DNA Technology

During the past 20 years there have been remarkable advances in the use of fluorescence to study DNA. Fluorescence methods are now used for DNA sequencing, detection of DNA hybridization, restriction enzyme fragment analysis, and fluorescence in-situ hybridization (FISH), and to detect polymerase chain reaction products. Molecular beacons can be used to detect messenger RNA within cells. DNA arrays can be used to monitor the expression of thousands of genes using a single microscope slide. Fluorescence was used to sequence the human genome that was reported in 2000.[1–2] Given these advances it is surprising to realize that DNA sequencing by fluorescence was first reported in 1986. It is not practical to describe the many specialized methods used to study DNA in molecular biology, genetics, and medical diagnostics. In this chapter we provide an overview of the dominant users of fluorescence in DNA analysis.

21.1. DNA SEQUENCING

DNA sequencing first became practical in 1977.[3–4] The original method involved chemical degradation of the DNA using conditions that were partially selective for one of the four bases. The DNA fragments were then separated by chromatography and the fragments detected using ^{32}P autoradiography. In the same year a method became available for generating fragments terminating in each of the four bases.[5] This method used termination of enzymatic DNA synthesis using dideoxynucleotides. The fragments were again detected using radioactivity. An overview of the history of DNA sequencing methods can be found in the informative text by Watson et al.[6]

The use of radioactive tracers is problematic with regards to cost, safety, and disposal. Also the use of radioactivity was not amenable to the degree of automation needed to sequence long DNA chains, chromosomes or an entire genome. DNA sequencing using fluorescence was first reported in 1986.[7–12] At present essentially all sequencing is done using fluorescence detection. It is unlikely that the human genome would have been sequenced without the use of fluorescence. DNA sequencing is now highly automated[11] and performed in numerous laboratories around the world.

21.1.1. Principle of DNA Sequencing

A number of slightly different methods are used for DNA sequencing, but all methods rely on the use of dideoxynucleotide triphosphate (ddNTP) to terminate DNA synthesis. The basic idea of sequencing using ddNTP terminators is shown in Figures 21.1 and 21.2. In DNA the nucleotides are linked in a continuous strand via the 5' and 3' hydroxyl groups of the pentose sugar. DNA is replicated by adding nucleotides to the 3' hydroxyl group. For sequencing a DNA strand with an unknown sequence is replicated using DNA polymerase. Replication is started from a primer location with a known sequence. The most commonly used primer is the M13 sequence, which is 17 nucleotides long. In the example shown in Figure 21.2 a single fluorescent primer is used to initiate the reaction. The sample contains DNA polymerase and the four deoxynucleotide triphosphates.

Within a short period of time the DNA polymerase molecules are randomly distributed along the unknown sequence. The strands being synthesized have a sequence complementary to the unknown strand. The reaction mixture is split into four parts, one part for each of the four bases. The DNA polymerase reaction is randomly terminated by adding one of the ddNTPs to each of the four parts of the reaction. The ddNTPs are added along the growing chain. The absence of a 3' hydroxyl group on the ddNTPs prevents further elongation and terminates the reaction. This results in a mixture of oligonucleotides of varying length. The different size oligomers are separated by poly-

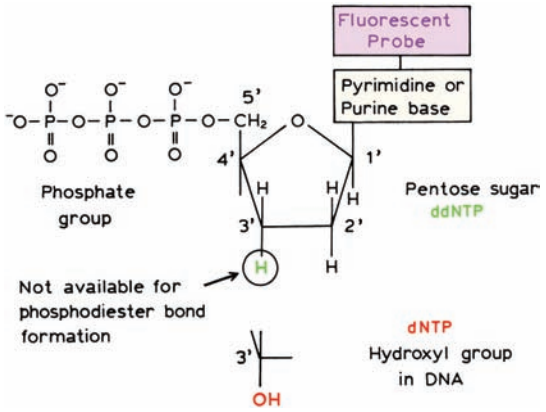

Figure 21.1. Schematic of a dideoxynucleotide triphosphate (ddNTP). Fluorescent and nonfluorescent ddNTPs are used for DNA sequencing, depending on the method. The 2' group is hydrogen in DNA, and is a hydroxyl group in RNA. In a ddNTP the 3'hydroxyl group is not present so the DNA chain cannot be continued.

acrylamide gel electrophoresis. Remarkably, numerous fragments differing by just one base pair can be resolved: up to several hundred bases. Each reaction mixture is electrophoresed in a separate lane. Each lane of the reaction mixture contains oligomers which are terminated with only one of the ddNTPs. The gels separate the DNA fragments according to size, so that the sequence can be determined from the fluorescence of the separated DNA fragments.

Because of the large number of sequences that need to be determined it is desirable to have the highest possible throughput. The throughput can be increased fourfold if the four DNA bases can be identified in a single lane of the chromatography gel. This can be accomplished using four fluorescent ddNTPs if each ddNTP contains a different fluorophore (Figure 21.3). In this case the reaction is initialized using a nonfluorescent primer. The reaction is terminated by addition to the four labeled ddNTPs. This inserts a labeled fluorophore that identifies the base at the 3' end of the terminated chains. The mixture can be analyzed in a single lane and the emission spectra used to identify the bases. Single- and four-lane sequencing represent the limiting cases, and many hybrid methods are also in use.

21.1.2. Examples of DNA Sequencing

A variety of fluorophores have been used for DNA sequencing: typically a set of four fluorophores, one for each base—A, C, G, or T. The fluorophores are typically select-

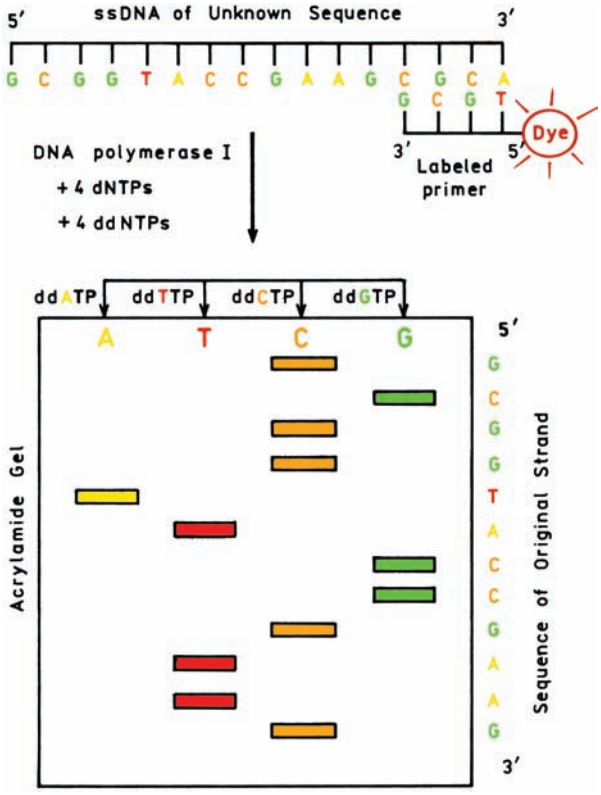

Figure 21.2. Four-lane DNA sequencing using nonfluorescent ddNTPs and a fluorescent primer for DNA synthesis.

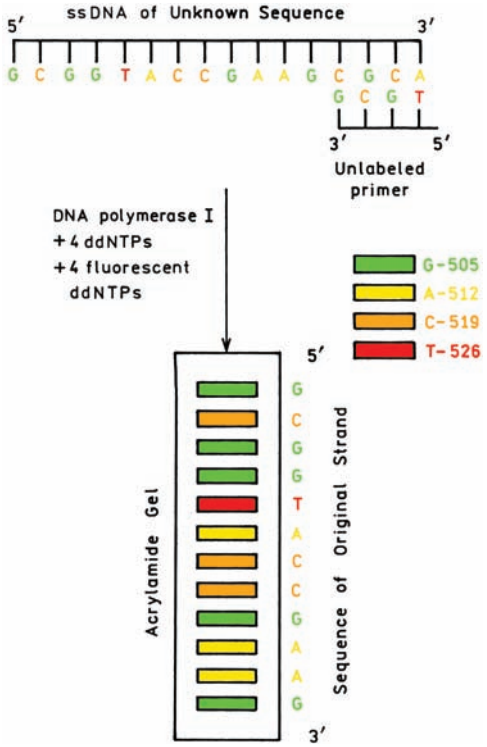

Figure 21.3. Single-lane DNA sequencing using a nonfluorescent primer and four fluorescent ddNTPs.

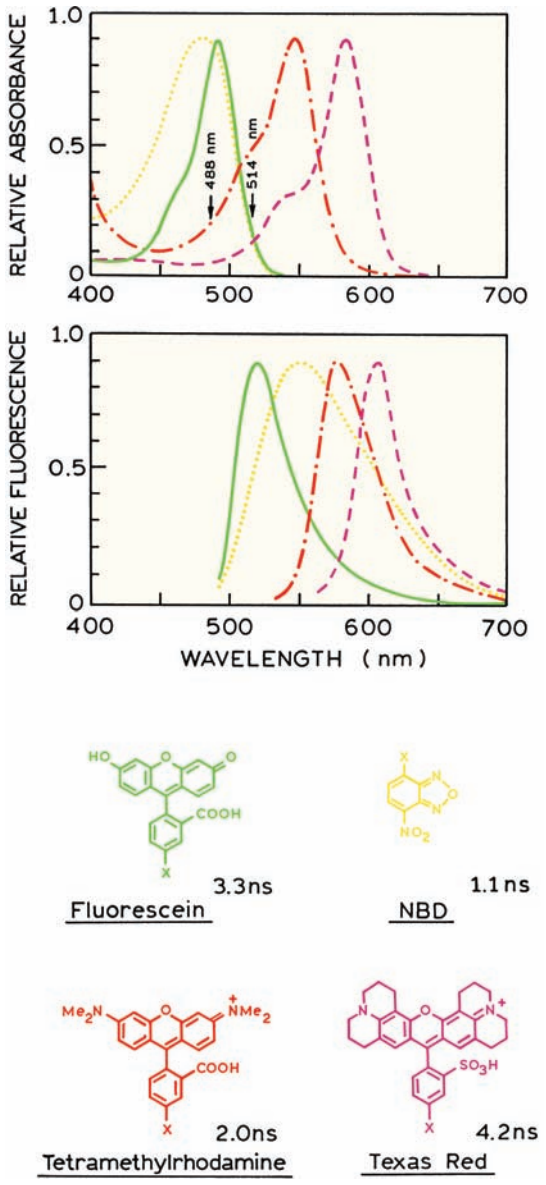

Figure 21.4. Fluorophores used for DNA sequencing with fluorescent primers. Top, absorption spectra; middle, emission spectra; bottom, probe structures. The X was linked to the 5' end of the DNA using an aliphatic amino group on the 5' terminus. Revised from [8]. Decay times are from [42] below.

ed so that all can be excited using the 488 nm line from an argon ion laser. The first set of fluorophores[8] used for DNA sequencing is shown in Figure 21.4. These fluorophores were attached to primers, which is different from the approaches shown in Figures 21.2 and 21.3. The four dyes could be excited at 488 nm, but the absorption of Texas Red and tetramethylrhodamine is obviously weak at 488 nm. For this reason it was necessary to use 514-nm excitation in order to obtain relatively equal intensities for all four probes. Another difficulty with these four dyes is the overlapping emission spectra. It was necessary to record intensities at more than one excitation and emission wavelength in order to identify the fluorophore. In spite of these difficulties the use of four fluorophores allowed use of a single gel column containing the mixture of labeled DNA fragments.

An improved series of dyes for use as fluorescent dideoxy terminators is shown in Figure 21.5. A hydroxyl group is not present on the 3' portion of the sugar, so that these nucleotide analogues are unable to elongate the DNA chain. These dyes displayed similar extinction coefficients at 488 nm, allowing the use of a single excitation wavelength (Figure 21.6). The fluorescence intensities of these fluorophores differ by less than a factor of two. The letters SF indicate succinylfluorescein, which was linked to the base. The numbers refer to the emission maximum of each fluorescein derivative. In the lower panel the letter refers to the base to which the fluorescein is attached.

The emission spectra in Figure 21.6 show that there is substantial overlap of the emission spectra at all useful wavelengths. While the overlap can be decreased using different dyes there is always some spectral overlap. These dyes were identified by measuring the emission intensities through filters (Figure 21.6, lower panel). The fluorescent intensities at each location in the gel are measured with a laser scanning instrument (Figure 21.7). The laser beam is scanned across the gel and the intensity ratios are measured using two filters and two detectors. The intensity ratios are used to identify the base.

21.1.3. Nucleotide Labeling Methods

A wide variety of chemical structures have been used to covalently label DNA.[13–14] One typical linkage was shown in Figure 21.5, which showed acetylene linkages between the fluorophores and the nucleotide bases. Other typical structures are shown in Figure 21.8. Probes can be attached to the 5' end of DNA via a sulfhydryl group linked to the terminal phosphate. Amino groups can also be placed on the terminal phosphate. The 5' phosphate can be made reactive with iodoacetamide probes by attaching a terminal —PO_3S residue. Alternatively, fluorophores have been linked to the bases themselves, typically opposite to the base recognition hydrogen bonding side of the base. This type of attachment is used to label the internal DNA bases.

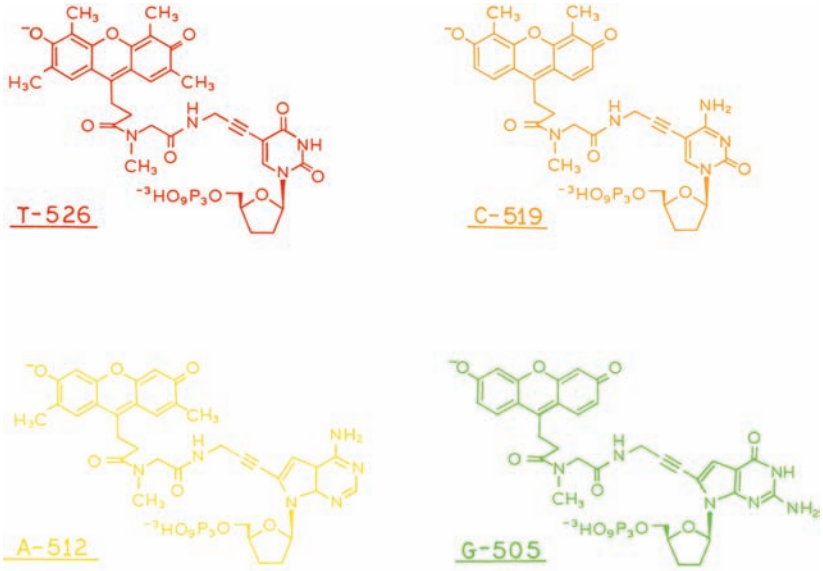

Figure 21.5. Fluorescent chain-terminating dideoxynucleotides. The letters refer to the DNA base, and the numbers refer to the emission maximum. Reprinted with permission from Prober JM, Trainor GL, Dam RJ, Hobbs FW, Robertson CW, Zagursky RJ, Cocuzza AJ, Jensen MA, Baumeister K. 1987. A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides. *Science* **238**:336–343 [9]. Copyright © 1987, American Association for the Advancement of Science.

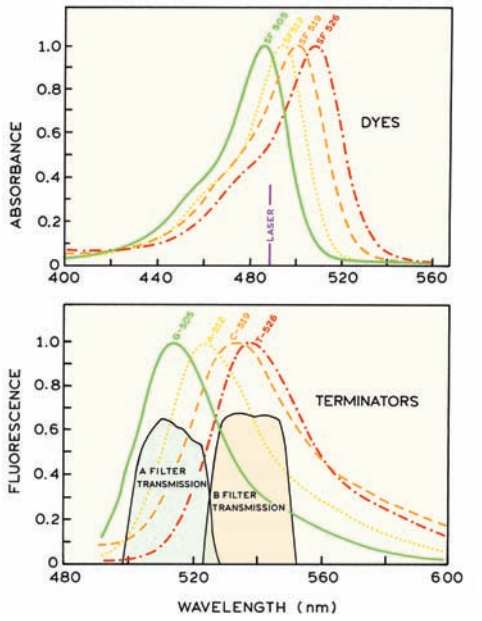

Figure 21.6. Absorption (top) and emission spectra (bottom) of the fluorescent chain terminating dideoxynucleotides in Figure 21.5. The absorption spectra are of the succinyl fluorescein (SF) dyes, prior to coupling to the amine reactive ddNTPs. Reprinted with permission from Prober JM, Trainor GL, Dam RJ, Hobbs FW, Robertson CW, Zagursky RJ, Cocuzza AJ, Jensen MA, Baumeister K. 1987. A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides. *Science* **238**:336–343 [9]. Copyright © 1987, American Association for the Advancement of Science.

21.1.4. Example of DNA Sequencing

It is informative to see some actual data from DNA sequencing,[15–16] which was accomplished using the fluorophores shown in Figure 21.9. These probes show moderately distinct emission spectra (Figure 21.10), so that single-lane sequencing should be possible. However, it is difficult to excite these four fluorophores using a single excitation wavelength.[17] Two laser sources were used at 488 and

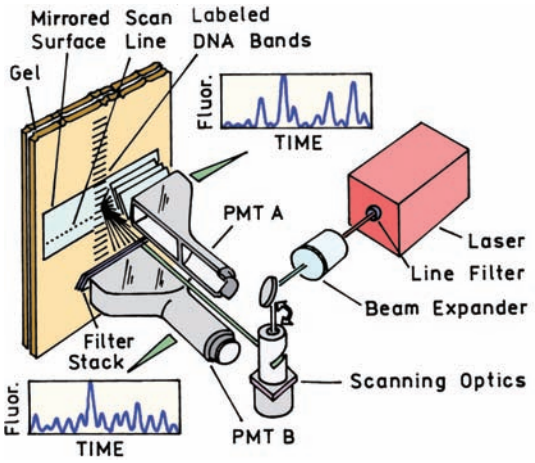

Figure 21.7. Apparatus for wavelength ratio intensity measurements from DNA gels. From [9].

