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Reviews

Analytical Ancestry: "Firsts" in Fluorescent Labeling of Nucleosides, Nucleotides, and Nucleic Acids

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BACKGROUND: The inherent fluorescent properties of nucleosides, nucleotides, and nucleic acids are limited, and thus the need has arisen for fluorescent labeling of these molecules for a variety of analytical applications.

CONTENT: This review traces the analytical ancestry of fluorescent labeling of nucleosides, nucleotides, and nucleic acids, with an emphasis on the first to publish or patent. The scope of labeling includes (*a*) direct labeling by covalent labeling of nucleic acids with a fluorescent label or noncovalent binding or intercalation of a fluorescent dye to nucleic acids and (*b*) indirect labeling via covalent attachment of a secondary label to a nucleic acid, and then binding this to a fluorescently labeled ligand binder. An alternative indirect strategy involves binding of a nucleic acid to a nucleic acid binder molecule (e.g., antibody, antibiotic, histone, antibody, nuclease) that is labeled with a fluorophore. Fluorescent labels for nucleic acids include organic fluorescent dyes, metal chelates, carbon nanotubes, quantum dots, gold particles, and fluorescent minerals.

SUMMARY: Fluorescently labeled nucleosides, nucleotides, and nucleic acids are important types of reagents for biological assay methods and underpin current methods of chromosome analysis, gel staining, DNA sequencing and quantitative PCR. Although these methods use predominantly organic fluorophores, new types of particulate fluorophores in the form of nanoparticles, nanorods, and nanotubes may provide the basis of a new generation of fluorescent labels and nucleic acid detection methods.

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In science, being the first to invent or describe a method or a composition of matter or expound a valid theory carries significant prestige. Tangible rewards for being first may include granting of a limited-term monopoly in the form of a patent or international recognition and the award of major scientific prizes *(1)*. Determining who was first is not always straightforward, however. History is replete with corrections and conflicts on this highly charged and often commercially sensitive topic, as is evident from the controversy that surrounds such familiar concepts as calculus and items such as the slide rule, laser, and telephone *(2–5)*.

In this review, we trace the origins of fluorescent labeling of nucleic acids, tracking the evolution of ideas and emergence of techniques and examining the associated intellectual property via issued patents. Our focus is on who was first to describe or discover a particular type of compound or technique or property of matter. This information is of particular significance for inquiries into the validity of patents through anticipation and obviousness analysis *(6)*. As others have noted about earliest dates, however, they "have a way of becoming unfixed as the history of the subject is further studied" and "there is no way of knowing what future students will unearth" *(7)*. The scope of the article is limited to labels that produce fluorescence upon irradiation with excitation energy of the appropriate wavelength. We do not consider phosphorescent labels, labels that can be converted to a fluorophore (e.g., fluorescein diacetate nanocrystal labels) *(8)*, or labels that act on other substances to produce fluorescent products (e.g., alkaline phosphatase label and a fluorogenic substrate).

The scientific literature is now enormous. PubMed includes more than 17 million citations back to the 1950s *(9)*, the CAplus database contains more than 27 million patent and journal articles *(10)*, and more than 7 million US patents have been issued *(11)*. We have searched extensively in this massive collection of abstracts, papers, reviews, and books; however, an ever-present danger is that we have overlooked an obscure publication or a public disclosure at a scientific meeting captured in an abstract book that did not make its way into a library or into a public database. Likewise,

