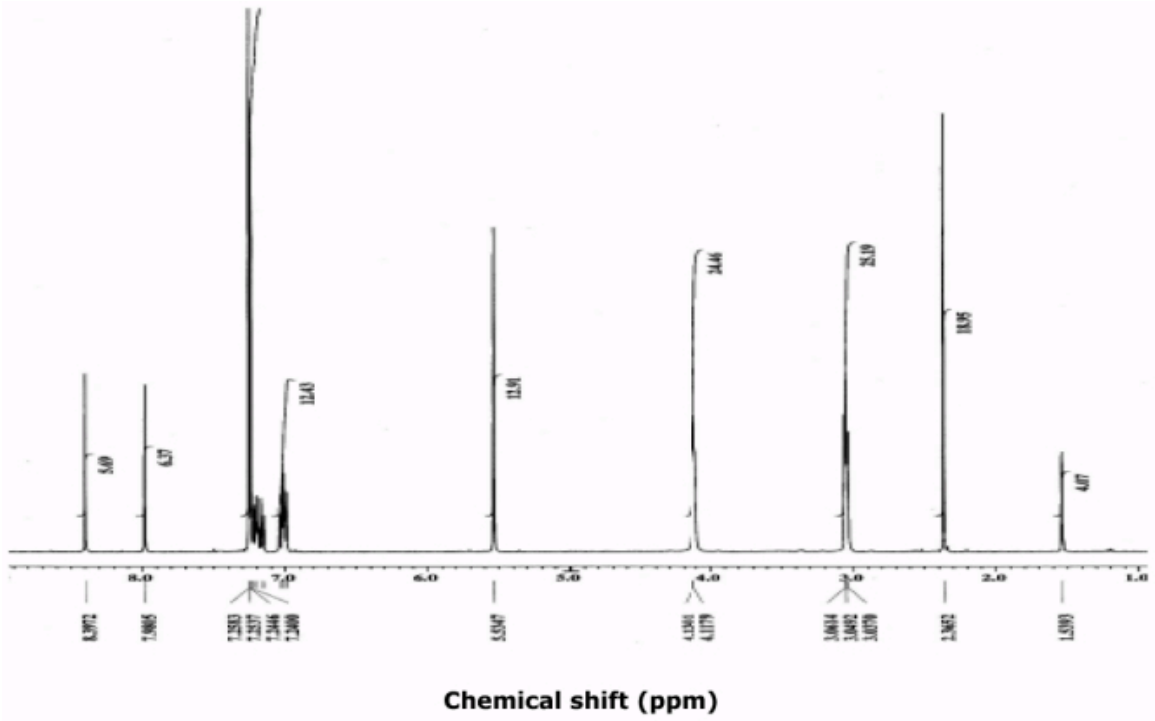
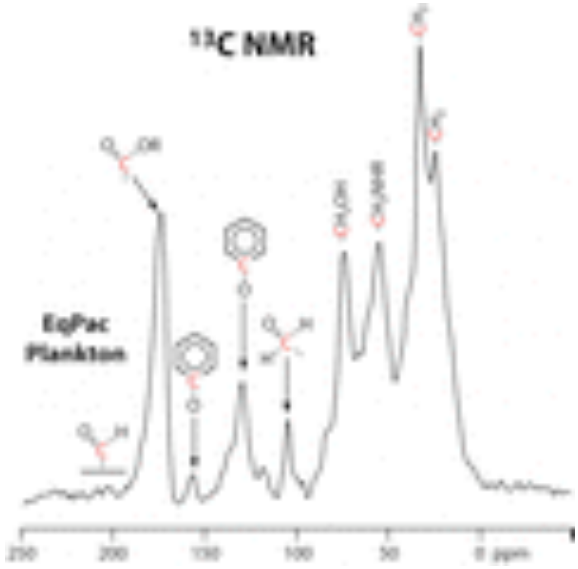


Figure 3. UV Absorption Spectrum of fluorescent nucleic acids.

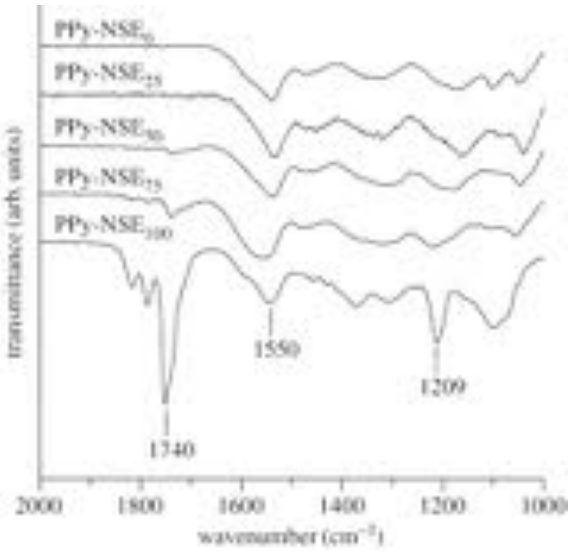
H^1 NMR for DAPI



C^{13} NMR for DAPI



IR for DAPI


PPy-NSE50
PPy-NSE25
PPy-NSE50
PPy-NSE25
PPy-NSE100
transmittance (arb. units)
1550
1209
1740
2000
1800
1600
1400
1200
1000
wavenumber (cm⁻¹)