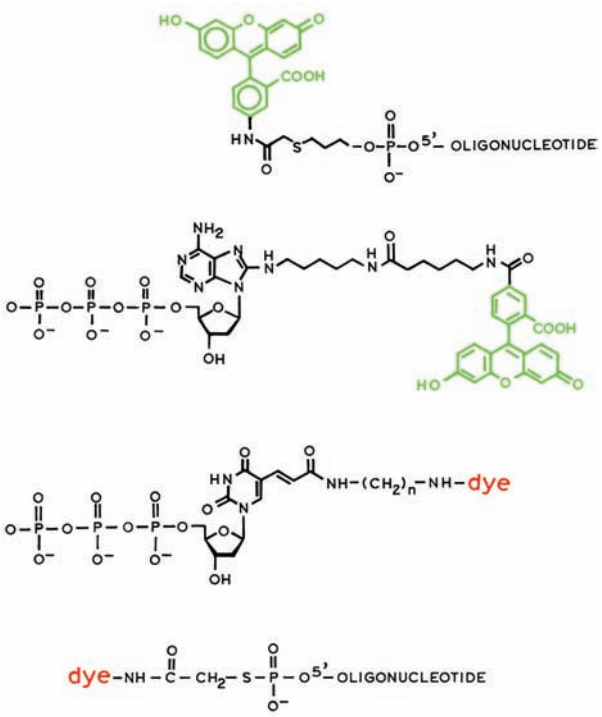

Figure 21.8. Methods to label DNA. In the top structure DNA is labeled with a fluorescent primer. The two structures in the middle show labeling of DNA using labeled nucleotide triphosphates. In the bottom structure DNA can also be labeled on the 5' end via a thiophosphate linkage (bottom). Revised from [13] and [14].

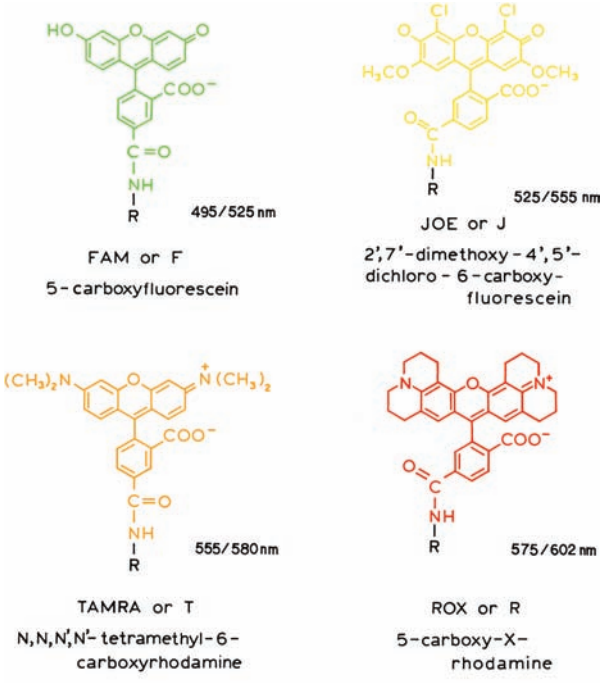

Figure 21.9. Fluorophores used as energy-transfer DNA sequencing probes. The two wavelengths are the excitation and emission maxima. Revised from [15].

543.5 nm (Figure 21.11), and the emission was observed at four wavelengths. The excitation wavelength was selected using a spatial filter or sector wheel. The emission was observed using four emission filters. The primers were labeled with the fluorophores and sequencing was accomplished using a single capillary tube. The lower panel shows the intensity tracers for each combination of excitation and emission wavelength that uniquely identifies each fluorophore and base.

21.1.5. Energy-Transfer Dyes for DNA Sequencing

In the previous example it was necessary to use two laser wavelengths to obtain comparable intensities from the four dyes. For sequencing it is desirable to have dyes that display distinct emission spectra and similar intensities with a single excitation wavelength. This is difficult to accomplish using a single fluorophore. Donor–acceptor pairs have been designed to fulfill these requirements.[18–25] One set of energy-transfer primers for sequencing was constructed using the fluorophores shown in Figure 21.9. The emission spectra of these four probes are moderately distinct (Figure 21.10), suggesting allowance of sequencing in a single lane. However, the intensities are unequal when excited at a single wavelength of 488 nm, which is why two excitation wavelengths are used in Figure 21.11. The donors and acceptors were covalently linked within the Förster distance

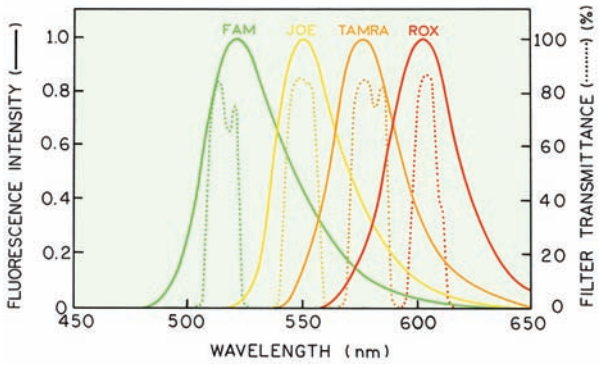

Figure 21.10. Emission spectra of the four probes used for construction of the energy-transfer primers. Reprinted with permission from [16]. Copyright © 1994, American Chemical Society.

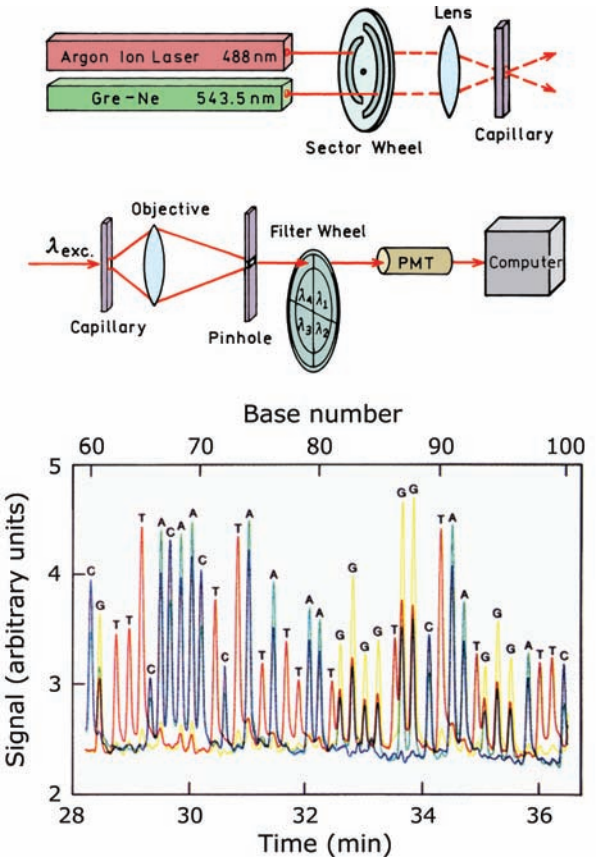

Figure 21.11. DNA sequencing using two excitation wavelengths and the fluorophores shown in Figure 21.10. Revised and reprinted with permission from [17]. Copyright © 1991, American Chemical Society.

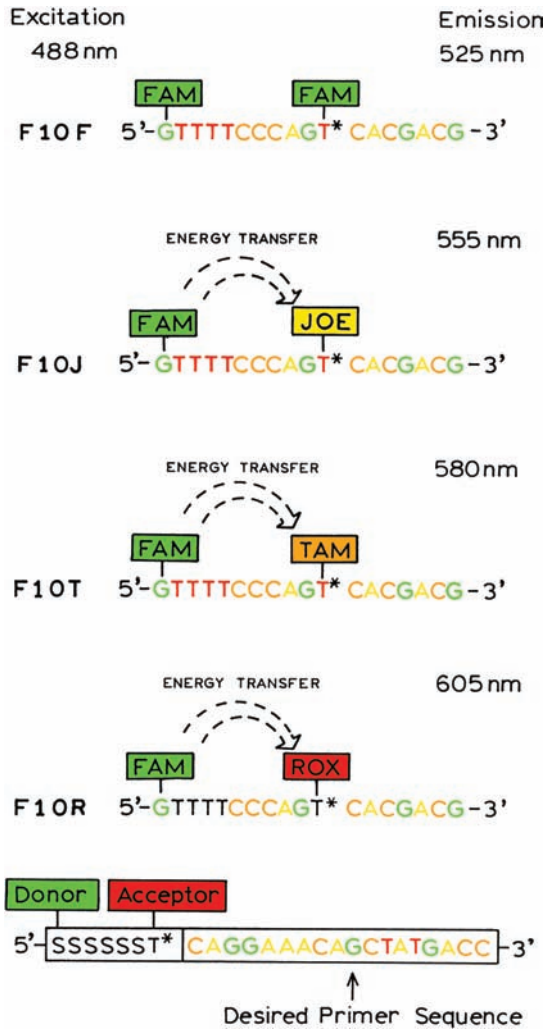

Figure 21.12. Energy-transfer primers used for DNA sequencing. F10F, F10J, F10T, and F10R are energy-transfer primers in which the donor and acceptor (see Figure 21.9 for the structures of F, J, T, and R) have been covalently linked using reactive oligonucleotides. Excitation is at 488 nm, and the emission wavelengths are indicated on the right-hand side of the figure. As shown at the bottom of the figure, donor–acceptor pairs can also be placed on sugar polymers (—SSS—) prior to the primer sequence. Revised from [15] and [16].

(R_0) using reactive oligonucleotides (Figure 21.12 top) or DNA-like sugar polymers without the nucleotide bases (bottom). The lengths of the oligonucleotide linkers were adjusted to obtain the amount of energy transfer so that the acceptor intensities are comparable to excitation at 488 nm.

The energy-transfer cassettes displayed nearly equal intensities when excited at 488 nm (Figure 21.13). The emission spectra are moderately well separated, which is easier to see in the normalized emission spectra (Figure 21.14). The bases can be readily identified by measurement at the four emission wavelengths, which allows DNA sequencing with capillary electrophoresis using a single 488 nm excitation wavelength. However, Figure 21.14 shows that the emission spectra overlap, and that there is residual emission from the donors which contributes to the intensities at shorter wavelengths. Hence, there is still a need for improved dyes for DNA sequencing. Another set of energy-transfer primers has been reported based on the Bodipy fluorophore.[25] These primers are claimed to show more distinct emission spectra than shown in Figure 21.13, but the Bodipy fluorophores are thought to be less photostable. Given the need for sequencing, there will be continued development of probes with improved spectral properties.

21.1.6. DNA Sequencing with NIR Probes

The genomes of many organisms are being sequenced, which must be accomplished as rapidly and inexpensively

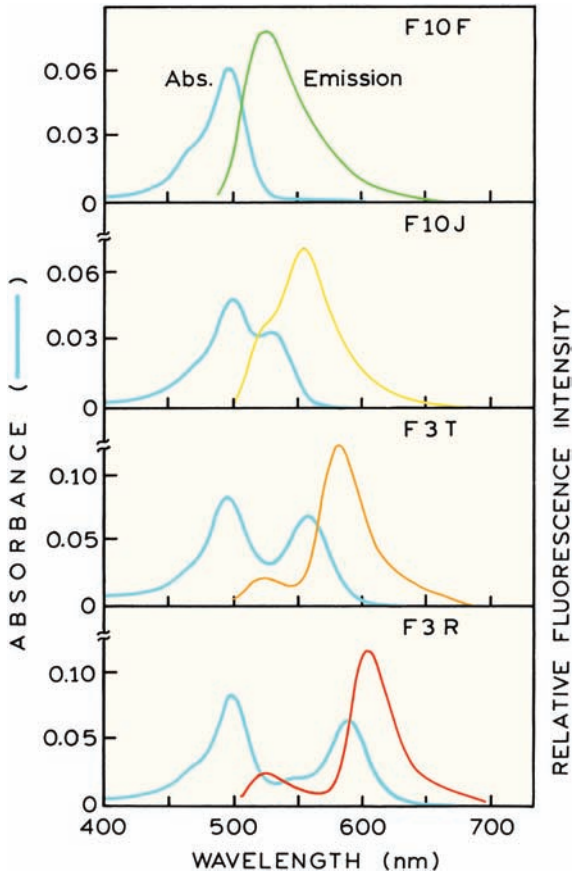

Figure 21.13. Absorption and emission spectra of the four energy-transfer primers showing the relative fluorescence intensity excitation at 488 nm. See Figure 21.9 for the structures of F, J, T, and R. The emission spectrum for each primer pair was determined in solutions having the same absorbance at 260 nm. From [15].

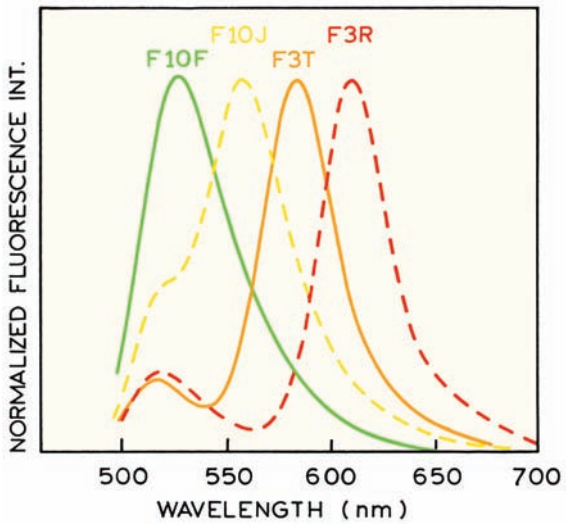

Figure 21.14. Normalized emission spectra of the energy-transfer DNA probes in Figure 21.12. From [15].

as possible. One method to decrease the cost is to use semiconductor laser diodes, which are now available for many wavelengths. These lasers consume little power and can operate for up to 100,000 hours between failures.[26] An additional advantage of red and NIR excitation is the lower autofluorescence from biological samples, gels, solvents, and optical components. Several groups have described NIR dyes for DNA sequencing.[27–31] One such DNA primer is shown in Figure 21.15. Excitation can be accomplished in the NIR at 785 nm, and emission occurs at 810 nm. Such dyes often display small Stokes shifts, which can result in difficulties in rejecting scattered excitation. The Stokes shift can be increased using donor–acceptor pairs, as shown in Section 21.1.5. The quantum yield of the NIR probe shown in Figure 21.15 is low (0.07), and considerably less than that of fluorescein (0.90). Nonetheless, the detection limit was 40-fold lower for this NIR probe, primarily because of the decreased background signal.[27]

Most sequencing instruments have been designed around the spectral properties of available probes. However, there are significant advantages in designing the probes prior to the instrumentation. For instance, the probes shown in Figure 21.4 require an argon ion laser at 488- and 514-nm excitation. Synthesis of the NIR DNA primer allowed design of a sensitive and reliable sequencer (Figure 21.16). The long-wavelength absorption maximum allowed use of a 785-nm laser diode as the excitation source.[27] The long-wavelength emission could be efficiently detected with an avalanche photodiode. The excitation beam is incident on the glass plate at the Brewster angle to minimize scattered light. This sequencer illustrates the effectiveness of including probe design as an integral part of the instrument design process. Because only a single fluorophore is used the NIR sequencing is done using four lanes.

At present a set of four NIR probes for single-lane DNA sequencing does not seem to be available, but the spectral properties of many NIR dyes are known.[32–33] Most NIR probes are used as primers with nonfluorescent dideoxy terminators. It is difficult and challenging to develop probes for use in DNA sequencing. The dyes must display similar intensities at a single excitation wavelength, and must not alter too greatly the electrophoretic mobility of the labeled DNA fragments. While it seems possible to have four distinct red-NIR dyes for DNA sequencing, this has not yet been accomplished.

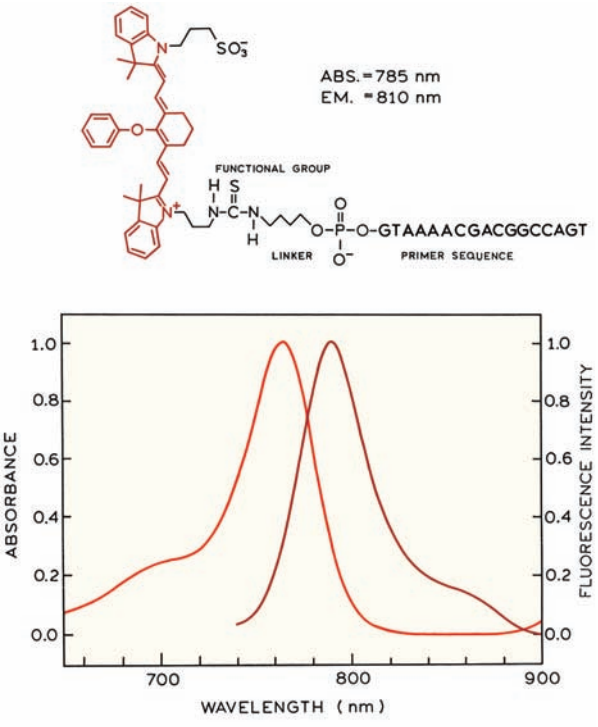

Figure 21.15. Structure, absorption, and emission spectra of an NIR DNA primer. Revised from [27].