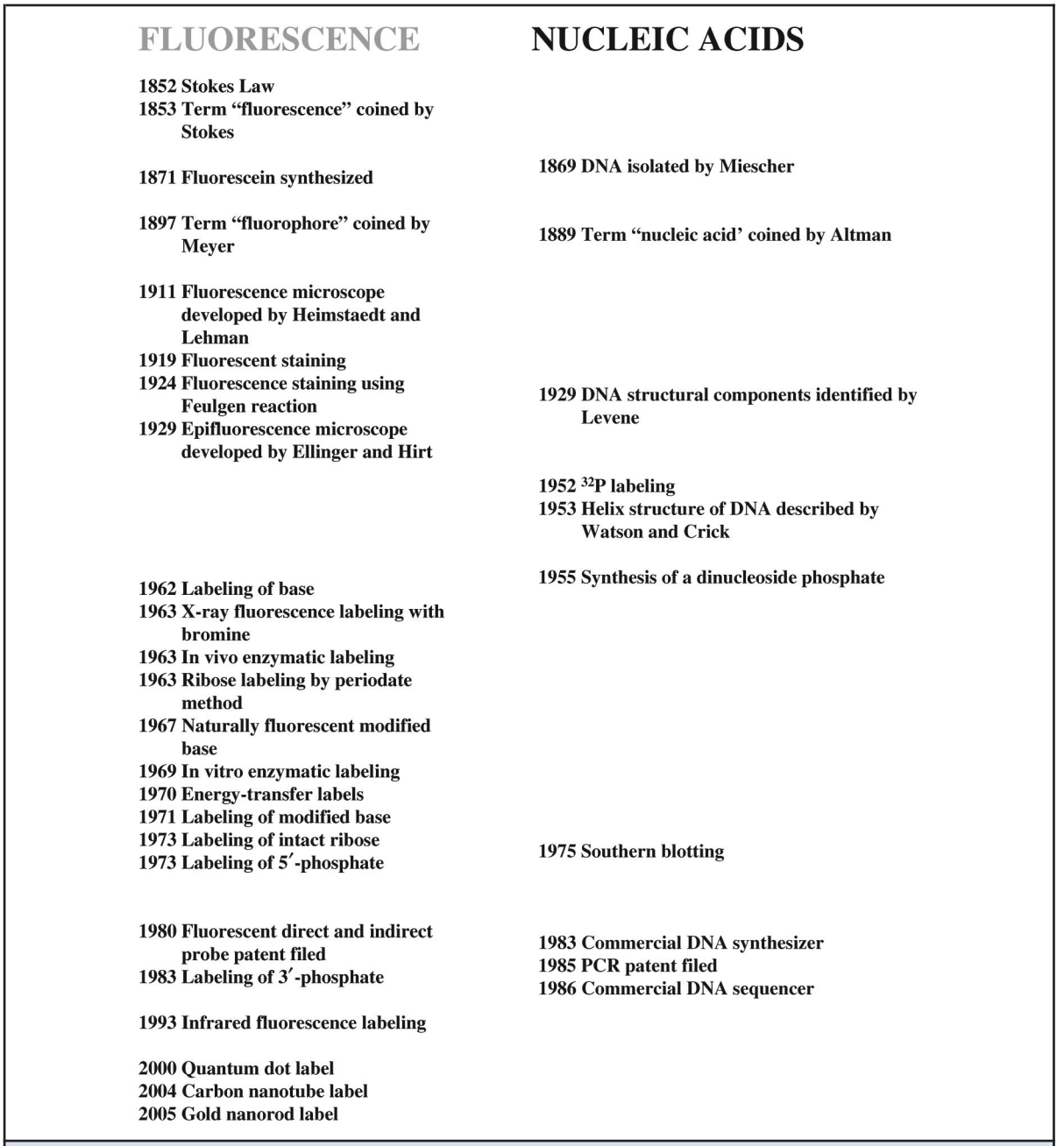

Fig. 1. Landmarks in fluorescence and nucleic acid chemistry.
P, patent priority.

we have inevitable linguistic biases, and publications in some languages may have escaped our scrutiny.

FLUORESCENCE IN NUCLEIC ACID ANALYSIS

Fluorescence was observed in antiquity, but the science of fluorescence dates back to work by Sir George Stokes (Stokes Law of Fluorescence: the wavelength of fluorescence emission is greater than that of the exciting radiation) who coined the term "fluorescence" in 1852 (Fig. 1) *(12)*. The term "fluorophore" to describe a chemical group associated with fluorescence was coined by Richard Meyer in 1897 *(13)*. Other important landmarks were the synthesis of the fluorescent dye, fluorescein, by Adolph von Baeyer in 1871 *(14)*

and the development of the fluorescence microscope in 1911 by Heimstadt and Lehman *(15)* and the epifluorescence microscope in 1929 by Ellinger and Hirt *(16)*. The range of fluorescence emission spans the electromagnetic spectrum from the x-ray (<10 nm) through the visible (380–750 nm) to the infrared (IR)[4] (630 nm to 3000 μm) regions of the spectrum. Nucleic acids have been labeled with fluorophores with emissions in the x-ray *(17)*, visible *(18, 19)*, and IR *(20, 21)* regions of the spectrum.

As the discovery of fluorescence precedes that of nucleic acids, the starting point of our inquiries was the history of nucleic acids. The nucleic acid DNA was first isolated in 1869 by Friedrich Miescher *(22)*. However, following its successful separation into a protein and an acid molecule, his pupil, Richard Altmann, named it "nucleic acid" in 1889 *(23)*. The structural components (the 4 bases, the sugar, and the phosphate chain) were identified in 1929 by Phoebus Levene, and he showed that the components of DNA were linked in the order phosphate-sugar-base *(24)*. He called each of these units a nucleotide and proposed that the DNA molecule consisted of a string of nucleotides linked together via the phosphate "backbone" of the molecule. Subsequently, in 1953, Watson and Crick solved the 3-dimensional structure of the DNA molecule and showed it to be a double helix *(25)*.

The study of the fluorescence of nucleic acids and the development of fluorescently labeled nucleic acids is set against a historical background of fluorescent methods for bioanalysis *(26)*. Fluorescence was already established by the late 1940s for both in vitro *(27)* and in vivo applications *(28)* and had been in use in other areas of analysis since at least 1922, when Hadding used x-ray fluorescence to analyze minerals *(29)*. By the 1930s, patents had been granted covering fluorescence-detecting apparatus useful in the diagnosis of disease *(30)*, and during the 1960s and 1970s, numerous patents were issued on the use of fluorescence for analysis of cells *(31, 32)* and virus particles *(33)*. Filing of patents for fluorescent nucleic acid probes began in the early 1980s and included directly labeled probes *(34–37)* and indirectly labeled probes *(34, 35, 38, 39)*. Filings for the application of fluorescent labels in sequencing also began to appear in the 1980s for both direct *(40, 41)* and indirect *(42, 43)* labeling.

Study of the fluorescence of bases, nucleosides, nucleotides, and their polymers has a long history dating back to the early part of the 20th century *(44–46)*. However, the fluorescence of nucleic acids is weak and has not proved particularly useful analytically, except in the case of nucleic acids containing certain modified bases that are naturally fluorescent. The first to be described was a base designated as "Y" [wybutosine (yW)] *(47)* in L-phenylalanyl-tRNA^{Phe}, and this has been chemically modified to another fluorescent form of the base by treatment with ammonium carbonate *(48)*. Subsequently, other fluorescent modified bases (e.g., pseudo uridine, 4-thiouridine, dihydrouridine, N4-acetylcytidine, 7-methylguanine, 7-methylinosine) were discovered *(49–53)*.

In view of the limited fluorescent properties of nucleic acids, the application of fluorescence in nucleic acids analysis has followed several pathways (Fig. 2). Direct labeling can be achieved by covalent labeling of nucleic acids with a fluorescent label or noncovalent binding (staining or intercalation) of a fluorescent dye to nucleic acids (Fig. 2A, B). Indirect labeling can be achieved by first covalently attaching a secondary label to a nucleic acid and then binding this to a fluorescently labeled ligand binder (Fig. 2C). Alternatively, a nucleic acid can be bound to nucleic acid binder molecule (e.g., antibody, antibiotic, histone, antibody, nuclease) that is labeled with a fluorophore (Fig. 2D).

