CHAPTER FOUR

Fluorescence Technologies for Monitoring Interactions Between Biological Molecules \textit{In Vitro}

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Abstract

Over the last two centuries, the discovery and understanding of the principle of fluorescence have provided new means of characterizing physical/biological/chemical processes in a noninvasive manner. Fluorescence spectroscopy has become one of the most powerful and widely applied methods in the life sciences, from fundamental research to clinical applications. \textit{In vitro}, fluorescence approaches offer the potential to sense in real-time extra and intracellular molecular interactions and enzymatic reactions, which constitutes a major advantage over other approaches to the study of biomolecular interactions. This technology has been used for the characterization of protein/
protein, protein/nucleic acid, protein/substrate, and biomembrane/biomolecule interactions, which play crucial roles in the regulation of cellular pathways. This chapter reviews the different fluorescence strategies that have been developed for sensing molecular interactions in vitro at both steady- and pre-steady-state levels.

**ABBREVIATIONS**

ANS 1-anilinonaphthalene-8-sulfonate  
AP5A \(\alpha,\gamma\)-di[adenosine-5’] pentaphosphate  
CPP cell-penetrating peptide  
FRET fluorescence resonance energy transfer or Förster resonance energy transfer  
GFP green fluorescent protein  
GUV giant unilamellar vesicles  
LUV large unilamellar vesicles  
Mant methylanthranylate  
NBD nitrobenzofurazan  
RT reverse transcriptase  
SUV small unilamellar vesicles

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**1. INTRODUCTION TO FLUORESCENCE**

Over more than a century, fluorescence methodologies have undergone tremendous development, from physical and biological to clinical applications. Indeed, since the first observations of fluorescence reported by Sir John Frederick William Herschel in 1845 from a quinine solution under sunlight, the phenomenon of fluorescence has been continuously investigated. In 1852, Sir George G. Stokes proposed the term “fluorescence” in honor of the blue-white fluorescent mineral fluorite/fluorspar. Nowadays, fluorescence spectroscopy has become one of the most widely applied technologies in the study of biological processes.

**1.1. Fluorescence principle**

The simplest definition of fluorescence could be formulated as the emission of light by a substance that has absorbed light or electromagnetic radiation. Today, fluorescence might be defined as a luminescent process in which sensitive molecules emit light from electronically excited states induced by a physical (absorption of light), mechanical (friction), or chemical mechanism. On the basis of Herschel’s and Stokes’ observations, Jablonski was the first to give a clear and scientific explanation of this phenomenon. The Jablonski diagram indicates the relationship between the ground state \((S_0)\), the excited
singlet states ($S_1$, $S_2$), and the excited triplet state ($T_1$), and the resulting fluorescence and phosphorescence emission (Fig. 4.1).

Most aromatic molecules with delocalized electrons are theoretically able to undergo luminescence and fluorescence phenomena. They can be associated to different sources in biological molecules, from natural intrinsic fluorescent probes (tryptophan or natural fluorescent protein; GFP, RFP, etc.) to small synthetic chemical dyes (Cyanine, Alexa, Atto, etc.). Fluorescence technology constitutes an ideal noninvasive approach to monitor and characterize in detail specific interactions between biological molecules. A large number of interactions can be investigated at both steady-state and kinetic levels using either intrinsic or extrinsic fluorescence probes. Depending on the type of interaction and the context, several fluorescence-based methods are available, including solvatochromism, anisotropy, and fluorescence resonance energy transfer (FRET) (Fig. 4.2). This chapter focuses on the different applications of fluorescence technology to monitor specific events in biology for both fundamental and mechanistic issues.

1.2. Solvatochromism and resonance energy transfer

1.2.1 Solvatochromism

Most fluorescent molecules can be considered environmentally sensitive probes since there are several environmental parameters that can affect their fluorescent properties. These environmental factors include the solvent,
inorganic and organic compounds, temperature, pH, and the concentration of the fluorescent molecules. The effects of these parameters vary widely from one fluorophore to another. In particular, the absorption and emission spectra, as well as the quantum yield, can be affected by the polarity of the environment. This phenomenon, more generally known as “solvatochromism,” can be defined as the ability of a fluorescent molecule to undergo changes in position and/or intensity of absorption or emission bands according to solvent polarity variation.\(^5\) The solvatochromic effect, hypsochromic (blue) or bathochromic (red) shifts, refers to a strong dependence of the absorption and emission spectra on solvent polarity (Fig. 4.3). Since the polarities of the ground and excited states of a fluorophore are different, a change in solvent polarity will induce different stabilization of the ground and excited states and thus a modification of the energy between these electronic states.\(^5\) Consequently, variations in the position, intensity, and shape of the absorption and emission spectra can be used as a direct indicator of solvent changes and local environment modification.