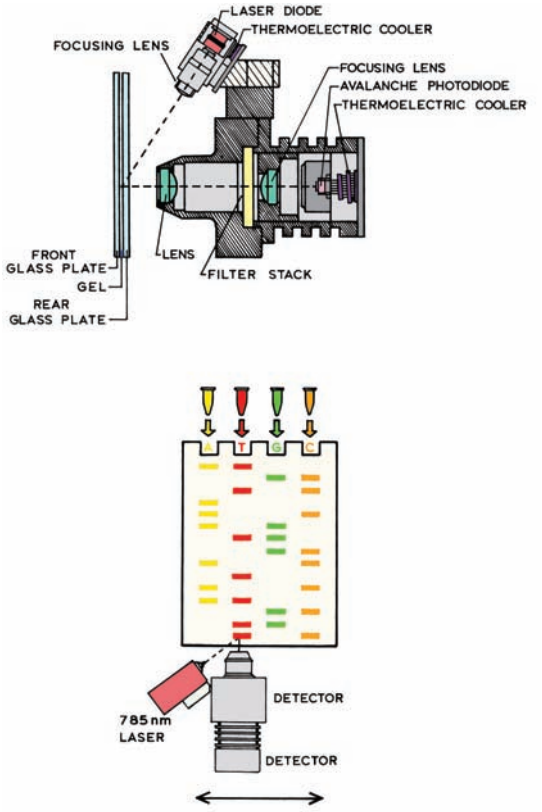

Figure 21.16. NIR DNA sequencer. Redrawn with permission from LiCor Inc.

21.1.7. DNA Sequencing Based on Lifetimes

It is difficult to obtain four dyes with similar absorption spectra and different emission spectra, which allows determination of all four bases on a single gel column. The use of decay times, instead of emission maxima, offers an alternative method to identify the bases. An additional advantage of lifetime-based sequencing is that the decay times are mostly independent of intensity. If decay times are used to identify the bases, the emission spectra can overlap, possibly making it easier to identify suitable fluorophores. Furthermore, the instrumentation for time-resolved measurements has become simpler, less expensive, and more reliable (Chapter 4), so that rapid and continuous lifetime measurements is relatively simple to implement. Progress has been made on lifetime-based sequencing.[34–41] The decay times for the initially used DNA sequencing dyes have been measured in polyacrylamide gels under sequencing conditions.[42] These decay times are listed on Figure 21.4. While the decay times are different for each dye, pulsed light sources at 488 and 514 nm were not available at that time. Hence the efforts on lifetime-based sequencing were focused on longer wavelength dyes. A set of lifetime DNA dyes excitable at 636 nm has been developed (Figure 21.17).[43] The decay times are seen to range from 3.6 to 0.7 ns. Methods have been described for "on-the-fly" lifetime measurements of labeled DNA primers in capillary electrophoresis,[44–46] and there is continuing progress on the instrumentation[47–48] and probes[49–50] for lifetime-based sequencing. Capillary gel electrophoresis is being used in place of slab gels, providing more rapid separations with improved resolution.[51] It is now possible to identify up to one thousand bases in a single separation,[52–53] and there is continuing development of new formats and instruments for high throughput DNA sequencing.[54–58]

21.2. HIGH-SENSITIVITY DNA STAINS

There are numerous applications that require detection of DNA and DNA fragments. One example is analysis of DNA fragments following digestion with restriction enzymes. Frequently one wishes to know whether a DNA sample is from a particular individual, or whether an individual car-

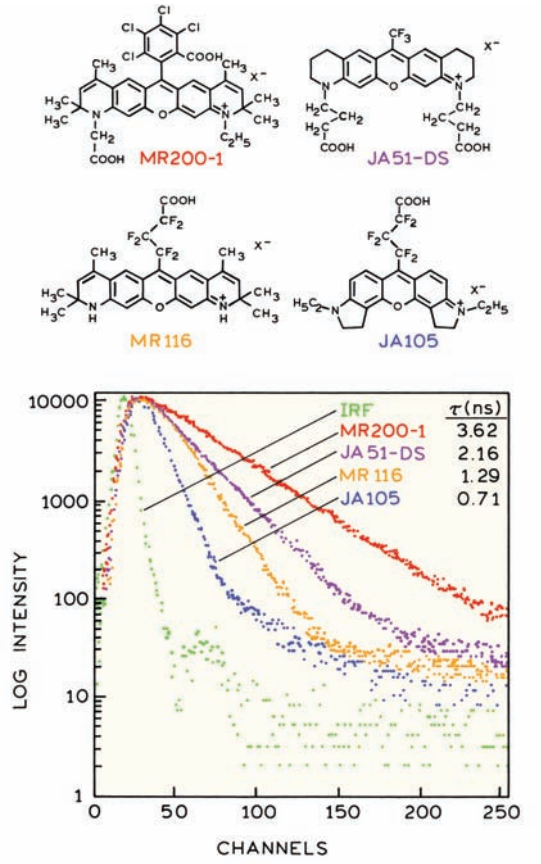

Figure 21.17. Structure and intensity decays of dyes for lifetime-based sequencing. Excitation was with a pulsed laser diode at 636 nm. IRF, instrument response function. Revised from [43].

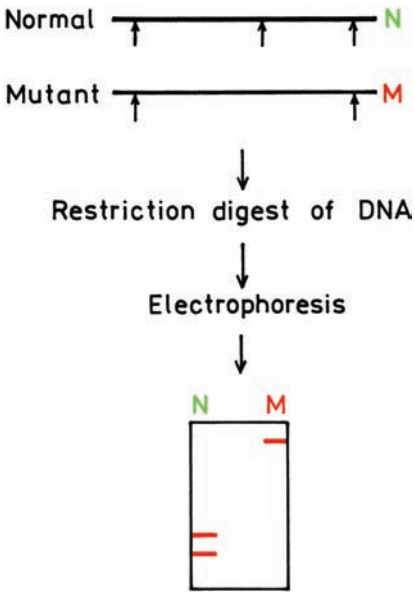

Figure 21.18. Analysis of DNA restriction fragments. The arrows indicate three cleavage sites in normal DNA (N), one of which is missing in the mutant DNA (M). The smaller fragments at the end were not detected.

ries a particular gene. This determination does not require sequencing and can be accomplished by examination of the DNA fragments formed by enzymatic degradation of DNA by restriction enzymes. A large number of restriction enzymes are known, each of which is specific for a particular base sequence, but they sometimes recognize more than one sequence. Generally, the enzymes are specific for relatively long sequences of four to nine base pairs, so that relatively small numbers of DNA fragments are formed. A schematic of a restriction fragment analysis is shown in Figure 21.18. The normal DNA has three restriction enzyme sites, and the mutant is missing one of these sites. Following digestion and electrophoresis, the mutant DNA shows one larger DNA fragment, whereas the normal DNA showed two smaller DNA fragments.

Typically samples of DNA are examined using one or more restriction enzymes. The fragments are different for each individual due to sequence polymorphism occurring in the population. These different size fragments are referred to as restriction fragment length polymorphisms (RFLPs), which do not represent mutations but rather the usual diversity in the gene pool. Following enzymatic digestion the fragments are separated on agarose gels. Originally the DNA was detected using ^{32}P and autoradiography. Today detection is accomplished mostly by fluorescence.

Detection of DNA using stains has a long history, starting with staining of chromatin with acridine dyes. The situation was improved by the introduction of dyes such as ethidium bromide and propidium iodide, which fluoresce weakly in water and more strongly when bound to DNA.[59] DNA on gels is detected by exposing the gels to ethidium bromide (EB). When using EB the gel typically contains micromolar concentrations of EB to ensure that the DNA binds significant amounts of EB. Because of the micromolar binding constants, sensitivity can be low because of background from the free dyes.

21.2.1. High-Affinity Bis DNA Stains

There are now a number of greatly improved dyes which have high affinity for DNA and almost no fluorescence in water. Some of these dyes are dimers of acridine or ethidi-

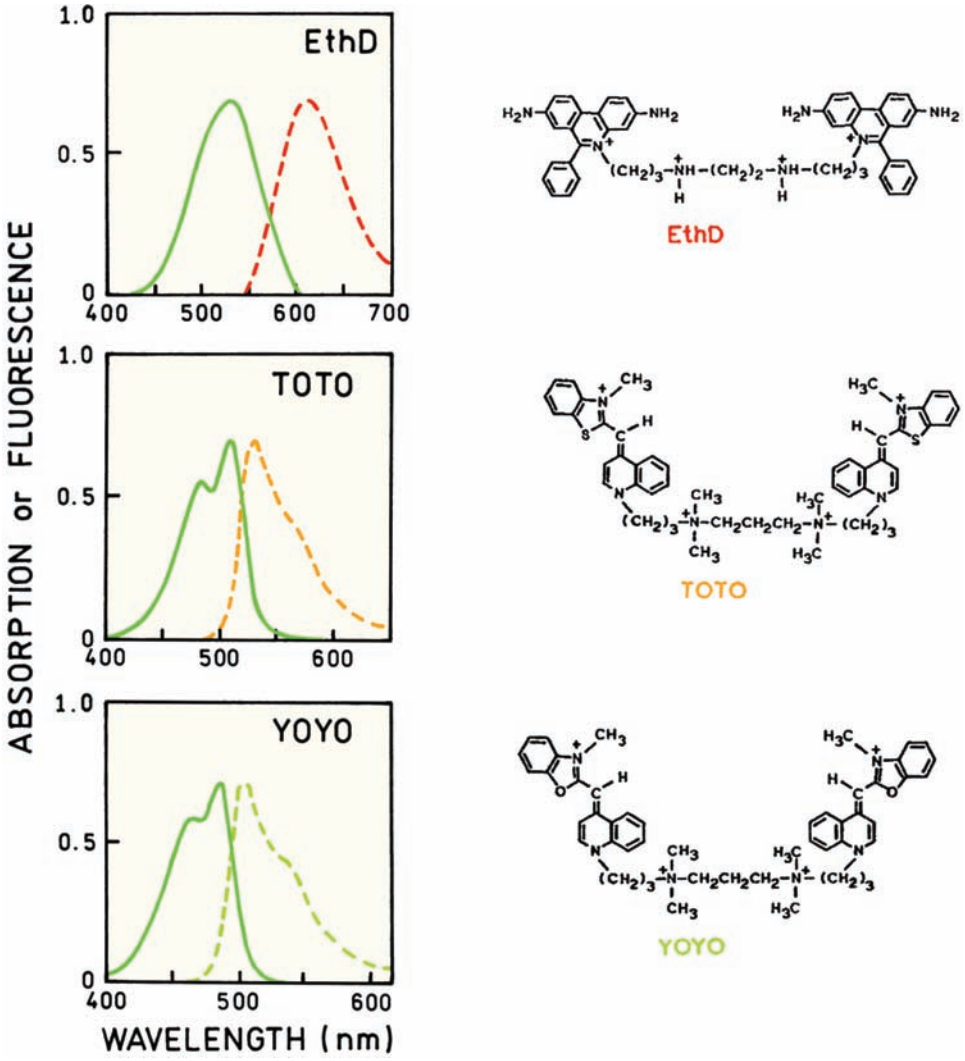

Figure 21.19. Chemical structures of high-affinity DNA dyes: absorption (dashed) and emission (solid) spectra of the dyes bound to DNA. The relative enhancements of the fluorescence of the dyes on binding to DNA are (top to bottom) 35, 1,100, and 3,200. Data from [68].

um bromide.[60–61] The ethidium dimer was found to bind DNA 10^3 to 10^4 more strongly than the monomer.[62] The homodimer of ethidium bromide (Figure 21.19) was found to remain bound to DNA during electrophoresis. This result is surprising because the positively charged dye is expected to migrate in the opposite direction from the DNA. This result suggests the dyes do not dissociate from DNA on the timescale of electrophoresis. The DNA fragments can be stained prior to electrophoresis and it is not necessary to maintain a micromolar concentration of free dye. The DNA gels display little background fluorescence, and the DNA fragments can be detected with high sensitivity.

The usefulness of the EB homodimer resulted in further development of DNA dyes with high affinity for DNA.[63–70] The structures of several high-affinity dyes are shown in Figure 21.19. These dyes are positively charged and display large enhancements in fluorescence upon binding to DNA. The EB homodimer displays an enhancement of 35-fold, and TOTO-1 displays an enhancement of 1,100-fold. The name TOTO is used to describe thiazole homodimers. The use of these dyes with pre-stain DNA fragments provided a 500-fold increase in sensitivity as compared with gels stained with ethidium bromide after electrophoresis.[68] These dyes are widely used as DNA stains, and ana-

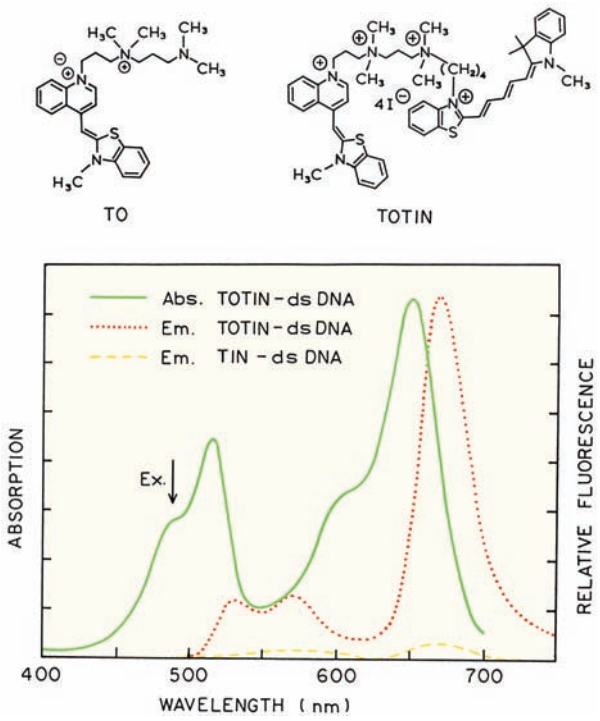

Figure 21.20. **Top**: Structures of thiazole orange (TO) and the thiazole orange–thiazole–indolenine–heterodimer TOTIN, an energy-transfer dye for staining of DNA. **Bottom**: Absorption (solid) and emission (dotted) spectra of TOTIN and emission spectrum of TIN (dashed); the structure on the right side of TOTIN, bound to double-stranded DNA. Excitation was at 488 nm. Reprinted with permission from [72]. Copyright © 1995, Academic Press Inc.

logues with slightly longer excitation and emission wavelengths are also available. Different DNA samples can be stained with different dyes prior to electrophoresis. The dyes do not exchange between the DNA strands, allowing the samples to be identified from the spectral properties. This allows molecular weight standards to be electrophoresed in one lane on the gel with the unknown sample.

21.2.2. Energy-Transfer DNA Stains

The bis dyes shown in Figure 21.19 display favorable properties, but it is desirable to have dyes excitable at 488 nm with larger Stokes shifts. Such dyes were created using donor–acceptor pairs.[71–72] One such dye is shown in Figure 21.20. The thiazole dye on the left (TO) serves as the donor for the thiazole–indolenine acceptor (TIN) on the right. TOTIN remains bound to DNA during electrophoresis. The half-time for dissociation is 317 min.[71] TOTIN can be excited at 488 nm, and displays emission from the TIN moiety near 670 nm. For excitation at 488 nm the emission from TIN alone is much weaker (dashed). TOTIN also allows red excitation at 630 nm with laser diode or HeNe sources.

21.2.3. DNA Fragment Sizing by Flow Cytometry

DNA fragment sizing is usually performed almost exclusively on slab or capillary gels. These methods are typically limited to fragment sizes up to 50 kb in length, and the size resolution is highly nonlinear. Flow cytometry is a method in which cells flow one by one through an area illuminated by a laser beam. Information about the cells is obtained using fluorescent labels. The development of high-affinity DNA stains allows DNA fragment sizing using flow technology.[73–81] The amount of dye bound by the DNA fragments is proportional to the fragment length. Longer DNA fragments bind more dye. The DNA fragments are analyzed in a flow system similar to that used for flow cytometry. This approach allows measurement of the size and number of DNA fragments, without physical separation of the fragments by chromatography or electrophoresis.

An example of DNA fragment sizing by flow cytometry is shown in Figure 21.21. The DNA was from bacteriophage λ, which was digested with the HindIII restriction enzyme.[80] The DNA was stained with TOTO-1, and excited by an argon ion laser at 514 nm. As the TOTO-1 stained DNA passes through the laser beams the instrument records a histogram based on the size of the photon bursts (Figure 21.21, top). For this combination of DNA and restriction enzyme the size of the DNA fragments was known. The photon burst size correlated precisely with fragment size (Figure 21.21, bottom). The photon burst size was found linear with fragment size up to 167 kb.[79] Given the expense and complexity of DNA gels, it seems probable that DNA analysis by flow cytometry will become more widely used in the near future. Flow analysis of DNA has already been used to measure DNA damage[73] and for rapid identification of photogens.[74]

21.3. DNA HYBRIDIZATION

Detection of DNA hybridization is widely useful in molecular biology, genetics, and forensics. Hybridization occurs during polymerase chain reaction (PCR) and fluorescence in-situ hybridization. A variety of methods have been used to detect DNA hybridization by fluorescence. Several possible methods are shown schematically in Figure 21.22.

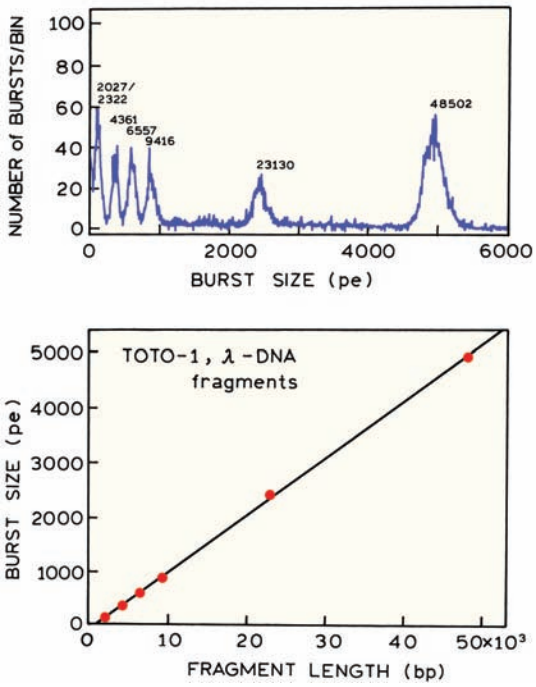

Figure 21.21. **Top**: Histogram of photon burst sizes of TOTO-1 stained DNA from a HindIII digest of λ-DNA. **Bottom**: Correlation of the photon burst size with DNA fragment length. Excitation was at 514 nm from an argon ion laser, and emission was observed through a 550-nm interference filter. Modified and reprinted from [80]. Copyright © 1995, American Chemical Society.