MOTIVATION FOR FLUORESCENCE LABELING

By and large, the motivation for combining fluorescence and nucleic acids has been to provide a nonisotopic label (tag or marker or reporter group) that has a detectable signal to study nucleic acid sequence, structure, structural dynamics, protein and ligand interactions, or hybridization with other nucleic acids (probing) *(34)* (fluorescent labeling of the broad class of receptors, specifically nucleic acids, has been described *(54)*). The impetus for synthesizing fluorescent nucleoside or nucleotide analogs has been for photoaffinity labeling, preparing coenzyme analogs, improving detectability in chromatographic analysis, and rendering DNA fragments detectable in polyacrylamide gel electrophoresis as part of dideoxy DNA sequencing protocols, or in quantitative PCR reactions.

It should be appreciated that the genesis of some fluorescently labeled nucleic acids, nucleotides, or nucleosides has not always been motivated by a specific desire to develop fluorescently labeled materials. For example, nucleic acids fluorescently labeled at the C-8 of guanine bases have been isolated or synthesized in studies of the mechanism of the interaction of DNA and carcinogens such as 2-acetylaminofluorene (AAF) *(55)*.

[4] Nonstandard abbreviations: IR, infrared; yW, wybutosine; AAF, 2-acetylaminofluorene; NIR, near infrared; CNT, carbon nanotube; tRNA, transfer RNA; sRNA, soluble RNA; 5BrU, 5-bromouracil; TdT, terminal deoxynucleotidyl transferase; TMR, tetramethylrhodamine; YOYO, oxazole yellow homodimer; TO, thiazole orange; TOTO, thiazole orange homodimer; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; TMV, tobacco mosaic virus.

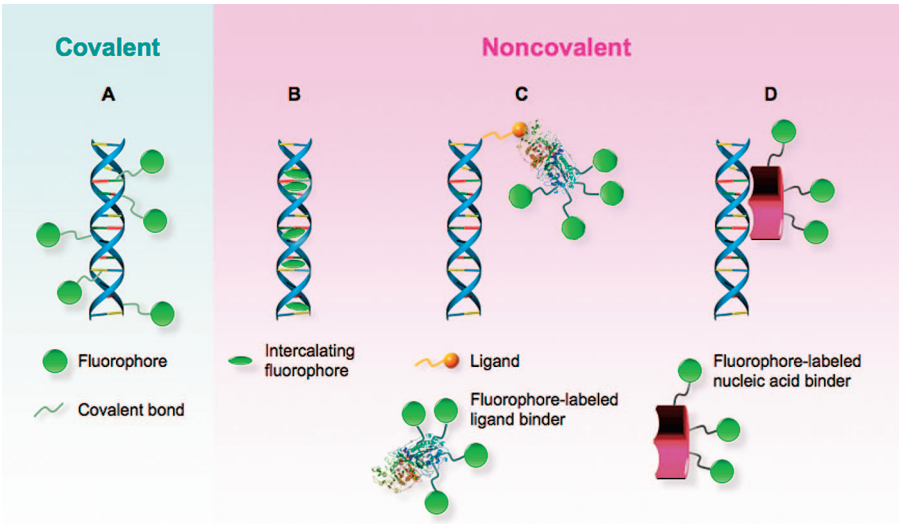

Fig. 2. Covalent (A) and noncovalent (B–D) fluorescent labeling strategies.

(A), Direct covalent labeling. (B), Staining or intercalation. (C), Indirect labeling via binding of a fluorescently labeled ligand binder to a ligand covalently attached to a nucleic acid. (D), Indirect labeling via binding of a fluorescently labeled nucleic acid binder directly to a nucleic acid.

SCOPE AND SELECTION OF FLUORESCENT LABELS

The vast majority of fluorescent nucleic acid labeling studies have used organic fluorescent dye molecules (e.g., fluorescein, rhodamine); however, fluorescent metal chelates that have a long-lived time-resolvable signals (e.g., europium chelates) and various organic (e.g., carbon nanotubes) and inorganic (e.g., quantum dots, gold particles, fluorescent minerals) particles have also been used. In most cases, the fluorescent labels used for nucleic acids had been previously used as labels in immunoassays.

Considerations in the choice of the fluorophore include factors such as fluorescence quantum yield, Stokes shift, fluorescence emission spectrum (including time-resolvability) and ability to use several fluorophore labels simultaneously, susceptibility to photobleaching, and reduction of background interference, as in the case of time-resolved fluorescent labels based on lanthanide chelates *(37, 56)* and cyanine *(57)* and metal *(20, 21)* chelate-based IR labels.

At a pragmatic level, the choice of the fluorophore has been guided by its availability and ease of attachment, and in this regard various activated fluorescein molecules have enjoyed considerable popularity (e.g., fluorescein isothiocyanate). The signal from a fluorescent label is determined in part by the fluorescence quantum yield of the fluorophore, and, the near unity quantum yield of fluorescein underlies the popularity of this label. The fluorescent signal emitted from a fluorophore depends directly on the intensity of the excitation light. As the excitation light intensity increases, however, there is a tendency for organic molecules to decompose, and this leads to photobleaching and loss of fluorescent signal. Inorganic fluorophore labels such as quantum dots are not prone to photobleaching, and this has spurred their application in nucleic analysis. The Stokes shift is another important characteristic of a fluorophore label. A large Stokes shift is advantageous because it minimizes interference by the excitation light in the measurement of the fluorescence emission. Stokes shifts of up to 200 nm are possible with seminaphthofluorone type dyes (Stokes shift for fluorescein is approximately 20 nm) *(58)*. An emission wavelength in the near infrared (NIR) can be advantageous for a fluorophore label, as this minimizes interfering fluorescence from biological samples, reduces scattering, increases tissue penetration for the signal, and allows the use of low-cost laser diode excitation sources *(37, 56)*. Also, the overall structure of a fluorophore label can influence the ability of a labeled nucleic acid to serve as a substrate for an enzyme. This has been a critical factor in the development of labeled nucleotides for sequencing using a polymerase, and the linker that attaches a fluorophore label to a nucleotide has a pronounced effect on the effectiveness of a DNA polymerase to incorporate a fluorescent nucleotide *(59)*.