In proteins, although the aromatic residues Trp, Phe, and Tyr can potentially be used as fluorescent probes, Trp is by far the most used intrinsic probe. Tryptophan is an important intrinsic solvatochromic probe that can be useful as a natural sensor of the conformational state of a protein or for assessment of the nature of its environment.\(^6\)–\(^9\) The indole group of the side chain confers maximum absorbance near 280 nm and maximum emission intensity located between 310 and 350 nm on the tryptophan residue depending on the environment. The use of denaturants, surfactants, or
other amphiphilic molecules generally induces changes in the environment, providing information on the surrounding of tryptophan and thereby of proteins.\textsuperscript{10} If a globular protein containing several tryptophans in its “hydrophobic” core is denatured, the environment of tryptophans is modified and a shift of the emission spectrum maximum to a longer wavelength (bathochromic/red shift) is observed. This effect arises from the exposure of tryptophan to an aqueous environment as opposed to a hydrophobic protein core. In contrast, the addition of a surfactant such as phospholipid vesicles to a protein that contains a tryptophan exposed to the aqueous solvent will induce a shift of the emission spectrum maximum to a shorter wavelength (hypsochromic/blue shift) if the tryptophan becomes embedded in the phospholipid vesicle. Thus the transfer of tryptophan residues from a polar to a less polar environment such as the membrane bilayer is usually associated with a blue shift. In addition, this blue shift may be accompanied by an increase in the quantum yield of tryptophan (Fig. 4.3).

An alternative approach to the intrinsic fluorescence of tryptophan is the covalent attachment of an extrinsic fluorophore to a single site on the target biomolecule. Indeed, there are a large number of fluorescent probes with photochemical properties that are more attractive than tryptophan and which are also sensitive to environmental changes.\textsuperscript{5,11} By conjugating these to proteins, nucleic acids, or lipids, the sensitivity and quality of information provided by fluorescence spectrometry can be clearly improved. Synthesized for the first time in 1871 by Adolf von Baeyer,
fluorescein was one of the first environment-sensitive probes, exhibiting pH-dependent excitation and emission wavelengths.\textsuperscript{12,13} Although fluorescein and its chemical analogs are still used to label specific sites of target biomolecules, other probes have been developed with improved fluorescence stability, quantum yield, or solvatochromism. For example, Prodan, NBD (nitrobenzofurazan), Coumarin, Nile red, and their derivatives constitute well-known environment-sensitive dyes that are usually applied to sense changes in the environment proximal to the target.\textsuperscript{11}

1.2.2 Resonance energy transfer
At the beginning of the twentieth century, Jean Perrin was the first scientist to consider the interaction through space between molecules.\textsuperscript{14} He proposed that the excitation energy is transferred from one molecule to another through interactions between oscillating dipoles of closely spaced molecules. Based on this observation, Theodore Förster developed the theoretical basis of resonance energy transfer, that is, the “Förster resonance energy transfer” or FRET.\textsuperscript{15,16} FRET generally occurs between a donor (D) molecule in the excited state and an acceptor (A) molecule in the ground state (Fig. 4.4A). The donor molecules typically emit at shorter wavelengths that overlap with the absorption spectrum of the acceptor (Fig. 4.4B). Energy transfer occurs without the appearance of a photon and is the result of long-range dipole–dipole interactions between the donor and the acceptor. When both molecules are fluorescent, the term “fluorescence resonance energy transfer” is used instead, although the energy is not transferred by fluorescence.\textsuperscript{17} The rate of energy transfer depends on the spectral overlap of the emission spectrum of the donor with the excitation spectrum of the acceptor, the quantum yield of the donor, the relative orientation of the donor and acceptor transition dipoles, and the distance between the donor and acceptor molecules. The distance dependence of FRET allows for measurement of the distances between donors and acceptors.\textsuperscript{17} Indeed, when the energy transfer efficiency ($E$) is measured, the distance ($r$) between the two fluorophores can be calculated, according the equation

$$E = \frac{R_0^6}{R_0^6 + r^6}$$

where $R_0$ is the so-called Förster distance at which the efficiency of transfer equals 50%. This latter point is attractive with regard to the identification of molecular interactions \textit{in vitro} or in a biological context. Thus the choice of a
specific acceptor/donor couple to associate with interacting partners can constitute a powerful tool to demonstrate both dynamic and in situ interactions as well as conformational changes.

1.2.3 **Fluorescence polarization**

Fluorescence Polarization was first described in 1926 by Perrin. This specific type of polarization is based on the observation that fluorescent molecules in solution, excited with plane-polarized light, will emit light back in a fixed plane if the molecules remain stationary during the excitation of the fluorophore; the emitted light remains “polarized.” However, molecules rotate and tumble, and the planes in which light is emitted can be very different from the plane used for initial excitation; the emitted light
is then “depolarized” (Fig. 4.5). With regard to intermolecular interactions, if a molecule is bound by a larger ligand, its effective molecular volume is increased and its rotation is slowed so that the emitted light is in the same plane as the plane-polarized light of excitation (Fig. 4.5). In this case, both the bound and unbound states of the molecule have an intrinsic polarization value: a high (polarized) value for the bound state and a low (depolarized) value for the unbound state. Fluorescence polarization is a weighted average of the two values, providing a direct evaluation of the fraction of molecule/ligand binding. Thus fluorescence polarization measurements are also indicative of the formation of larger molecule/ligand complexes.