One commonly used method is to detect an increase in RET when complementary donor and acceptor labels hybridize (upper left). The presence of complementary DNA sequences can be detected by increased energy transfer when these sequences are brought into proximity by hybridization.[82–89] This can occur if the complementary strands are labeled with donors and acceptors. An example of this approach was shown in Figure 1.27. Energy transfer can also occur if the donor- and acceptor-labeled oligonucleotides bind to adjacent regions of a longer DNA sequence (Figure 21.22). An example of this approach is shown below in Figure 21.36. Hybridization can be detected if a donor intercalates into the double-helical DNA, and transfers to an acceptor-labeled oligonucleotide. The use of an intercalating dye has been extended to include a covalently attached intercalators, whose fluorescence increases in the presence of double-stranded DNA (d).[90–91] One example is shown in Figure 21.23, in which the acridine dye is covalently linked to the 3' phosphate of an oligothymidylate. Upon binding to a complementary adenine oligonucleotide the acridine fluorescence increases about twofold. Hybridization can be competitive where the presence of increased amounts of target DNA competes with formation of donor–acceptor pairs.[92] The acceptor can be fluorescent, or it can be nonfluorescent, in which case the donor appears to be quenched. A competitive assay (Figure 21.22) was

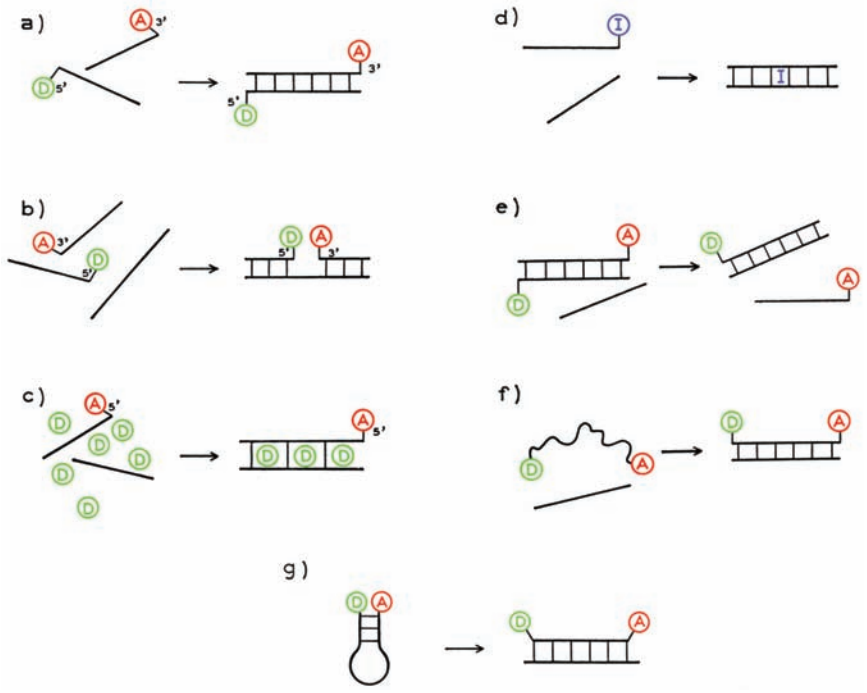

Figure 21.22. Methods to detect DNA hybridization by energy transfer. D, donor; A, acceptor or quencher; I, intercalating dye.

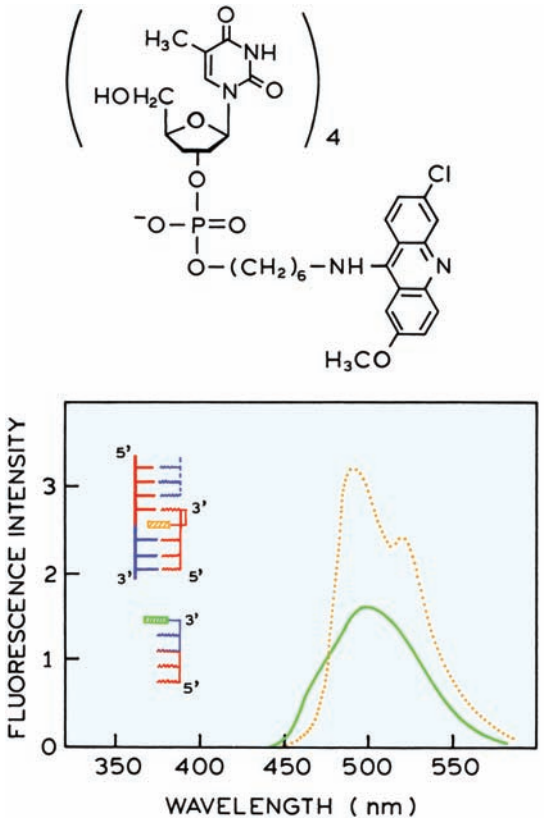

Figure 21.23. DNA hybridization detected by a covalently bound intercalating probe. Fluorescence intensity of the probe, an acridine dye covalently linked to the 3'-phosphate of an oligothymidylate (dashed); fluorescence intensity of the probe upon binding to a complementary adenine oligonucleotide (dotted). From [91].

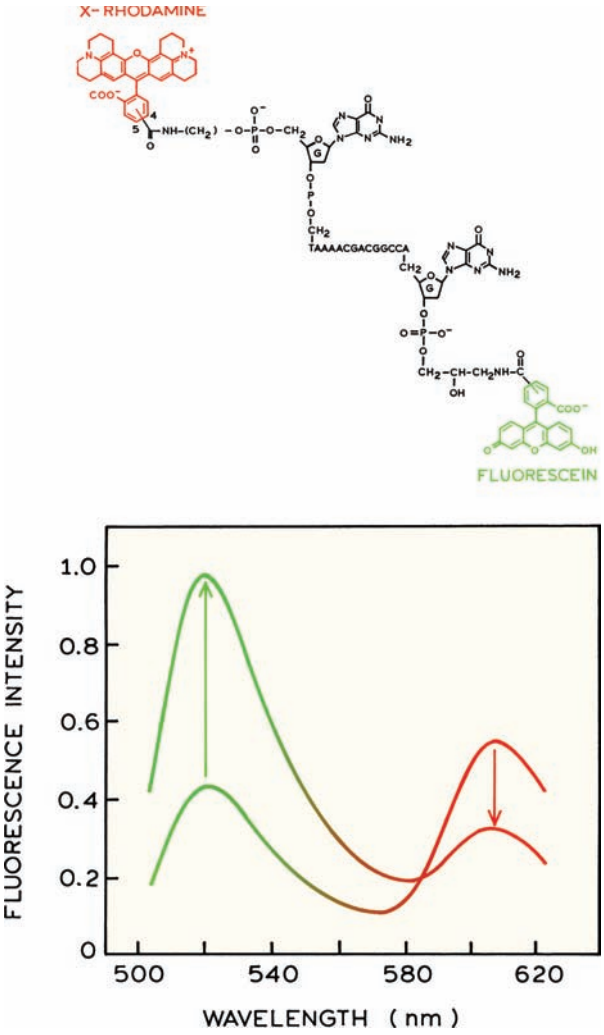

Figure 21.24. Detection of DNA hybridization with a single donor- and acceptor-labeled oligonucleotide. The increase in donor emission and decrease in acceptor emission occurred upon binding of the oligonucleotide to its complementary strand is shown. From [93].

performed with complementary DNA strands in which the opposite strands were labeled with fluorescein and rhodamine.[93] Hybridization of the strands resulted in quenching of the donor fluorescence. Increasing amounts of unlabeled DNA, complementary to one of the labeled strands, resulted in displacement of the acceptor and increased donor fluorescence. Such arrays can be useful in amplification reactions in which the DNA is thermally denatured during each cycle.

21.3.1. DNA Hybridization Measured with One-Donor- and Acceptor-Labeled DNA Probe

Most DNA hybridization methods (Figure 21.22) require two probe DNA molecules, one labeled with donor and the other with acceptor. Assays can be based on a single donor- and acceptor-labeled probe DNA.[93] One example is shown in Figure 21.24, in which single-stranded DNA is labeled on opposite ends with a donor and acceptor, respectively. In the absence of the complementary strand the single-stranded probe DNA is flexible. This allows the donor- and acceptor-labeled ends to approach closely, resulting in a high FRET efficiency. Upon binding of the single-stranded probe DNA to its complementary strand, the donor and acceptor become more distant due to the greater rigidity of double-stranded DNA. Hybridization can be detected by an increase in donor emission and a decrease in acceptor emission. There are many circumstances where an FRET assay would be simplified by the use of only a single probe molecule. The donor and acceptor concentrations are forced to remain the same, independent of sample manipulations, because they are covalently linked. This allows the extent of

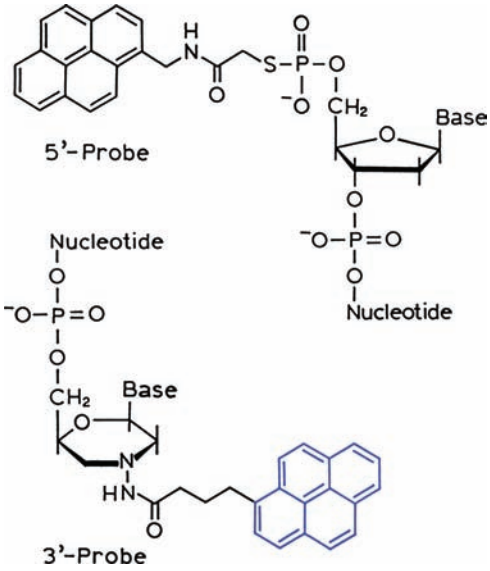

Figure 21.25. Pyrene-labeled oligonucleotide probes. Modified from [96].

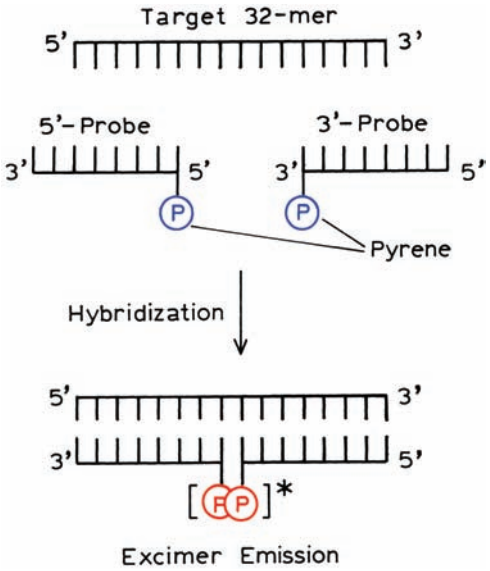

Figure 21.26. Principle of the excimer-forming DNA hybridization array. Modified from [96].

hybridization to be determined using wavelength-ratiometric measurements.

21.3.2. DNA Hybridization Measured by Excimer Formation

DNA hybridization can also be detected by pyrene excimer formation.[94–96] DNA probes were synthesized with pyrene attached to the 5' and 3' ends (Figure 21.25). It is well known that one excited pyrene molecule can form an excited-state complex with another ground-state pyrene, forming an excimer. This complex displays an unstructured emission near 500 nm as compared to the structured emission of pyrene monomer near 400 nm. The use of excimer formation to detect DNA hybridization is shown in Figure 21.26. The assay requires two DNA probes that bind to adjacent sequences on the target DNA. In this example the correct target DNA is a 32-mer oligonucleotide. If both the 5'- and 3'-pyrene probe bind to target DNA, the pyrene

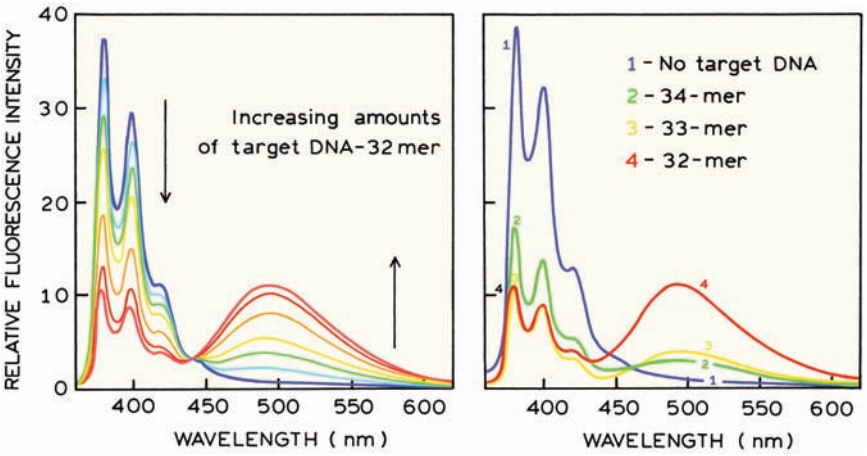

Figure 21.27. Effect of target DNA (32-mer) and mismatched target DNA (33- and 34-mer) on the emission from DNA probes labeled with pyrene at the 3' and 5' ends. The target DNA 32-mer and the mismatched target DNA have the sequence 5'-AGAGGGCACGGATACC*GCGAGGTGGAGC-GAAT-3', where the asterisk denotes the location of one or two extra thymine residues in the 33- and 34-mer, respectively. Modified from [96].

monomers will be in close proximity, resulting in excimer emission. Emission spectra of a mixture of the 3' and 5' probes are shown in Figure 21.27. In the absence of target DNA the emission is near 400 nm and characteristic of a pyrene monomer. Titration with increasing amounts of target DNA results in increasing emission from the excimer near 500 nm.

This hybridization assay based on excimer formation is sensitive to precise matching of the target sequence with the probe sequence. Just one extra thymine residue in the target DNA, between the pyrene sites on the probe DNA, eliminates most of the excimer emission. This property of the assay is distinct from a hybridization assay based on FRET. In the case of FRET the donor–acceptor interaction occurs over long distances, so that the additional distance of one base would not abolish FRET. In contrast, excimer formation is a short range interaction that requires molecular contact between the pyrene monomers. For this reason it is sensitive to small changes in the pyrene-to-pyrene distance.

21.3.3. Polarization Hybridization Arrays

DNA hybridization has also been detected using fluorescence anisotropy.[97–99] Hybridization is detected by the increase in anisotropy when labeled DNA binds to its complementary strand. These assays are analogous to the fluorescence polarization immunoassays. Polarization or anisotropy measurements have the favorable property of being independent of the intensity of the signal and dependent on the molecular weight of the labeled molecule. Also polarization measurements do not require separation steps.

DNA hybridization arrays based on polarization have been reported using the fluorescein probes,[100–101] as well as a more novel NIR dye.[99] The structure of an NIR dye, LaJolla Blue™, is shown in Figure 21.28. The central chromophore is a phthalocyanine, which displays the favorable property of absorbing in the NIR, and in this case was excited by a pulsed laser diode at 685 nm. The phthalocyanines are poorly soluble in water, and hence the central silicon atom was conjugated to polar groups to increase the water solubility and prevent aggregation.

Polarization values of the LaJolla Blue™ oligonucleotide are shown in Figure 21.29. The dye-DNA probe was mixed with either complementary (●) or non-complementary DNA (▲, ■). The polarization increases upon mixing with the complementary strand, but not with the non-complementary oligomers. This result suggests that polarization measurements can be used to monitor the production of complementary DNA by PCR and related amplification methods. However, the change in polarization is not large, which is probably because the fluorophores have substantial motional freedom when present in both single-stranded and double-stranded DNA. A unique aspect of the data in Figure 21.29 is the use of pulsed excitation and gated detection after the excitation pulse. This was done to avoid detection of scattered light and/or background fluorescence from the sample.

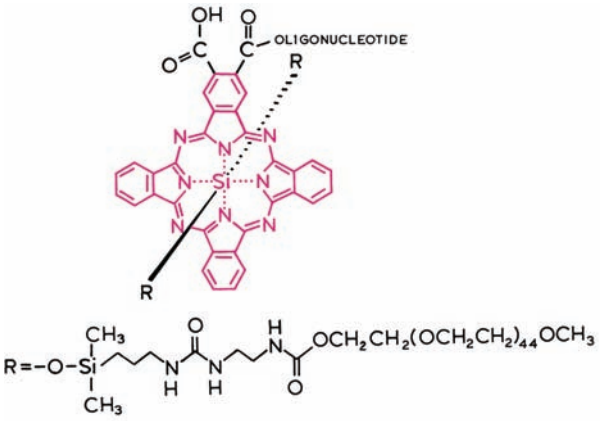

Figure 21.28. Structure of the LaJolla Blue™–oligonucleotide. Revised and reprinted with permission from [99]. Copyright © 1993, American Association for Clinical Chemistry.

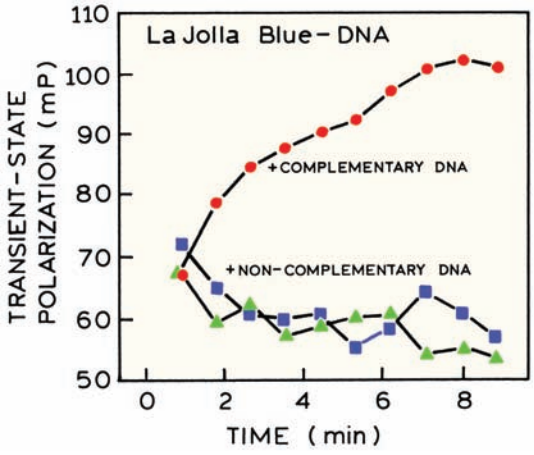

Figure 21.29. Fluorescence polarization DNA hybridization array. One mP is equivalent to 0.001 polarization units. The probe DNA was mixed with complementary (●) or non-complementary DNA (▲, ■). The excitation source was a pulsed laser diode at 685 nm. The emission at 705 nm was detected after the excitation pulse. Modified from [99].