An interesting trend has been the impact of micro- and nanotechnology on direct and indirect fluorescent labeling of nucleic acids in the form of micro- or nanosized organic or inorganic particles. The scope of

this strategy includes quantum dots *(60–62)*, metal nanoparticles and nanorods *(63)*, carbon nanotubes (CNTs) *(64, 65)*, dye-doped core-shell particles *(66)*, dyed latex particles *(67, 68)*, and liposomes or polymer shells filled with fluorescent particles (e.g., quantum dots, dyed polymer beads, and naturally occurring minerals such as eucryptite) *(69)*.

Fluorescent metal chelates. A chelate-based strategy for fluorescent labeling presents an interesting aspect of determining who was first to describe a particular type of fluorescent labeling. A patent published in 1977 describes labeling of "target substance" with rare-earth complexes *(70)*, and another patent with a priority in 1979 describes labeling of antigens with fluorescent metal chelates *(71)*. Because a nucleic acid such as DNA is an example of a target substance and is an antigen, these generic claims could be viewed as a disclosure of fluorescent metal chelate labeling of a nucleic acid. Likewise, another patent (priority 1981) describes the fluorescent lanthanide chelate labeling of a "biologically active substance," which of course would include a nucleic acid *(72)*. Subsequently, lanthanide chelates were specifically described in a patent (priority 1981) as labels for DNA in a sandwich hybridization assay *(37)*.

Quantum dot. A quantum dot is a nanocrystal composed of periodic group II–VI (e.g., CdSe, CdS), II–V (InP, InAs), or IV–VI (e.g., PbTe, PbS) materials. It can contain as few as 10–50 atoms and have a diameter as small as 2–10 nm *(73–75)*.

Advantages of quantum dots compared with conventional organic fluorescent dyes include high quantum yield (bright signal), less susceptibility to photobleaching, and a fluorescence emission wavelength that is directly related to the diameter of the quantum dot. A quantum dot is usually coated with a shell to improve quantum efficiency and stability. The shell surface of a "core/shell" quantum dot can be functionalized by treatment with organic molecules (e.g., silanes) that provide points of attachment for nucleosides or nucleotides *(60, 61)* or DNA *(62)*. Early suggestions for the use of quantum dots are to be found in articles published in 1998 *(76, 77)*. Interestingly, quantum dots had been attached to nucleic acids such as transfer RNA (tRNA) as part of a quantum dot synthesis scheme in which the tRNA, dispersed in a gel matrix, acted as an ion-exchange/nucleation site for formation of the quantum dots (e.g., AgO, CdS) *(78)*.

Metal nanoparticle or nanorod. A starting point for gold as a fluorescent label was the description of the fluorescence of bulk gold by Mooradian in 1969 *(79)*. Subsequently, the fluorescence of gold nanoparticle clusters and nanorods *(80, 81)* was demonstrated. In hindsight, all of the early work using colloidal gold–labeled nucleic acids *(82)* could be considered fluorescent labeling, as could the DNA self-assembly of gold nanoparticle studies *(83)*, albeit in neither case was the gold detected by fluorescence. Recently, however, fluorescent gold nanorod labels (17 nm diameter, 230 nm long; emissions at 743 and 793 nm) have been used to label DNA. The nanorod label was attached to thiolated DNA via Au-SH binding *(80)*.

Carbon nanotube. A CNT is a nanometer-diameter cylindrical carbon molecule, and as discovered in 2002, it fluoresces in the near-infrared part of the spectrum *(84)*. A CNT can be derivatized to contain carboxyl groups, and these serve as points of attachment for fluorescent labeling of nucleic acids *(64, 65)*.

DIRECT COVALENT LABELING OF NUCLEIC ACIDS WITH A FLUORESCENT LABEL

There are several sites on a nucleic acid molecule at which covalent attachment is possible, including attachment sites on the sugar, the phosphate, and the different purine and pyrimidine bases (Fig. 3) *(85)*. Most labeling reactions are designed to target a specific location on a nucleic acid, but indiscriminate covalent labeling is possible using nitrenes that react at random with other molecular species by interatomic radical insertion reactions involving, for example, carbon–hydrogen, oxygen–hydrogen, and nitrogen–hydrogen bonds. This highly reactive functional group has been used to attach an ethidium fluorophore to DNA by reacting the DNA with ethidium azide *(86)*. This strategy is analogous to other photochemically generated free radical–based labeling reactions developed in the 1960s for proteins *(87)*.