21.3.4. Polymerase Chain Reaction

Polymerase chain reaction (PCR) is an important advance for DNA technology.[102–105] PCR allows almost unlimited amplification of DNA. Small amounts of DNA isolated from forensic evidence, DNA libraries, or archaeological sites can be replicated many times to obtain useful amounts for further studies. The progress of a PCR reaction can be followed by any probe that detects the presence of double-helical DNA. These methods include probes like Syber Green, which are only fluorescent in the presence of double-stranded DNA or molecular beacons (Section 21.4) that bind to the amplified DNA and become fluorescent. The most widely used approach is based on energy transfer and is called Taqman. This name refers to the use of Taq DNA polymerase, which is stable at the high temperatures needed to denature the double-stranded DNA prior to each round of amplification. The sample initially contains a D–A oligonucleotide, in which the donor fluorescence is quenched.[103–104] During the PCR reaction this D–A strand is displaced and cleaved by DNA polymerase, which displays some nuclease activity as well as polymerase activity. Upon cleavage of the D–A pair, the donor becomes distant from the acceptor and thus more fluorescent (Figure 21.30). PCR assays based on fluorogenic donor–acceptor pairs are presently used in commercial instruments. The oligonucleotide sequence in the D–A pair is complementary to a portion of the DNA to be amplified. This type of assay is an extension of the concept of fluorogenic probes described in Chapter 3, wherein the molecule becomes more fluorescent as the result of enzymatic cleavage.

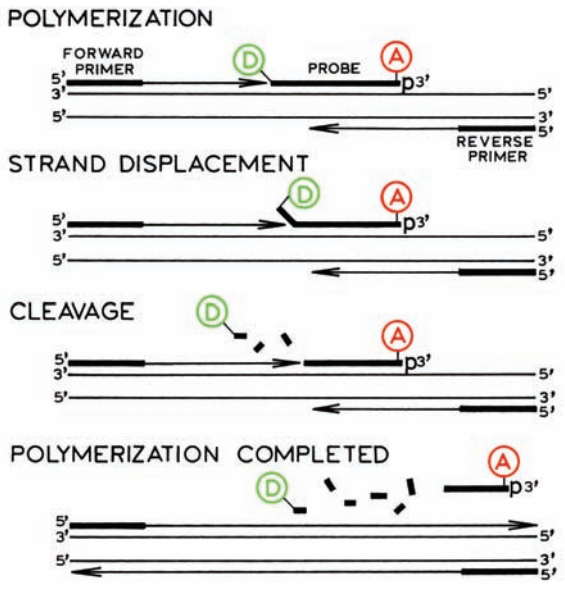

Figure 21.30. Release of donor quenching during polymerase chain reaction. From [103].

21.4. MOLECULAR BEACONS

21.4.1. Molecular Beacons with Nonfluorescent Acceptors

In DNA or genetic analysis it is frequently necessary to detect the presence of a single gene in a sample containing the entire genome. This can be accomplished by identifying and detecting a base sequence that is unique for a particular gene. Detection of such sequences in a mixture of DNA can be accomplished using molecular beacons.

Molecular beacons were introduced in 1996[106–107] and have become widely used in biotechnology and the biosciences. A schematic of a typical molecular beacon is shown in Figure 21.31. A molecular beacon contains a fluorophore (donor)–quencher (acceptor) pair, a loop region, and a stem region that contains two short complementary sequences. The loop region contains a base sequence that is complementary to a target sequence. In the absence of target DNA the complementary sequences on each end hybridize, brining the fluorophore and quencher in close contact. Binding to target DNA results in extension of the beacon and increased fluorescence.

Molecular beacons possess a number of characteristics that are favorable for their use in biotechnology, diagnostics and genetic analysis. Molecular beacons allow detection of the target sequence without any separation steps. It was found that hybridization of beacons to target sequences is

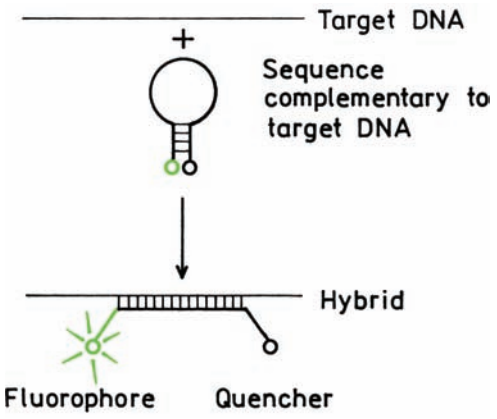

Figure 21.31. Schematic of a typical molecular beacon.

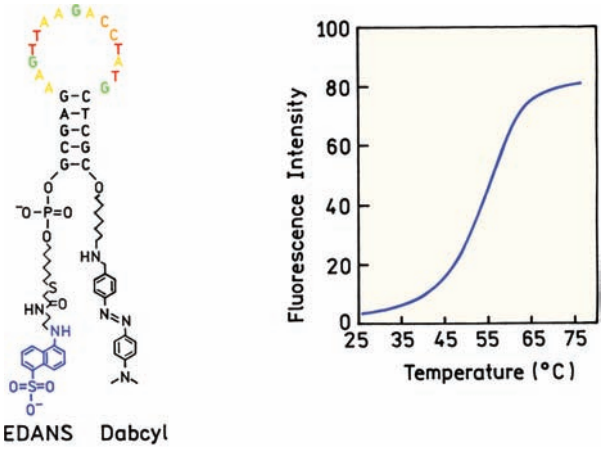

Figure 21.32. Structure and thermal unfolding of a molecular beacon. From [106].

more specific than hybridization of linear DNA to a similar size sequence. The beacon can be almost completely specific for a target sequence and discriminate against sequences with a single base mismatch. The beacons rapidly unfold and fold as the temperature is increased and decreased, allowing their use with real-time detection in polymerase chain reaction (PCR). Molecular beacons can also be used as intracellular probes for DNA or mRNA.

Figure 21.32 shows the sequence and structure of a molecular beacon.[106] The stem region contains five base pairs and the loop is complementary to a 15-base sequence in the target DNA. The fluorophore EDANS is a dansyl derivative. The quencher is Dabcyl, which is an RET acceptor. At low temperatures the beacon is hybridized and almost nonfluorescent. Upon heating the beacon unfolds and the EDANS emission increases 25-fold. Figure 21.33 shows the fluorescence intensities of a molecular beacon upon addition of the complementary sequence, and sequences with a single base mismatch or deletion. The intensity increases significantly for the perfectly matched sequence. The photograph of the UV-illuminated beacon shows a bright visible emission in the presence of the target and no visible emission in the absence of target DNA. Molecular beacons display a high on–off contrast ratio as well as high specificity.

Molecular beacons are used to follow PCR amplification (Figure 21.34). In this example the beacon contained a loop sequence that is complementary to a middle segment of an 84-base long amplicon. The intensities are measured after the reaction mixture is cooled to the annealing temperature at 50°C. At higher temperatures all the beacons are unfolded and not hybridized, so the intensity represents the total number of beacons in the sample. When the sample is annealed the intensity depends on the number of target sequences. This number increases with each PCR cycle, resulting in a higher intensity at the annealing temperature.

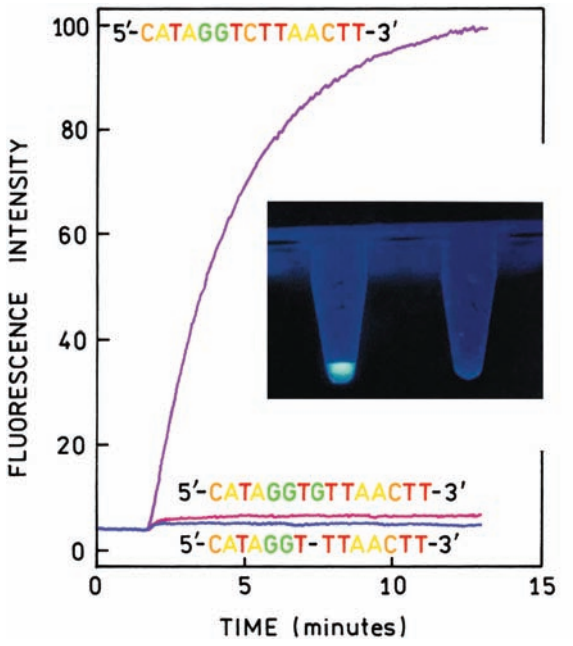

Figure 21.33. Fluorescence intensity of the molecular beacon in Figure 21.32 upon addition of the complementary oligo, or oligos with a one-base mismatch or deletion. The photo shows the UV-illuminated molecular beacon in the presence (left) and absence (right) of the complementary oligomer. Revised from [106].

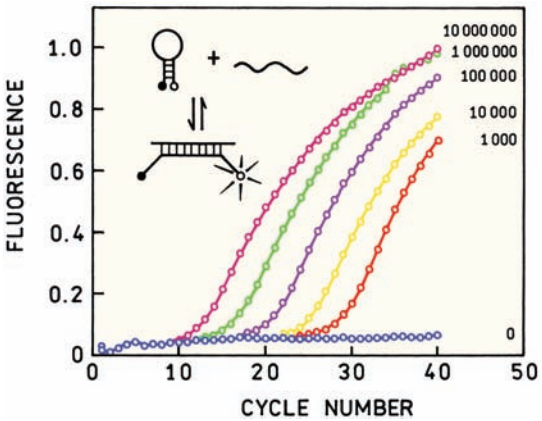

Figure 21.34. Use of a fluorescein–dabcyl molecular beacon to follow PCR amplification. The numbers on the right are the initial number of template molecules. Revised from [107].

The cycle at which the fluorescence becomes detectable depends on the number of amplicons at the start of the amplification.

A somewhat surprising result with molecular beacons is that dabcyl quenches fluorophores with emission from blue to red wavelengths.[107] This quenching appears to be independent of spectral overlap between the emission and the dabcyl absorption. Quenching occurs even when there is no obvious spectral overlap. This is a favorable result because a single type of quencher can be used with a wide variety of fluorophores. The reasons for universal quenching by dabcyl are not completely known and may be the result of complex formation between the fluorophore and quencher.[108] Dabcyl is not the only quencher used in molecular beacons. Molecular beacons have been reported which use a variety of quenchers including pyrene.[109] Molecular beacons have also been based on intercalation into the double helix.[110]

21.4.2. Molecular Beacons with Fluorescent Acceptors

The previous section described molecular beacons with a single emitting species. Molecular beacons can also be made using fluorescent acceptors.[111–115] The beacon shown in Figure 21.35 has a Cy3 donor and a Cy5 acceptor. In this beacon one of the probes is located within the oligomer rather than at one of the ends. Upon addition of the target sequence the extent of energy transfer decreased, resulting in an increase in the donor emission and a decrease in the acceptor emission. For a molecular beacon with two fluorophores the intensity ratio is independent of the total molecular beacon concentration. Additionally, the ratio can be used to determine the concentration of the target sequence, if the concentration of the beacon is known.

Molecular beacons with a quencher or fluorescent acceptor serve different purposes. A molecular beacon of the type shown in Figure 21.35 may not be useful for detection of a small quantity of target in a sample containing other DNA. A small amount of target DNA will result in a small change in the intensity ratio, which may not be detectable. In contrast, a molecular beacon of the type shown in Figure 21.31 displays emission against a dark background, allowing low concentrations of target to be detected. However, the intensity data alone do not reveal the concentration of target DNA.

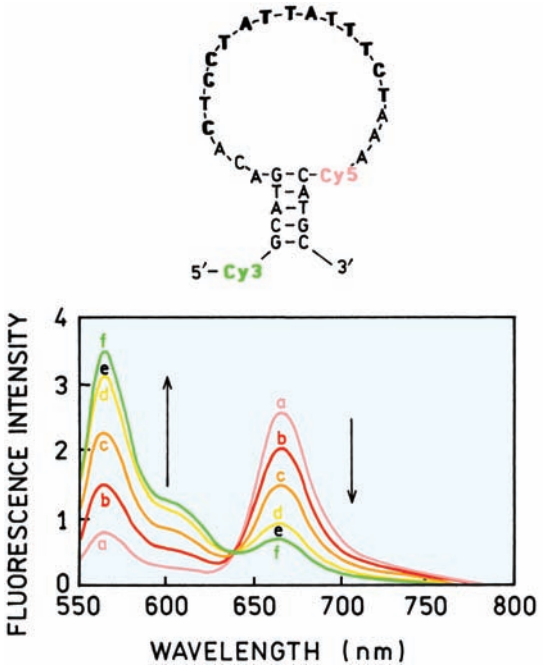

Figure 21.35. Molecular beacon based on RET between a Cy3 donor and a Cy5 acceptor. The arrows indicate increasing concentrations of the target sequence. Revised and reprinted with permission from [113]. Copyright © 2004, American Chemical Society.

21.4.3. Hybridization Proximity Beacons

Molecular beacons can be highly specific, but it can be important to decrease the number of false positives. This can be accomplished by using molecular beacons which hybridize close to each other on the target sequence (Figure 21.36). The beacons are designed so that RET occurs between a donor on one beacon and an acceptor on the other beacon.[116] Both beacons are quenched in the absence of target DNA. Specificity is increased because RET will only occur when both beacons are bound. Binding of the donor beacon alone or the acceptor beacon alone will increase the donor or acceptor intensities, but it will not increase the extent of RET.

Figure 21.37 shows emission spectra of the donor and acceptor beacons. In the absence of target DNA both the donor and acceptor are quenched and the signal is close to zero. If only the donor beacon binds to the target the donor emission is high. The acceptor emission remains low, even in the presence of target DNA, because the acceptor absorbs weakly at the excitation wavelength. When both beacons are bound to the target the acceptor emission increases due to RET. The donor is partially quenched by RET to the

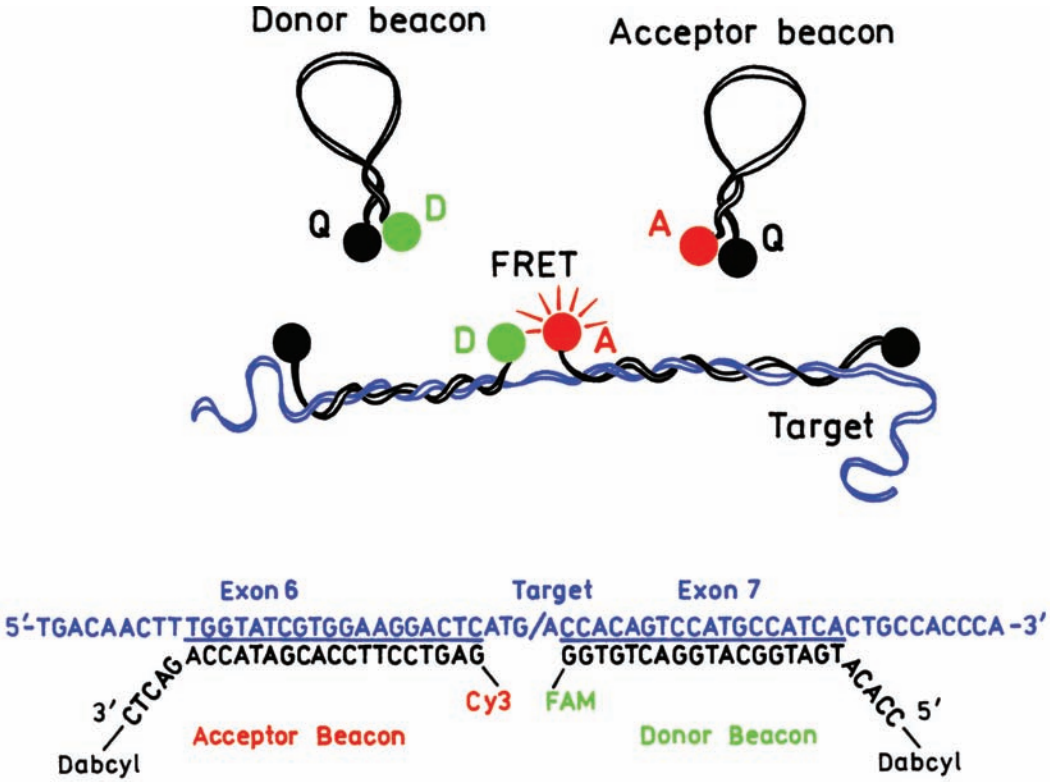

Figure 21.36. Donor and acceptor molecular beacons for a hybridization proximity array. Reprinted with permission from [116]. Copyright © 2003, American Chemical Society.

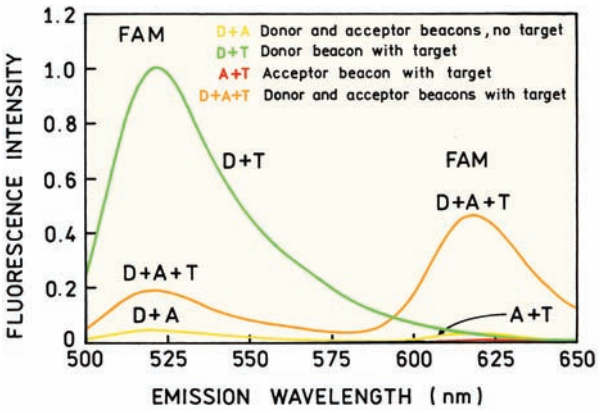

Figure 21.37. Emission spectra of the donor and acceptor beacons in Figure 21.36 in the absence and presence of target DNA. Revised and reprinted with permission from [116]. Copyright © 2003, American Chemical Society.

nearby acceptor. Emission from both the donor and acceptor is only seen when both beacons bind to the same target sequence. This type of beacon could be made even more specific using a lanthanide donor and detection of the sensitized acceptor emission

21.4.4. Molecular Beacons Based on Quenching by Gold

Gold surfaces and colloids are becoming more widely used in bioassays because of the well-developed chemistry for linkage to the surface, the ease of colloid preparation, and the chemical stability of the surfaces. Gold is an highly effective quencher of fluorescence.[117–119] Quenching probably occurs by RET to the gold surface, but other mechanisms may also be present. Because of the strong quenching gold can provide a large on–off ratio for molecular beacons.[120–121] A molecular beacon on a gold surface is made by binding a labeled oligomer to the surface by a sulfhydryl group.[121] In the absence of target DNA the rhodamine label is quenched (Figure 21.38). In the presence of target DNA the rhodamine moves away from the gold surface and becomes fluorescent. Surface-bound molecular beacons could have a different sequence at each position on an array,

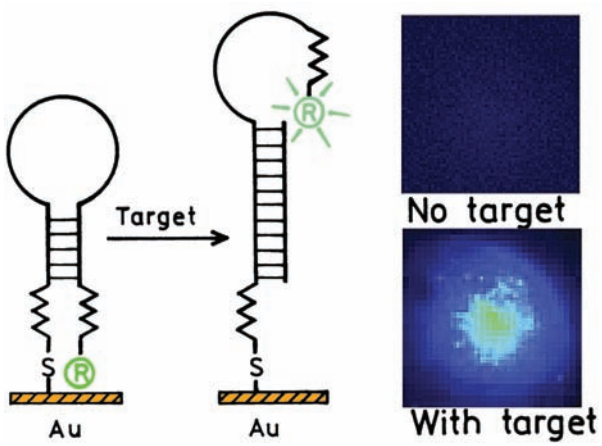

Figure 21.38. Surface-bound molecular beacon with quenching by a gold surface. The CCD photons shows epifluorescence confocal microscope image of the surface-bound beacon in the absence (top) and presence (bottom) of the target sequence. Reprinted with permission from [121]. Copyright © 2003, American Chemical Society.

allowing detection of a good number of sequences by the spatial localization of complementary sequences on the array.

21.4.5. Intracellular Detection of mRNA Using Molecular Beacons

An ability to monitor gene expression in a single cell would be of great value in cell biology. However, detection of specific messenger RNAs within a cell is a challenging task. Staining with nucleic acid probes will label both DNA and RNA. Even if a stain is specific for RNA, it will stain all the RNA, not just the desired gene product. Molecular beacons can be used to monitor specific mRNAs in living cells.[122–124]

Figure 21.39 shows light and fluorescence images of mammalian kidney cells.[124] The light image shows a cluster of five cells. One cell was microinjected with a molecular beacon specific for β-actin mRNA. The fluorophore was TAMRA and the quencher dabcyl. The images were recorded with an intensified CCD camera so the different colors represent different intensities. The fluorescence images taken at 3-minute intervals show a progressive increase in fluorescence intensity. Only the single microinjected cell showed this emission. Control experiments showed that a nonspecific molecular beacon did not display a time-dependent increase in intensity. This control experiment indicates that the increase in intensity is due to the mRNA for β-actin and not the result of hydrolysis of the molecular beacon. This result shows that molecular beacons can be used to study gene expression in living cells.

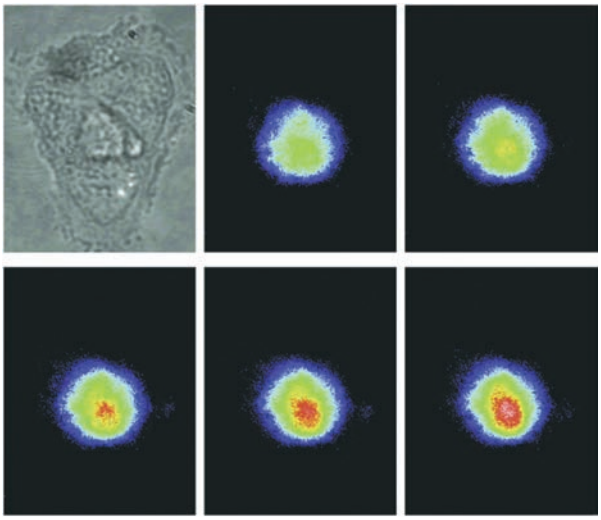

Figure 21.39. Light and fluorescence images of kangaroo rat kidney cells. The fluorescence images are taken at 3-minute intervals following microinjection of a molecular beacon specific for β-actin mRNA. The molecular beacon contained TAMRA and a dabcyl acceptor. Revised and reprinted with permission from [124]. Copyright © 2001, American Chemical Society.

21.5. APTAMERS

Molecular beacons are used to detect the presence of specific sequences in biological samples. Specially designed sequences of DNA can also be used to detect other molecules that are not nucleic acids. These nucleic-acid sequences that bind to specific molecules are called aptamers. Specific detection by aptamers depends on specific interactions with the analyte as well as base pairing between different parts of the aptamer.

The concept of an aptamer is best illustrated by a specific example.[125–126] Figure 21.40 shows an aptamer that binds cocaine. This aptamer contains a fluorescein donor and a dabcyl acceptor. Cocaine binds to a central region of the aptamer, which then forms additional base pairs between the two ends of the aptamer. This folding brings the donor and acceptor closer together and results in quenching of fluorescein by the dabcyl acceptor (Figure 21.41). Addition of closely related molecules does not result in donor quenching.

Aptamers provide a general approach to the design of reagents with high affinity for the desired species.[127–131] Aptamers can be made from DNA or RNA. The specificity

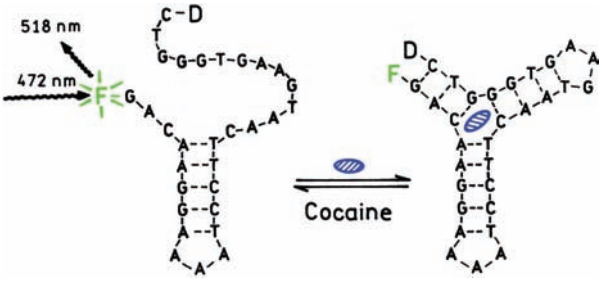

Figure 21.40. Structure of DNA aptamer that binds cocaine; F is fluorescein and D is dabcyl. Reprinted with permission from [126]. Copyright © 2001, American Chemical Society.

of aptamers can be as high as that obtained with antibodies. The base sequence of an aptamer determines its binding specificity. The sequence is determined by a procedure called Selex: selective enrichment of ligands by exponential enrichment. The procedure starts with a library of random DNA or RNA sequences that are flanked by the primer sequences used for polymerase chain reaction (PCR). The library is enriched for the molecule of interest, typically by binding to a chromatography column that contains this molecule. The oligomers that bind to the column are eluted, and amplified by PCR, followed by additional rounds of enrichment and selection. Finally the enriched library is cloned and sequenced, followed by selection of those sequences with the optimal binding affinity for the molecule of interest. Aptamers have been designed for a variety of molecules including cAMP,[132] adenosine,[133] steroids,[134] carbohydrates,[135] and the protein HIV reverse transcriptase.[136–137] Aptamers can contain more than one oligonucleotide, as shown for the aptamer that binds rATP (Figure 21.42). The aptamer consists of two oligonucleotides, one labeled with

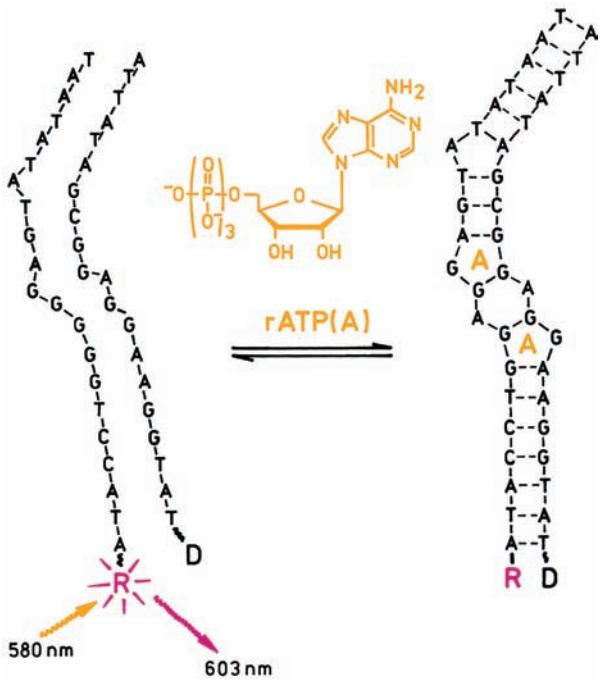

Figure 21.42. DNA aptamer specific for rATP; R is rhodamine and D is dabcyl. Reprinted with permission from [125]. Copyright © 2000, American Chemical Society.

a rhodamine donor and the other with a dabcyl acceptor.[125] Addition of rATP results in binding of the two oligonucleotides to two rATP molecules. This binding results in quenching of the rhodamine donor, which only occurs in the presence of rATP and not the other ribonucleotides (Figure 21.43).

Aptamers can be designed for proteins as well as for small molecules. Platelet-derived growth factor (PDGF) stimulates cell division and cell proliferation, and is a

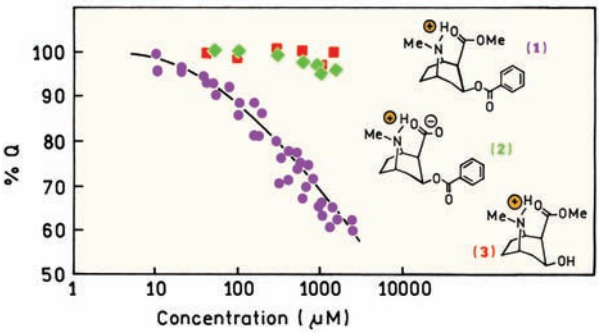

Figure 21.41. Donor intensity of the cocaine-binding aptamer in the presence of cocaine (1, ●), benzoyl-ecgonine (2, ♦) and ecgonine methyl ester (3, ■). Reprinted with permission from [126]. Copyright © 2001, American Chemical Society.

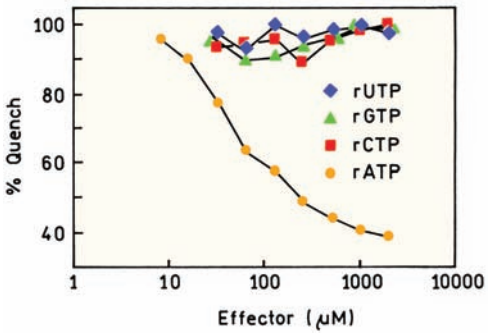

Figure 21.43. Rhodamine donor intensities of the aptamer shown in Figure 21.42 in the presence of ribonucleotides. Reprinted with permission from [125]. Copyright © 2000, American Chemical Society.

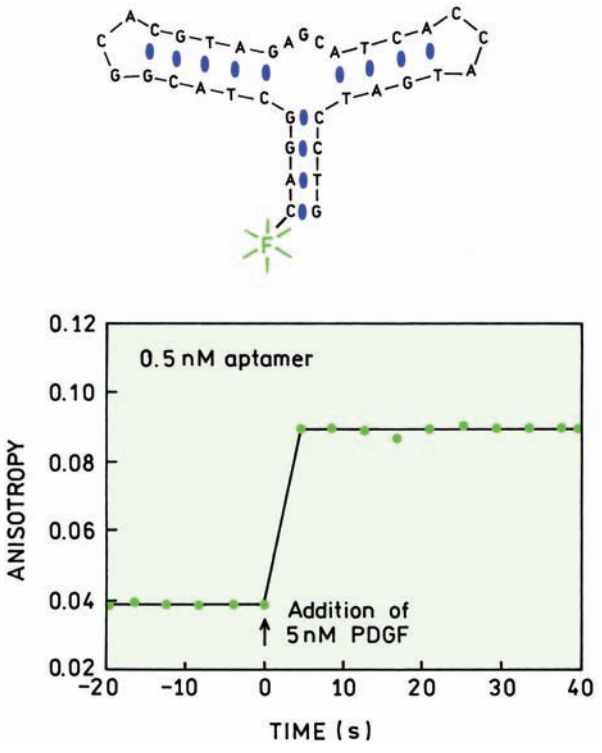

Figure 21.44. Structure of a fluorescein-labeled aptamer specific for PDGF. The lower panel shows the fluorescein anisotropy. Revised and reprinted with permission from [138]. Copyright © 2001, American Chemical Society.

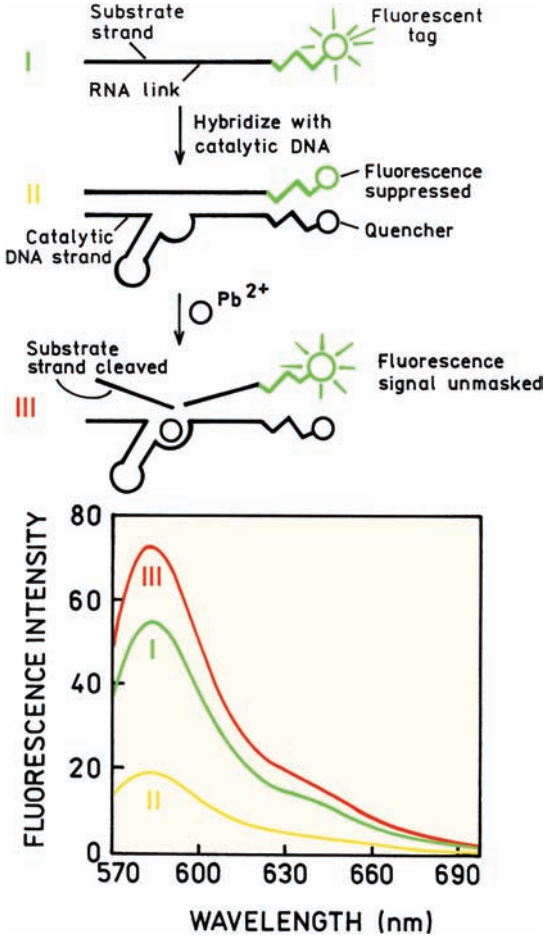

Figure 21.45. Detection of Pb^{2+} using a DNAzyme. The fluorophore is TAMRA and the quencher is dabcyl. From [142].

potential protein marker for cancer diagnosis. PDGF is typically detected using ELISA or radiotracer methods. Figure 21.44 shows a fluorophore-labeled aptamer specific for PDGF. Upon addition of PDGF the anisotropy increases more than twofold.[138] This result shows that other fluorescence parameters can be used with an aptamer, not just RET. The twofold increase in anisotropy is probably larger than could be obtained with a fluorescein-labeled antibody. The molecular weight of IgG is near 150,000, and an Fab fragment has a molecular weight near 50,000. For proteins of this size the fluorescein anisotropy would be near its maximal value before binding to PDGF. The smaller size of the aptamers and their high degree of flexibility in the absence of ligand should result in similar anisotropy changes in other aptamers.

21.5.1. DNAzymes

The uses of aptamers have been extended to include DNA sequences that have enzymatic activity,[139–140] analogous to the activity displayed by ribozymes. A combination aptamer-DNAzyme was developed for detection of lead ions.[141–142] The aptamer contained two parts that were labeled with a TAMRA donor or a dabcyl acceptor. These two oligomers spontaneously hybridized, which resulted in quenching of TAMRA by RET (Figure 21.45). Upon addition of Pb^{2+} the DNAzyme undergoes autocatalytic cleavage to release the fragments of the cleaved oligomer. The donor intensity increases when the quencher oligomer is released, which can be used to perform assays for lead. Aptamer technology may evolve to create a new class of sensors with high specificity and enzymatic activity.

21.6. MULTIPLEXED MICROBEAD ARRAYS: SUSPENSION ARRAYS

In Section 21.9 we will describe detection of DNA sequences using two-dimensional arrays of capture oligomers

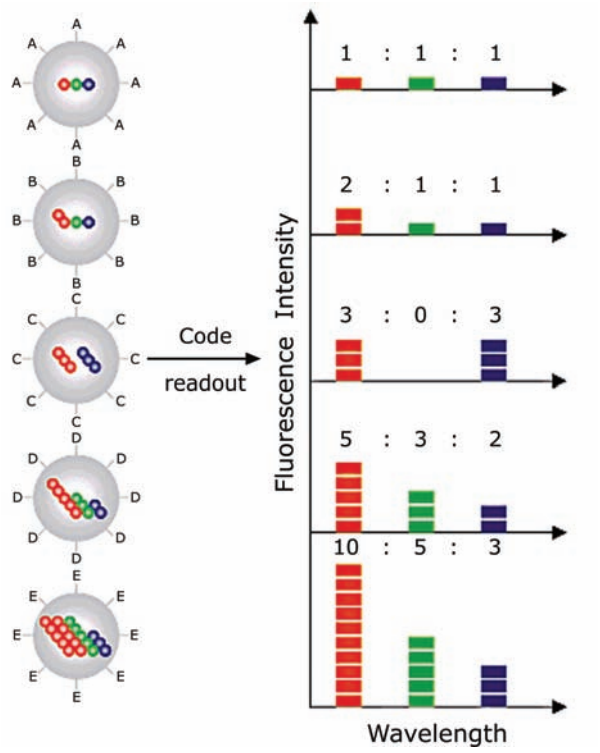

Figure 21.46. Optical coding of microbeads based on emission intensity and wavelengths. From [147].

on a glass support. These arrays are expensive to produce and the surface-localized molecules require long times to reach equilibrium binding. An alternative for multiplex assays is the use of optically coded beads or suspension arrays.[143–147] This approach is based on beads with unique optical signatures. The surface of each bead contains molecules that bind to a single analyte or single DNA oligomer.

The concept of a suspension array is shown in Figure 21.46. In this example the polymer beads contain varying amounts of semiconductor nanoparticles or quantum dots (QDs) with different emission wavelengths. QDs are described in Chapter 20. If it is possible to distinguish ten different intensity levels and six wavelengths then one million unique codes can be created. In practice the number of detectable unique codes is likely to be less. Suspension arrays can also be created using different types of fluorophores.[146] However, the width of emission spectra usually limits the number of unique wavelengths. Quantum dots are well suited for multiplex assays.[148] The narrow emission spectra allows a reasonable number of different emission wavelengths, as is shown by a photograph of QD suspensions illuminated with a UV lamp (Figure 21.47). The photostability of QDs is also important for a multiplex assays because the relative intensities as well as the wavelengths are used to identify the beads.