Labeling the sugar. It appears that the first example of covalent labeling of a nucleic acid at any position involved the ribose sugar and can be attributed to Feulgen in 1924 *(18, 88)*. Acid hydrolysis of DNA causes depurination, and the liberated aldehyde group of the ribose sugar is then available to react with the amine group of a pararosaniline (fuchsin) Schiff reagent. A specifically fluorescent Feulgen method was published by Ornstein et al. in 1957 *(89)* and was based on acriflavine as the Schiff-type aldehyde reagent; this produced a green fluorescent staining of nuclei in tissue sections. The scope of this reaction was subsequently expanded to other fluorogenic Schiff reagents by Kasten et al. *(90)*. The first example of covalent labeling of a nucleic acid in solution, as opposed to a tissue section, was described in 1958 by Kissane and Robins *(19)*. They were interested in developing a fluorometric assay for DNA in brain tissue. Their method entailed depurination of the DNA followed by

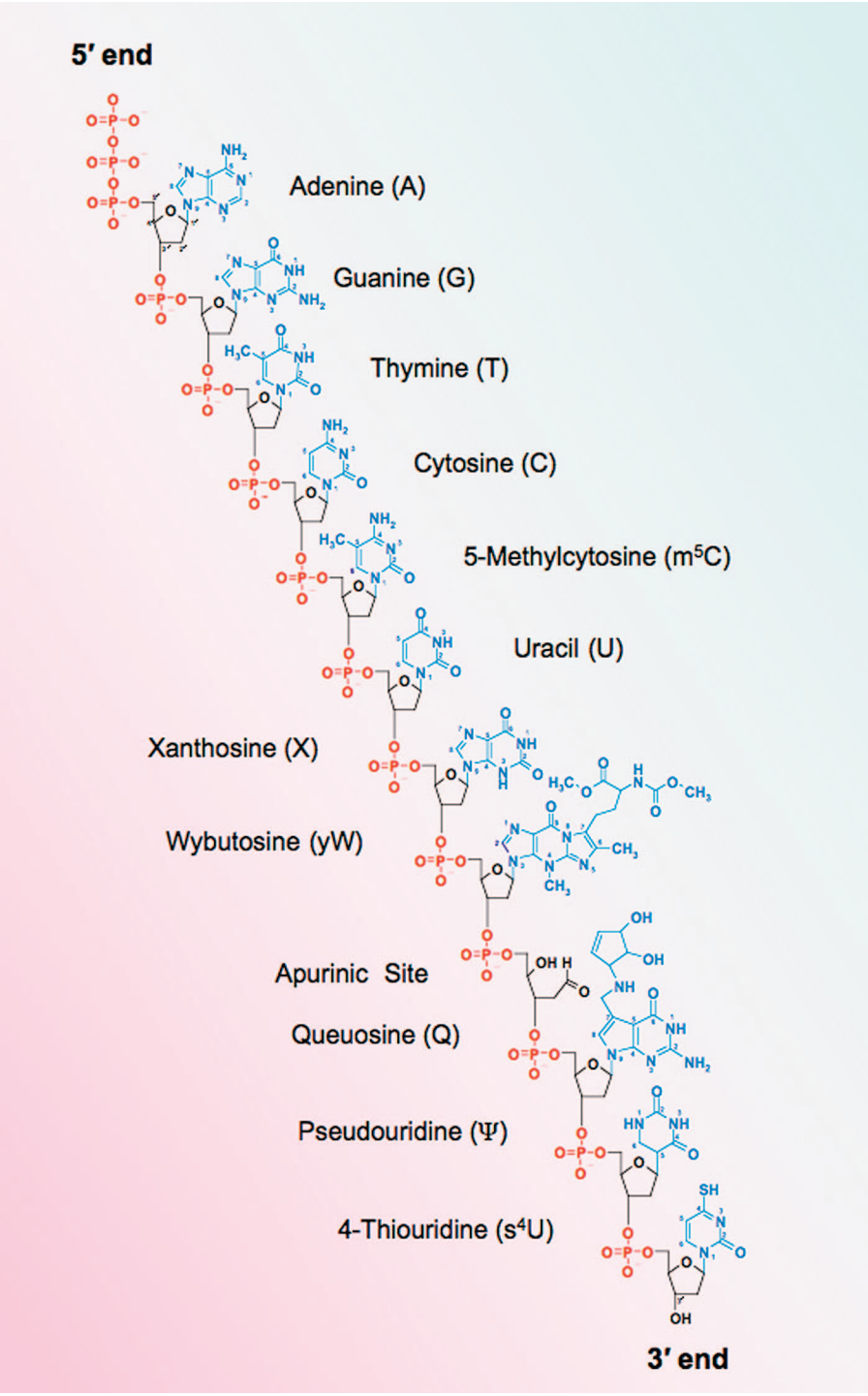

Fig. 3. Sites of attachment for fluorescent labels on a nucleic acid.

reaction of the aldehyde group in the deoxyribose with 3,5-diaminobenzoic acid to produce a fluorescent Schiff base product.

An alternative nondepurinating labeling method was described by Churchich in 1963 *(91)*. Periodate-oxidative ring opening of the ribose sugar ring of solu-

ble RNA (sRNA) and subsequent reaction of the aldehydes produced with the reactive amino groups of acriflavine (3,6-diamino-10-methylacridine) gave fluorescently labeled sRNA. This was used to determine the relaxation time of the sRNA by a fluorescence polarization method.

Fluorescent labeling of an intact sugar at the 3′ or 5′ position dates back to 1973 *(92)*. Thymidine, blocked at the 3′ or 5′ position, was reacted with α-naphthyl isocyanate to produce thymidine 3′- or 5′-naphthylcarbamate. Subsequently, the scope of the fluorophores attached to these sugar ring positions was expanded to include other well-known fluorophores such as anthracene *(93)* and dansyl *(94)*. Alternative strategies use 5′-*N*-protected 5′-amino phosphoramidites, and after deprotection, the amino group is reacted with an activated fluorescent dye (e.g., fluorescein isothiocyanate) *(95)*.

Labeling via a reaction that bridges the 3′ and 5′ position was also developed in 1973 *(96)*. ATP was trinitrophenylated by simultaneous reaction at the 2′- and 3′-hydroxyl groups of the ribose sugar to give an ATP derivative that fluoresced in ethanol–water solutions.