Figure 21.47. Real-color photograph of ZnS-capped CdSe quantum dots excited with a near-UV lamp. From [148].

Suspension arrays can be used to rapidly detect the presence of DNA sequences in a mixture.[148] This is accomplished by attaching a different capture oligomer to each type of bead (Figure 21.48). The beads are mixed with the DNA sample. Each type of bead and hence each sequence is identified by its emission spectrum. If a labeled oligomer binds to a particular bead then an additional emission peak is seen from this bead. The mixture of oligomers can all be labeled with the same fluorophore because the bead identifies the sequence and the fluorophore emission indicates the presence or absence of the sequence in the sample.

Figure 21.49 shows emission spectra of several types of microbeads. The top panels show the bead with a 1:1:1 intensity ratio before and after incubation with the target sequence labeled with Cascade Blue. Emission from the target sequence is seen near 440 nm. The lower panels show detection of different target sequences with different microbeads. In practice it would be necessary to collect such data from a larger number of beads. This can be accomplished using flow analysis similar to that used in flow cytometry or DNA fragment size analysis (Section 21.2.3).

21.7. FLUORESCENCE IN-SITU HYBRIDIZATION

Fluorescence in-situ hybridization (FISH) is a widely used method in cell biology, medical testing, and genomics.[149–152] The concept of FISH is shown in Figure 21.50. The DNA to be tested, typically metaphase chromosomes, is exposed to fluorescently labeled probe DNA. The exposure conditions result in denaturation of the chromosomes and hybridization of the chromosomes with the probe DNA. The probe DNA has a base sequence directed toward one or

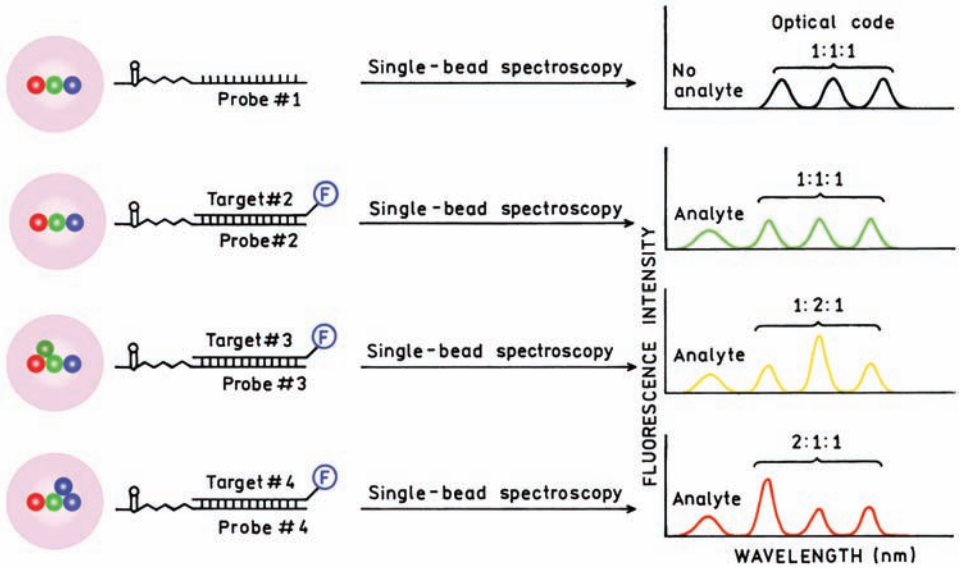

Figure 21.48. Schematic of a DNA hybridization array using QD-labeled microbeads. From [148].

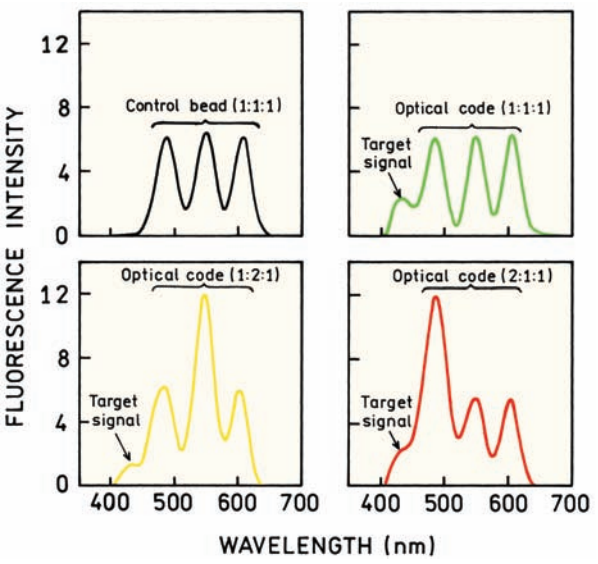

Figure 21.49. Emission spectra of a single type of microbead after equilibration with its target sequence. The target oligomer was labeled with Cascade Blue emitting near 440 nm using biotin-streptavidin chemistry. From [148].

more chromosomes. The probe DNA is labeled with one or more fluorophores. Following exposure to the probe DNA, one or more of the chromosomes become fluorescent. Alternatively, the probe DNA can be specific for the centromeric or telomeric region of chromosomes, in which case only the ends of the chromosomes become fluorescent.

DNA probes can also be specific for small regions of DNA representing one or several genes. FISH can also be used with dispersed DNA in interphase cells, typically to detect individual genes. When first introduced in-situ hybridization was performed using radioactive traces and radiography. At present in-situ hybridization is performed almost exclusively using fluorescence.

21.7.1. Preparation of FISH Probe DNA

Preparation of probe DNA to identify individual genes is relatively straightforward. A DNA oligomer with the gene sequence and appropriate primer sequences is synthesized. This task was aided by completion of the human genome in

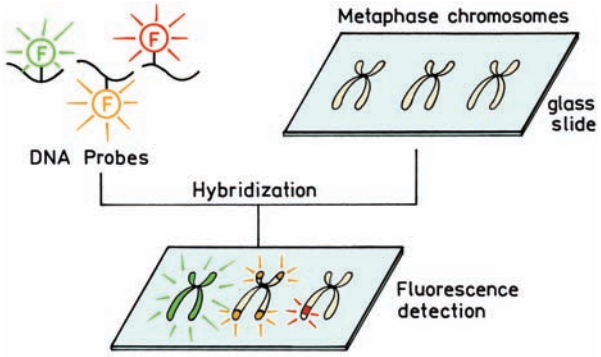

Figure 21.50. Schematic of fluorescence in-situ hybridization (FISH).

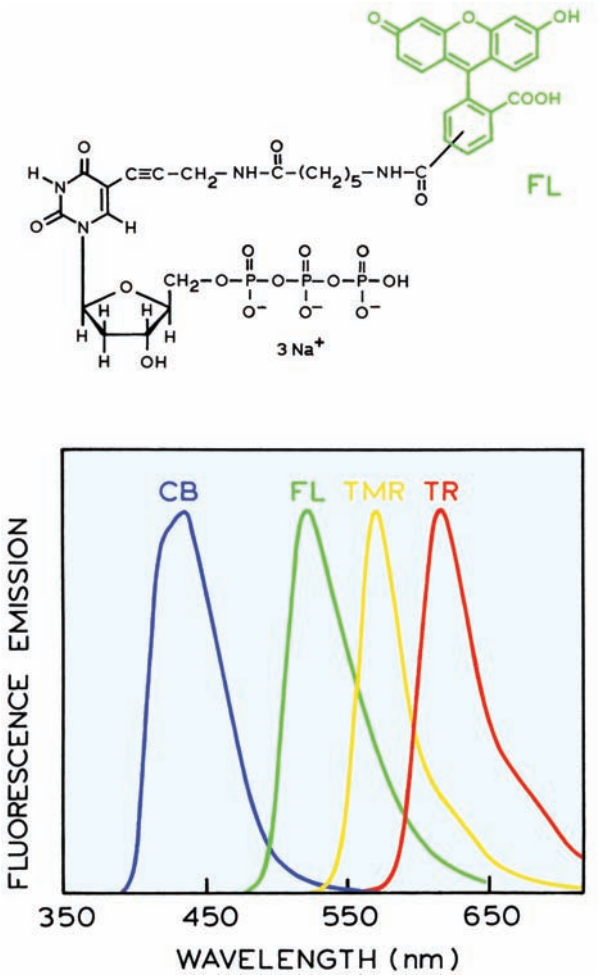

Figure 21.51. Emission spectra of fluorescent deoxynucleotide for incorporation into DNA FISH probes. The fluorophore can be fluorescein (FL), or one of a variety of other fluorophores such as tetramethyl rhodamine (TMR), Texas red (TR), or cascade blue (CB). From Molecular Probes literature.

2001. The DNA can be amplified using DNA polymerase and/or PCR. Fortunately, it is possible to incorporate a high density of fluorophores into the probe DNA. This is accomplished using labeled deoxynucleotide triphosphates (dNTPs). Several labeled dNTPs are shown in Figure 21.51. Note that these labeled bases contain the 3' hydroxyl group so that the DNA strand can be continued, in contrast to the ddNTPs shown in Figure 21.1. These nucleotides can be incorporated at reasonable densities without interfering with hybridization or base pairing. A wide variety of fluorophores can be used. The highest sensitivity has typically been found using rhodamines,[152] which are more photostable than fluorescein. These probes can be used to identify regions of a chromosome or the location of a gene within a chromosome (Figure 21.50).

More complex procedures are needed to prepare probe DNA to entirely label or paint a selected chromosome. This requires that the probe DNA contains a large number of different sequences so that the entire chromosome is labeled. At the same time the probe DNA cannot contain sequences that bind to the other chromosomes. This task is made more complex by the presence of repetitive sequences that are present in all the chromosomes. The preparation of probe DNA to paint individual chromosomes starts with isolation of the individual chromosomes using flow cytometry. The chromosomal DNA is then amplified using PCR in the presence of the labeled nucleotide. The DNA can also be labeled using nick-translation. In this procedure the DNA is incubated with DNAase I, DNA polymerase, as well as labeled and unlabeled nucleotides. The enzymes partially cleave the DNA, remove nucleotides, and replace the nucleotides with labeled nucleotides from the reaction mixture. This procedure also reduces the average size of the DNA fragments, which improves the rate of hybridization. Because of the repetitive sequences that appear in all the chromosomes, the probe DNA described above will bind to these regions in all the chromosomes. These sequences are removed by incubation with a competitor DNA sample that contains these sequences, forming double-helical DNA that contains the unwanted sequences. An excess amount of the competitive DNA is used to prevent binding of these sequences to the chromosomes.

FISH is usually performed on fixed cells on microscope slides. Prior to fixation the cells can be trapped in the metaphase by colchicine, which interferes with mitosis. Following fixation unwanted cellular components are removed with enzymes and/or solvents. Considerable experimentation and testing is needed to identify the detailed treatments needed to prepare useful samples.[150] The fixed preparations are then incubated with various solvents and at elevated temperatures to allow the probe DNA to hybridize with the chromosomes.

21.7.2. Applications of FISH

FISH has numerous applications in cell biology and genetic testing.[153–154] Some examples include studies of gene expression,[155] detection of the gene for Duchenne muscular dystrophy,[156] detection of the human papillomavirus thought responsible for cervical cancer,[157] and fetal sex determination from amniotic fluid.[158] The power of FISH

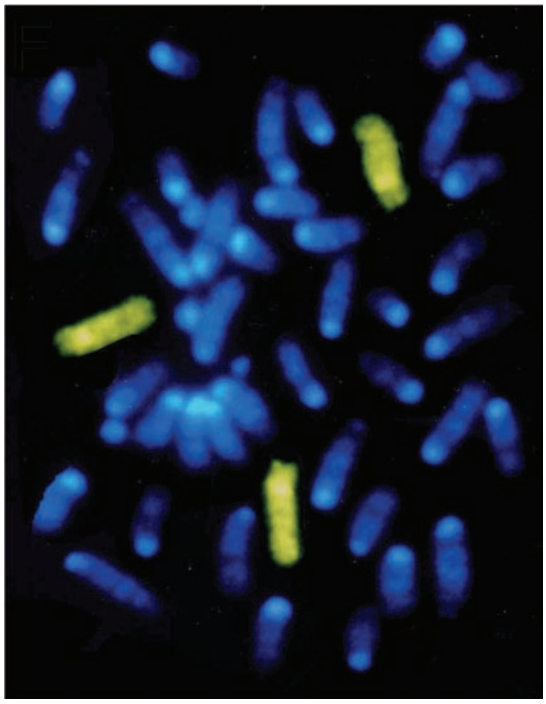

Figure 21.52. FISH of a human-hamster hybrid cell. All the chromosomes are stained uniformly using a nonspecific probe like DAPI. Three copies of human chromosome 4 were identified by a probe (yellow) specific for this chromosome. From [150]. Courtesy of Dr. Thomas Ried from the Center for Cancer Research (NCI/NIH).

can be illustrated by several examples. Figure 21.52 shows metaphase chromosomes from a human-hamster hybrid cell line.[150] All the chromosomes were stained with a nonspecific probe with blue emission. The chromosomes were exposed to a FISH probe specific for human chromosome 4. The yellow emission from this probe shows the cell contains three copies of this chromosome.

FISH can be used to locate individual genes instead of painting entire chromosomes (Figure 21.53). Methaphase mouse tumor chromosomes were stained with DNA probes specific for the immunoglobulin heavy-chain locus (red) or for the c-myc gene (green). In this case only small regions of the chromosome are labeled.[150] The interphase nucleus in the lower right released some of its DNA during sample preparation. FISH can also be applied to interphase nuclei when the DNA is not condensed into chromosomes. An interphase cell was stained with three gene-specific probes, one with green emission and two with red emission (Figure 21.54). The image shows that the gene labeled with the green probe is localized between the two other genes.

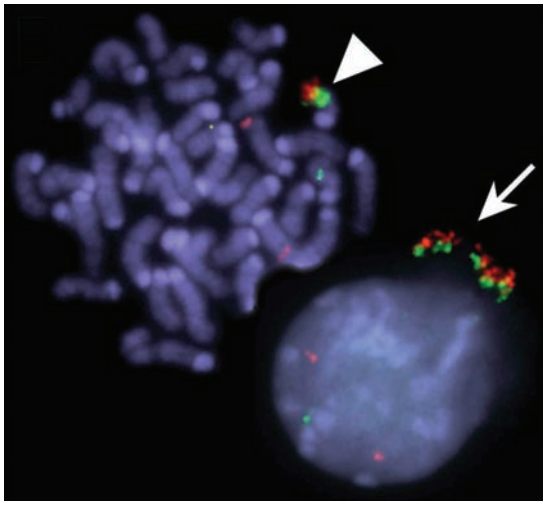

Figure 21.53. Mouse tumor metaphase chromosomes stained for the immunoglobulin heavy chain locus (red) and the c-myc gene (green). All the DNA was stained with a nonspecific blue fluorophore. Image courtesy of Dr. Thomas Ried from the Center for Cancer Research (NCI/NIH).

21.8. MULTICOLOR FISH AND SPECTRAL KARYOTYPING

Suppose it is necessary to identify each of the chromosomes by staining with FISH probes. Because of the width of the emission from most fluorophores it is not possible to visually identify more than about five fluorophores. Identifica-

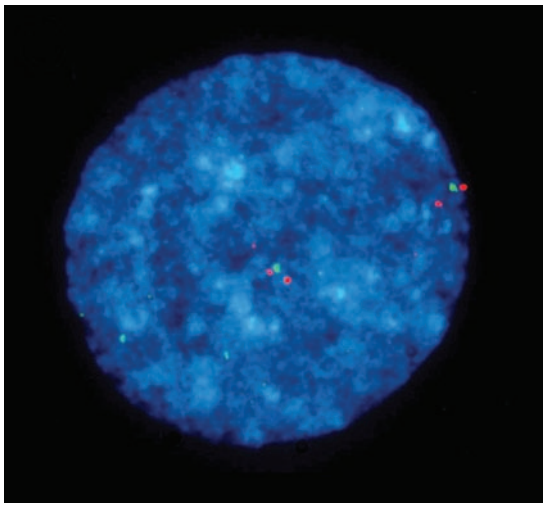

Figure 21.54. Labeling of an interphase nucleus with three gene-specific probes. Images courtesy of Dr. Thomas Ried from the Center for Cancer Research (NCI/NIH).

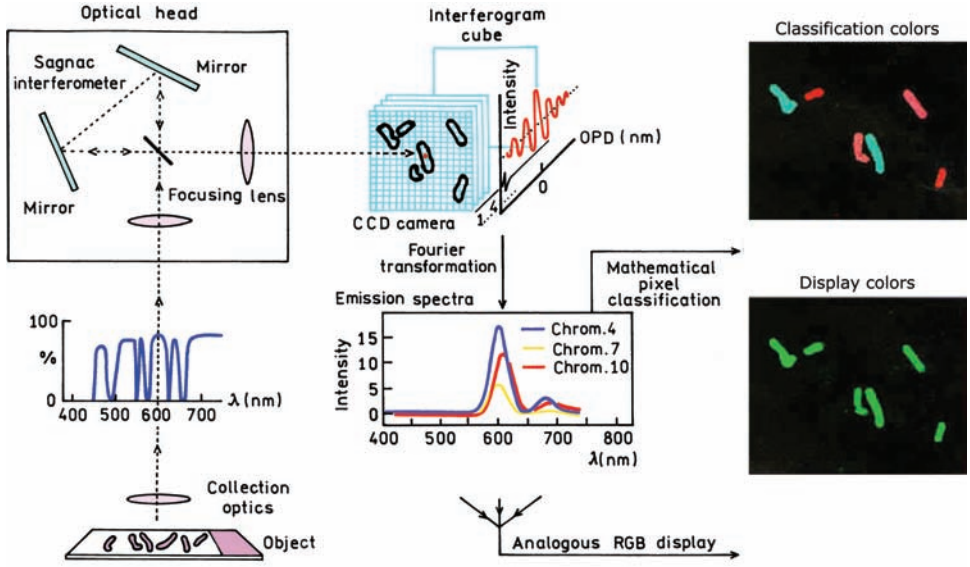

Figure 21.55. Principle of spectral imaging and karyotyping. The wavelength distribution of the emission is determined with an interferometer. The results can be displayed as true colors (display color) or pseudocolor (clarification color). From [165].

tion of all 24 chromosomes could be accomplished by sequential staining with different DNA probes, but this is impractical. This problem of chromosome identification was solved using mixtures of fluorophores in each DNA probe. The fluorophore mixtures are designed so that the emission spectrum of each mixture can be uniquely identified and assigned a pseudocolor. The DNA probe is now a complex mixture containing different sequences to label the entire chromosome and different fluorophore ratios to yield 24 individually identifiable emission spectra. The fluorophores are usually attached to the DNA in two different ways. Some fluorophores are attached directly, as shown in Figure 21.51. Some fluorophores are attached indirectly using protein linkers. This is accomplished using biotin or digoxigenin-labeled nucleotides. These nucleotides are then labeled with avidin or antibodies that have covalently linked fluorophores.

Two approaches are used to record the spectral information, multicolor FISH (m-FISH),[159–161] and spectral karyotyping (SKY).[162–165] In m-FISH the emission is imaged through several filters. These images are used to identify the mixture which stained each chromosome. In SKY the emission is imaged through an interferometer that is scanned to obtain the spectral information (Figure 21.55). The value of spectral labeling of the chromosomes is shown by the images on the right side of Figure 21.55. The display colors, which approximate the true colors, are essentially the same for all six chromosomes. However, three of the chromosomes are labeled with Cy3 and three with Texas Red. Use of the emission spectra allowed the identity of the chromosomes to be determined, and each type was assigned a different pseudocolor.

Spectral karyotyping has been extended to allow for identification of all 24 chromosomes (Figure 21.56). The display colors approximate the visual appearance of the painted chromosomes. The spectral distribution is used to identify each chromosome based on the known spectral distribution of the painting probes.[165] Each spectral distribution is assigned a pseudocolor that allows visual discrimination. The chromosomes can then be easily paired and analyzed.

Spectral karyotyping and m-FISH provide an approach to the study of abnormal cells.[162] Figure 21.57 shows a chromosomal image from an ovarian cancer. The image on the left is an inverted DAPI image, which is used because it approximates the Giemsa stains familiar to cell biologists. The middle panel is an RGB image created from separate images taken through three different emission filters which is intended to represent the visual image. The device shown in Figure 21.55 was used to identify the spectral signatures. The SKY images show that there have been many chromosomal rearrangements, which can be seen from chromosomes that are assigned multiple pseudocolors. SKY and m-FISH provide a means to detect chromosome abnormal-

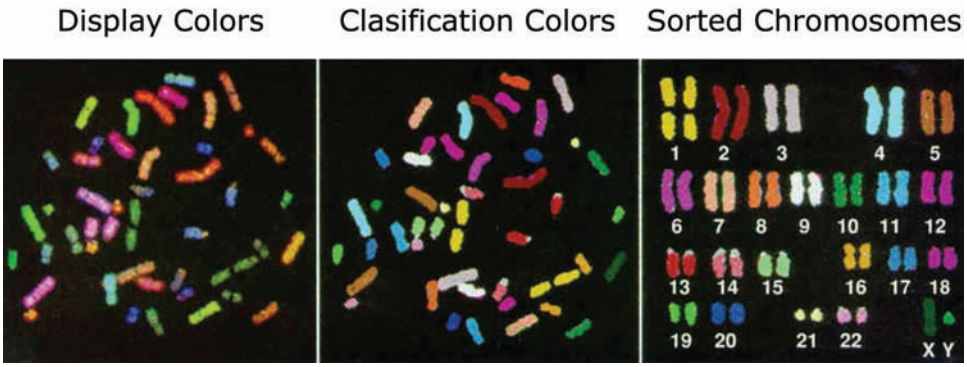

Figure 21.56. Spectral karyotyping of 24 human chromosomes using 24 pointing probes. From [165].

ities and rearrangements, as well as monitoring bone marrow cells following transplantation and cancer therapy.[166–172] The use of FISH relies on methods for labeling DNA, computerized imaging, and high, sensitivity CCD detection. FISH technology represents a combination of modern optics, molecular biology, and fluorescence spectroscopy, and promises to become a central tool in molecular medicine.

21.9. DNA ARRAYS

DNA arrays provide a method for parallel high-throughput analysis of gene expression. This capability has resulted in a paradigm shift in biological research. Traditional experiments in gene expression studied one or a few genes in an organism. Presently it is possible to simultaneously study the expression of thousands of genes in a single experiment.[173–180]

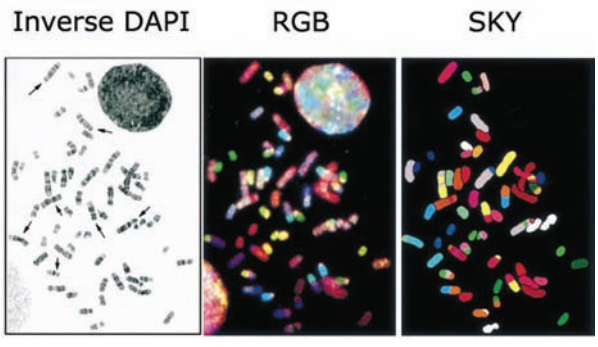

Figure 21.57. Chromosome images of an ovarian carcinoma. Left, inverted DAPI image. Middle, RGB image. Right, SKY classification image. From [162].

DNA arrays consist of regular arrays of DNA fragments or oligomers on a solid support, usually glass microscope slides. These slides can contain more than 20,000 different sequences or more in only a few square centimeters of area. There are two general methods to prepare arrays, by mechanical spotting of DNA solutions on slides or by light-generated arrays. Spotted arrays are now being produced in individual laboratories and in core facilities. Light-generated arrays are more expensive to produce and are usually manufactured commercially.

21.9.1. Spotted DNA Microarrays

Preparation of a DNA array is somewhat expensive and complex (Figure 21.58). DNA clones are prepared by one of several available methods.[180] Usually mRNA is isolated from the desired sample and used to create cDNA using reverse transcriptase. The use of mRNA or cDNA results in DNA fragments that represent the expressed genes. The use of mRNA or cDNA is generally preferable to using the entire genome, which contains many regions that are repetitive or not converted into gene products. The DNA clones are then spotted onto microscope slides. Prior to spotting, the slides are treated with polylysine or an aminosilane reagent to cover the surface with positive charges, which results in DNA binding to the surface. After drying, the slides are illuminated with UV light, which probably crosslinks the DNA to the surface. The surface is then treated with succinic anhydride to remove the positive charges on the surface, which would result in nonspecific binding. Spotting of the slides is accomplished using automated instruments designed for this purpose.[181] Spotting is usually done using small capillaries that make contact with the

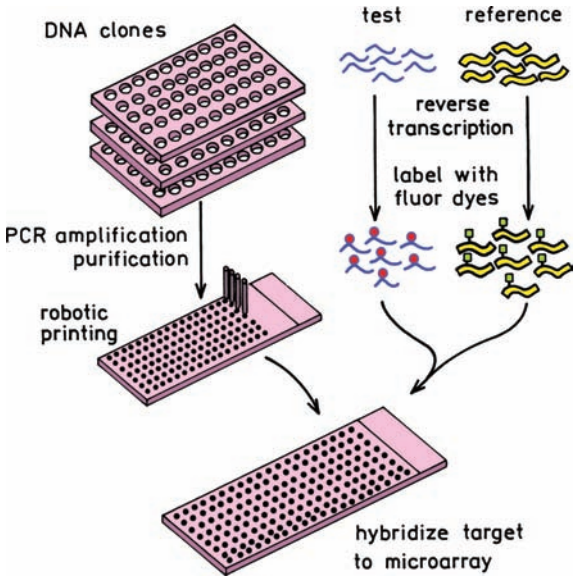

Figure 21.58. Use of DNA arrays for gene expression profiling. From [178].

slide (Figure 21.59). The microarrays can also be spotted using ink jet or bubble jet technology.[182–184]

DNA microarrays with a modest number of spots can be used for specific diagnostic tests or bioassays. Microarrays with a larger number of surface-bound oligomers are often used to measure profiles of gene expression.[185] A schematic of these experiments is shown in Figure 21.60. Messenger RNA is isolated from two samples that originate with the same organism or cell line, but which have been treated differently. One sample serves as the control. The other sample is stimulated to divide or is treated in a way that affects the cell. The mRNA is isolated from both samples and converted to cDNA. During synthesis the cDNA is labeled using fluorescent oligonucleotides, typically containing Cy3 and Cy5. These two samples of cDNA are then coated over all the spots and allowed to hybridize. The concentrations of cDNA are adjusted so that the amounts are less than that bound to the surface. Under these conditions the amount of labeled cDNA bound is roughly proportional to the amount in the samples.

The relative level of each cDNA is determined from the relative intensities of the two fluorophores on each spot of the array. Figure 21.61 shows a portion of an array. The color image is usually constructed from the relative intensities of the green Cy3 emission and the red Cy5 emission. In this experiment the CDKN1A gene is overexpressed in the sample relative to the control, and the MYC gene is underexpressed. These expression levels are seen from the red spot for CDKN1A and a green spot for MYC, or from the intensity traces for this row of spots (lower panel). The other spots are yellow, which indicates that the expression levels of these genes are the same in the control and the sample.

DNA arrays can contain large numbers of genes. The array in Figure 21.62 contains more than 9000 genes from the plant *Arabidopsis*. The color of each spot indicates the relative expression level of each gene. By using such arrays

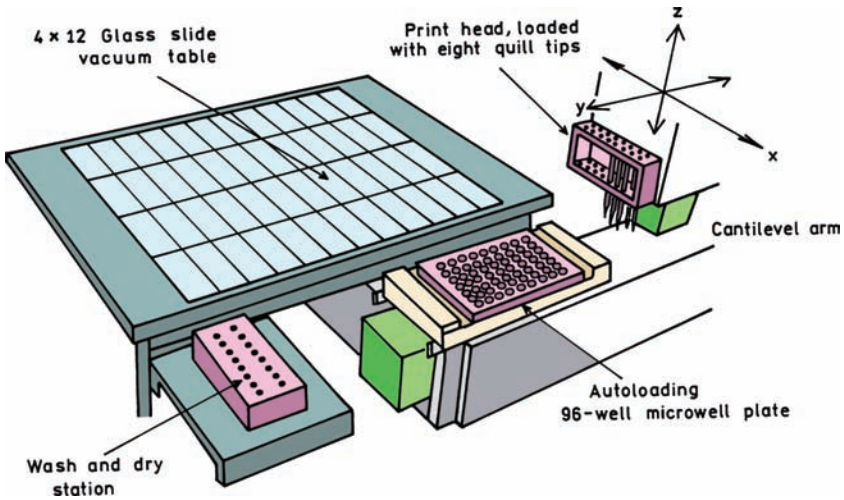

Figure 21.59. Apparatus for making spotted DNA microarrays. From [175].

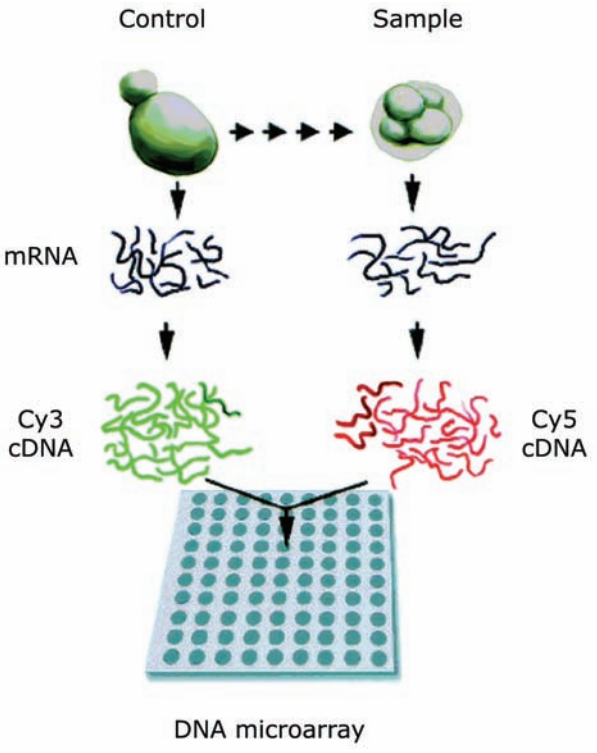

Figure 21.60. Analysis of gene expression using a spotted DNA microarray. Revised from [185].

it is possible to study how families of genes are activated in response to stimuli or at different phases of the growth cycle.

21.9.2. Light-Generated DNA Arrays

DNA arrays can also be made using a combination of photochemistry and photolithography.[187–189] This approach is shown in Figure 21.63. The surface is coated with a protected hydroxyl groups. Regions of the surface are deprotected by light and coupled to a nucleotide. Another region of the surface is then deprotected and another nucleotide is added. This process is repeated until the oligomers are 15 to 25 bases long. Up to 300,000 oligonucleotides can be synthesized and located in a 1.28 x 1.28 cm array. Typically a single gene is represented by about 20 oligomers. These light-generated arrays are manufactured by Affymetrix Inc. Gene chips are available for a number of organisms. It appears that the expression levels are determined by intensities at a single wavelength rather than by intensity ratios for different wavelengths. Light-generated arrays can also be made using micromirror arrays or digital light processors.[190–191]

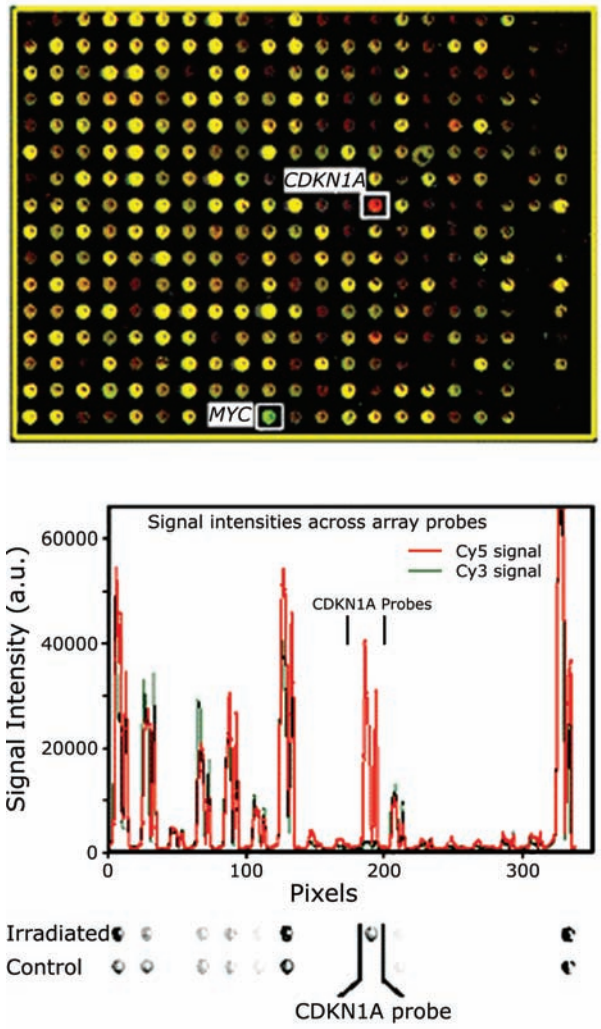

Figure 21.61. Color image of portion of a DNA array. The lower panel shows the intensity profiles of Cy3 (green) and Cy5 (red) across one row of the array. From [178].

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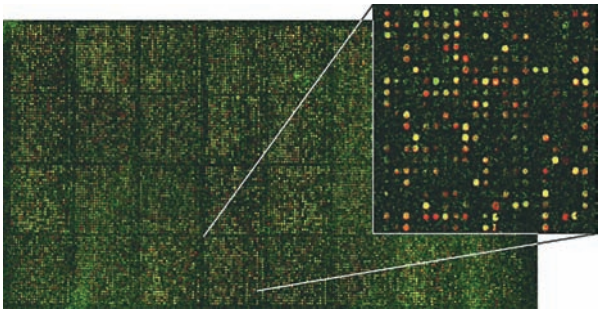

Figure 21.62. Gene expression by *Arabidopsis* plants using an 18,000-element DNA microarray. The control DNA was labeled with Cy5 and the sample was labeled with Cy3. From [186].

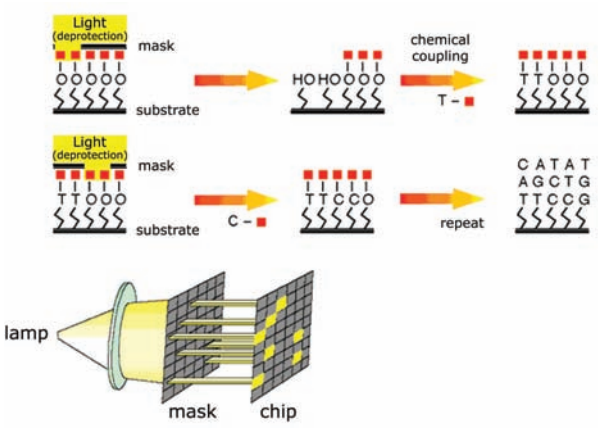

Figure 21.63. Light-directed synthesis of oligonucleotide arrays. From [189].

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PROBLEM

P21.1. *Spectra Observables Useful for DNA Sequencing*: Intensity, intensity-ratio, and lifetime measurements have been used for DNA sequencing. Suggest reasons why anisotropy and collisional quenching have not been used for DNA sequencing.