Labeling the phosphate. The first fluorescent labeling of the phosphate group at the 5′ position dates back to 1973 *(97)*. This was achieved by first synthesizing dansyl or anthraniloyl phosphoromorpholidate derivatives and then reacting these with the 5′-phosphate of tRNA. Labeling of the 3′-phosphate was reported by Gohlke et al. *(98)* as part of studies to make fluorogenic substrates for a ribonuclease assay (e.g., 2′,5′-bis-tert-butyldimethylsilyl 3′-uridine-4-methylumbelliferone-7-yl)phosphate).

In 1989, the scope of phosphate labeling was expanded by labeling an internucleotide phosphate *(99)*. Oligonucleotides synthesized to contain reactive phosphorthioate diesters at specific locations were dansylated to form fluorescent phosphorthioate triesters.

Labeling the base. Direct fluorescent labeling of a base can be traced back to work on the photoreaction between skin-photosensitizing furocoumarins and flavin mononucleotide *(100)* and the expansion of the reaction to pyrimidine bases of nucleic acids, such as thymine *(101–106)*. Usually, labeling of thymine is problematic because the 5-methyl substituent blocks the reactive 5-position. However, fluorescent labeling that involves reaction at the 5- and 6-positions of a thymine base or other pyrimidine bases is possible via a photochemically induced cyclo-addition reaction with various furocoumarins (e.g., 5-methoxypsoralen) *(103)*. Thymine has also been rendered fluorescent by an alkylation reaction to produce a 1-(2,3-dioxobutyl) thymine derivative *(107)*.

Subsequently, guanosine was labeled at the 8-position with *N*-acetoxyl-*N*-2-fluorenyl acetamide as part of studies on the reactions of the carcinogen *N*-acetoxy-*N*-2-fluorenylacetamide with guanosine *(55)*. Ring amine groups of guanine can also be labeled via reaction with a diazotized fluorescein derivative *(108)*.

Labeling of AMP or dAMP, or labeling of A in poly(A) [single stranded or complexed with poly(U)], was achieved in 1974 by reaction with 9-bromomethylanthracene *(109)*. Reaction occurred at the amino group at the 6-position and also at the 1-position in the case of the mononucleotide. Fluorescent labeling of adenosine and cytidine by a reaction that bridges the 1- and 6-position of adenine and the 3- and 4-position of cytidine to produce etheno compounds can be achieved via a cyclization reaction with chloroacetaldehyde. This was first described by Kochetov et al. *(85)* and developed for fluorescent labeling purposes by others *(110, 111)*.

The synthesis of a fluorescent 1-(2,3-dioxobutyl) uracil derivative was reported in 1978 *(107)*. Later, Saito et al. *(112)* adapted a photochemical alkylation reaction to make strongly fluorescent 5-pyrenyl uridine.

Labeling of aminoacylated nucleic acids. Some nucleic acids, such as tRNA, are modified by addition of an amino acid, and reactive chemical groups on the amino acid provide convenient sites for covalent attachment of a label. This is exemplified by the naphthoxyhpacetylation of the amino group in the amino acid moiety in tRNA using a 2-naphthoxy acetyl ester of *N*-hydroxysuccinimide. This synthetic procedure was described in 1968 using a series of tRNAs (e.g., $tRNA^{Asp}$) *(113)* and applied as a fluorescent labeling strategy several years later for $tRNA^{Ile}$ *(114)*.

Labeling of modified bases and nucleic acids. Several of the modified bases that occur in the nucleic acids of some organisms have chemically reactive side chains that are suitable for attachment of fluorescent labels *(115)*. The first examples of this type of labeling were reported in 1971. The thiol substituent in 4-thiouridine and 4-thiouracil was reacted with a fluorescent coumarin derivative to give the fluorescent sulfide *(116)*. Another contemporaneous route to a fluorescent tRNA derivative involves photodimerization of 4-thiouridine with cytidine, followed by sodium borohydride reduction of the photodimer *(117)*. Table1 lists other modified bases that have been fluorescent labeled (see review by Favre and Thomas *(118)*).

Labeling via enzymatic incorporation of a fluorescent analog or fluorescent base or modified base. Among the earliest examples is to be found in the work of Zeitz and Lee (1963) *(17)*. As part of their studies on the radiosensitivity of DNA, they replaced thymine with 5-

Table 1. Fluorescence and fluorescent labeling of nucleic acids.

Discovery (first use, publication, or patent)	Reference	Discovery (first use, publication, or patent)	Reference
Natural fluorescence		5- and 6-position	Musajo et al. *(103)*, Musajo et al. *(104)*
		U	
Free bases	Heyroth and Loofbrow *(44)*	1-position	Lee et al. *(107)*
Nucleosides and nucleotides	Udenfriend and Zaltman *(26)*	3-position	Yoshida et al. *(176)*
Oligonucleotides	Eisinger et al. *(168)*, Gueron et al. *(169)*	Amino acid group (tRNA)	
Polynucleotides	Konev *(45)*	Asp	Gillam et al. *(113)*
"Modified" or "odd" base		"Modified" or "odd" base	
Y	RajBhandary et al. *(47)*	4-thiouridine	Favre et al. *(51)*
4-Thio U	Lipsett *(170)*	Modified Y (yW)	Yoshikami and Keller *(48)*
N4-acetylcytidine	Pochon et al. *(53)*	9-methyladenine	Kochetov et al. *(85)*
Covalent fluorescent labeling		1-methylcytidine	Kochetov et al. *(85)*
Sugar		Pseudouridine (Ψ)	Yang and Söll *(50)*
Depurinated sugar	Feulgen and Rossenbeck *(18)*	2-thio-5-(*N*-methylaminomethyl)uridine	Yang and Söll *(50)*
Ring-opened sugar	Churchich *(91)*	Q (queuosine)	Yang and Söll *(49)*, Pingoud et al. *(177)*
Intact sugar		X [(3-(3-amino-3-carboxypropyl)U]	Schiller and Schechter *(178)*
2′ position	Hiratsuka and Uchida *(96)*	X-47	Faulhammer et al. *(179)*
3′ position	Bienvenüe and Tournon *(92)*	pre-Q_1	Kasai et al. *(180)*
5′ position	Bienvenüe and Tournon *(92)*	Under modified Y	Kuchino et al. *(181)*
Bridging 2′ and 3′ positions	Hiratsuka and Uchida *(96)*	Xanthosine	Macklin et al. *(182)*
Phosphate		Enzymatic incorporation of fluorescent nucleotides or analogs	
5′	Yang and Söll *(97)*	TdT-catalyzed	Rozovskaia et al. *(119)*
3′	Gohlke et al. *(98)*	T_4 RNA ligase–catalyzed	Richardson and Gumport *(121)*
Base		RNA polymerase–catalyzed	Ward et al. *(183)*, Ward et al. *(184)*
A		Polynucleotidyl phosphorylase–catalyzed	Zhenodarova and Klyagina *(185)*
Position not known	Pochon et al. *(53)*	DNA polymerase–catalyzed	Ried et al. *(186)*
1-position	Pochon and Perrin *(109)*	Avian myeloblastosis virus (AMV) reverse transcriptase	Prober et al. *(187)*
6-position	Pochon and Perrin *(109)*	Noncovalent labeling	
5- and 6-position	Barrio et al. *(110)*	Staining	
G		Eosin	von Provazek *(126)*
2-position	Jeffrey et al. *(171)*, Koreeda et al. *(172)*	Nucleic acid binder	
7-position	Casperson et al. *(173)*	Antibiotic	Crissman et al. *(153)*
8-position	Kriek et al. *(55)*	Histone	Lewis *(154)*
C		Antibody	Beiser et al. *(155)*
4-position	Draper and Gold *(174)*	Nuclease	Benjaminson et al. *(156)*
6-position	Pochon et al. *(52)*, Leng et al. *(175)*	Restriction endonuclease	Taylor et al. *(68)*
3 and 4-position	Barrio et al. *(110)*, Secrist et al. *(111)*	Poly(U)	Cheung et al. *(157)*
T			
3-position	Yoshida et al. *(176)*		

bromouracil (5BrU) in a DNA sample by growing *E. coli B* 15T– in a medium containing 5BrU, and then detected the bromine atom in the incorporated 5BrU by irradiating the sample with x-rays and detecting the fluorescence emission of the bromine at approximately 0.1040 nm.

Subsequently, the availability of various enzymes facilitated development of in vitro protocols for labeling nucleic acids. These were originally developed for nonfluorescent labeling and subsequently expanded to include fluorescent labeling of nucleic acids. In vitro fluorescent labeling was initially achieved with an *E. coli* RNA polymerase-catalyzed incorporation reaction using a d(A-T) template and the fluorescent analogs of ATP, formycin, 2-aminopurine, or 2,6-diaminopurine. These same analogs were also attached to the terminus of a tRNA molecule using tRNA-CCA pyrophosphorylase *(38, 39)*.

Other enzymes used for in vitro labeling include terminal deoxynucleotidyl transferase (TdT) that catalyzed the incorporation of fluorescent bases such as 3-*O*-acyl(fluorescein or rhodamine) UTP *(119)*. A variant on this procedure used TdT to incorporate 4-thiouridine at the 3′ end of DNA, and the thiol group of the incorporated 4-thiouridine was in turn labeled with fluorescein, eosin, or aminonaphthalene 1-sulfonic acid derivatives *(120)*. T_4 RNA ligase is also useful for fluorescent labeling. By using fluorescein and tetramethylrhodamine (TMR) derivatives of P^1-(6-amino-hex-1-yl)-P^2-(5′-adenosine), it was possible to introduce a fluorescent fluorescein or TMR label onto the 3′-hydroxyl group of RNA in good yield *(121)*.

NONCOVALENT LABELING OF NUCLEIC ACIDS WITH A FLUORESCENT LABEL

The 2 principal methods of noncovalent labeling are direct methods in which a fluorescent dye or particle binds to a single- or double-stranded nucleic acid (staining) (Fig. 2B) and indirect methods in which a fluorescently labeled nucleic acid binding agent (e.g., avidin or an antibody) binds to a secondary label (e.g., biotin, iminobiotin) covalently attached to the nucleic acid (Fig. 2C) or to a specific structure, e.g., an RNA:DNA hybrid (Fig. 2D).

Direct noncovalent binding of fluorescent dyes to nucleic acids (staining). The scope of dye-binding detection methods encompasses dyes that bind to nucleotides *(122)*, double- and single-stranded nucleic acid; dyes that have selectivity for double- vs single-stranded nucleic acid, DNA vs RNA *(123)*; and dyes that bind to the minor groove, e.g., Hoechst 33258 *(124)*, and the major groove, e.g., methyl green *(125)*, of DNA.

Study of the interactions between fluorescent dyes and nucleic acids traces back to the turn of the 20th century and studies on vital fluorochroming using eosin and erythrosin *(126)*, and this in turn has its origins in the colorimetric histochemical staining reactions pioneered by Raspail in the early 1800s *(127)*. Binding of the 10-methyl homolog of ethidium bromide to DNA was suggested in 1953 *(128)*, and the intercalative binding of acridine, proflavine, acridine orange, and ethidium bromide to nucleic acids was demonstrated over the next decade *(129–132)*. Ensuing years saw the introduction of superior variants of ethidium bromide, e.g., ethidium homodimer *(133)*; intercalating dyes based on oxazoles [e.g., oxazole yellow homodimer (YOYO)] and thiazoles [e.g., thiazole orange (TO) and thiazole orange homodimer (TOTO)] that showed greater fluorescent enhancement when bound to double-stranded DNA (dsDNA) *(134, 135)*; and dyes such as PicoGreen that show greater selectivity for dsDNA vs RNA or single-stranded DNA (ssDNA) *(136)*.

Sensitive quantitative fluorescent DNA detection in solution using ethidium bromide was described in 1964 *(131, 137, 138)*. However, the application of intercalating dyes to solid phase DNA detection, e.g., in agarose gels, is controversial *(139)*. Aaij and Borst described this method in 1972 *(140)*, inspired by the bright orange bands observed when DNA was separated in preparative CsCl-ethidium gradients, but an article in the following year has been more commonly cited *(141)*.

Single-stranded nucleic acids can also be stained. For example, acridine orange staining of single-stranded virus RNA [tobacco mosaic virus (TMV)] was described in 1961 *(142)*. Subsequently, other dyes that stain ssRNA, e.g., Cuprolinic blue–magnesium chloride *(143)*, dyes for ssDNA including TOTO and YOYO *(144)*, and also dyes such as Hoechst 33258 that are selective for dsDNA in the presence of RNA and for dsDNA in the presence of ssDNA *(145)* have been developed. Triple-stranded nucleic acids will also bind to ethidium bromide *(137, 138, 146)*.

Generally, intercalating dyes show no sequence selectivity, but some dyes, such as ethidium bromide, bind to A-T base pair–rich regions *(147)*, and TOTO binds preferentially to 5′-pyrimidine-pyrimidine-purine-purine-3′ motifs in dsDNA (5′-CTAG-3′ preferred binding site) *(148)*.

Indirect noncovalent labeling via binding of fluorescent binding agents to secondary-labeled nucleic acids. Secondary labeling of a nucleic acid with an antigen or hapten provides a general route to indirect DNA labeling through subsequent binding of the secondary label to a fluorescently labeled binding agent.

Biotin and iminobiotin are secondary labels that provide a route to indirect fluorescent labeling of nucleic acids. For example, biotin covalently linked to a nucleic acid can be bound to an antibiotin antibody that is labeled with a fluorophore *(34)*, a streptavidinylated particle such as a latex bead containing fluorophores *(149)*, or a silica particle. Alternatively, the biotin secondary label can be bound to rabbit antibiotin antibody, and this in turn immunocomplexed with a goat antirabbit IgG labeled with a fluorophore *(150)*.

Fluorescein is antigenic, and so a route to indirect fluorescent labeling a nucleic acid is to first label it with fluorescein and then react the fluorescein hapten with antifluorescein antibodies that have been labeled with a fluorophore, e.g., fluorescein. This strategy was applied to detect DNA using fluorescein labeled RNA. The further indirect labeling of the RNA using fluorescein-labeled antifluorescein antibodies produced a 5- to 10-fold amplification compared to direct detection of the fluorescein-labeled RNA probe *(151)*. A similar strategy has been developed for biotin and iminobiotin ligands that will bind to fluorophore-labeled avidin or streptavidin *(38, 39)*.

Somewhat analogous is the use of a dipalmitoylphosphatidyl secondary label that can be incorporated into the wall of a liposome that encapsulates a fluorescent dye. A dipalmitoylphosphatidyl-labeled DNA–containing liposome encapsulating sulforhodamine B exemplifies this strategy *(152)*.

Indirect noncovalent labeling via binding of fluorescent binding agents to nucleic acid hybrids. A range of macromolecules show binding affinity toward nucleic acid sequences. By labeling these macromolecules with a fluorophore, it is possible to achieve indirect labeling of a nucleic acid. The range of macromolecules includes antibiotics (e.g., olivomycins) *(153)*, histones *(154)*, antibodies *(155)*, nucleases (e.g., deoxyribonuclease) *(156)*, inactive restriction endonucleases (e.g., *EcoR*1) *(68)*, and of course, nucleic acids. For example, polyadenylated RNA can be labeled by hybridization to poly(U) (50–1000 bases) that is covalently attached to a dansylated 300-angstrom-diameter latex microsphere *(157, 158)*.

Fluorescently labeled monoclonal antibodies capable of distinguishing DNA-RNA hybrid complexes from single-stranded DNA and RNA and double-stranded DNA and RNA can be used to fluorescently label a DNA-RNA hybrid *(159, 160)*. This forms the basis of the monoclonal anti-DNA:RNA hybrid capture assay strategy *(161)*.

DEGREE OF LABELING AND LOCATION OF LABELS

Labeling methods have been developed that control the location and number of fluorescent labels attached to a nucleic acid. The ability to attach a fluorescent label at a specific location has assumed importance for probes used in energy transfer assays.

Double labeling has been achieved with 2 natural fluorescent bases (e.g., pseudoU + dihydroU) *(50)*, a natural fluorescent base plus labeled base (e.g., Y + 3′-acriflavine) *(162)*, or 2 labeled bases (e.g., 3′-acriflavine + 5′-anthrinoyl) *(50)*. More recently, double labeling of the same oligonucleotide has assumed particular importance in the context of energy transfer probes, in which the donor and acceptor labels are located sufficiently close to quench fluorescence signal generation. Such probes are now widely used in the form of TaqMan probes for quantitative PCR assays *(163)*, energy transfer primers for DNA sequencing *(164, 165)*, and molecular beacons *(166, 167)*.

Conclusions

Fluorescently labeled nucleosides, nucleotides, and nucleic acids continue to be important types of reagents for biological assay methods and underpin current methods of chromosome analysis, gel staining, DNA sequencing, and quantitative PCR. These methods use predominantly organic fluorophores, but nanotechnology is now offering new types of particulate fluorophores in the form of nanoparticles, nanorods, and nanotubes that may provide the basis of a new generation of fluorescent labels and nucleic acid detection methods.

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