Fluorescence anisotropy is usually determined by the measurements of fluorescence emission in parallel and perpendicular planes. The degree of polarization ($p$) or anisotropy ($r$) is calculated according to the following equations:

$$p = \frac{I_\parallel - L_T}{I_\parallel + L_T} \quad \text{and} \quad r = \frac{I_\parallel - L_T}{I_\parallel + 2L_T}$$

where $I_\parallel$ is the fluorescence emission measured in the plane parallel to the plane of excitation and $L_T$ is the fluorescence emission measured in the plane perpendicular to the plane of excitation. Anisotropy and fluorescence polarization approaches can be applied to study different biomolecular interactions.
1.3. Fluorescence for biological molecules

Over the last two decades, fluorescence spectroscopy technologies have been extensively applied to understand specific molecular interactions in different biological and biochemical pathways, including monitoring major cellular events and enzyme mechanisms and sensing cellular response leading to aberrant behavior. Fluorescence approaches allow the characterization in a non-invasive manner, at the steady- and pre-steady-state levels of protein/protein, protein/nucleic acid, protein/substrate, and biomembrane/biomolecule interactions as well as enzymatic assays or competitive immunoassays, which constitutes a major advantage over other methods used for this purpose. Although fluorescence spectroscopy technologies provide valuable answers to numerous questions concerning the identity, location, conformation, and environment of any protein or nucleic acid, the sensitivity of the technologies also requires taking into account the limitations of the approach and keeping in mind that every small change in a parameter may induce variations. Thus each specific approach has several advantages and limitations (Table 4.1).

<table>
<thead>
<tr>
<th>Technique</th>
<th>Advantages</th>
<th>Disadvantages and limitations</th>
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<tr>
<td>Fluorescence intensity</td>
<td>- Simple</td>
<td>- Little information for quality control</td>
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<td></td>
<td>- Suitable for fluorogenic assays</td>
<td>- Sensitive to inner-filter and autofluorescence interference</td>
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<td></td>
<td>- Readily miniaturized</td>
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<td>Fluorescence polarization/</td>
<td>- Simple and reasonably predictive</td>
<td>- Local motion effects</td>
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<td>anisotropy</td>
<td>- Insensitive to inner-filter effects</td>
<td>- Suitability limited by lifetime of dye, ligand size, and molecular weight change</td>
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<td></td>
<td>- Ratiometric technique</td>
<td>- Dynamic range limited</td>
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<td></td>
<td>- Improved well-level quality control</td>
<td>- Can suffer from autofluorescence</td>
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<td></td>
<td>- Suitable for small ligands (≤15 kDa)</td>
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<td>Fluorescence resonance</td>
<td>- Simple and reasonable predictable</td>
<td>- Requires multiple labels</td>
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<td>energy transfer (FRET)</td>
<td>- Suitable for short inter/intramolecular distances (≤5 nm)</td>
<td>- Sensitive to inner-filter and autofluorescence interference</td>
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<tr>
<td></td>
<td>- Range of available donors and acceptors</td>
<td>- Limited to short distances to obtain high signal changes</td>
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<td></td>
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<td>- Most dyes monitor only donor quenching</td>
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2. MONITORING PROTEIN/SUBSTRATE INTERACTIONS

Protein/partner interactions, such as enzyme/substrate, protein/nucleic acid, and protein/nucleotide, play a crucial role in the regulation of biological pathways at the extracellular, cellular, viral, or drug delivery level. Development of fluorescence technologies using both natural and synthetic probes has provided new and appropriate tools to decipher the nature, strength, and impact on the life cycle of a wide range of molecular interactions. Protein/ligand complexes can be probed by intrinsic tryptophan fluorescence at both steady-state and pre-steady-state levels. However, most proteins contain more than one tryptophan residue, which constitutes a limitation for the analysis of the specific interaction of one domain of the protein. Therefore, in most cases, extrinsic fluorescence constitutes an attractive alternative as a large panel of fluorescent dyes with well-suited spectral properties and more appropriate quantum yields for sensitive detection and single-molecule assays.

2.1. Protein/nucleotide interactions

Nowadays, fluorescent- or caged-nucleotide analogs are widely used as probes for enzyme activities and as sensors for screening of inhibitors and/or activators. Nucleotide chemistry has focused on the design of specific fluorescently labeled nucleotides to investigate either enzymatic parameters or interactions with partners (activator, inhibitor, etc.) of nucleotide binding proteins, such as nucleotide kinases, protein kinases, GTPases, ATPases, and so on. Nucleotides can be modified on their base (benzo-, etheno-), on the phosphate (XTP-γ-naphthalene, γ-[(6-amino) hexyl]-, γ-(sulfo-1-naphthyl)amide, methylnaphthylate (Mant)), and on the sugar (trinitrophenyl-, Mant-) moieties, depending on the nature/structure of the nucleotide binding site of the enzyme (Fig. 4.6). New chemistry has also been proposed using the amino hexyl linker, allowing labeling with a large panel of available dyes with the amino group (Fig. 4.6). The binding of nucleotide analogs to proteins leads to large modifications of their extrinsic fluorescence, which can be used as a sensor for the determination of enzymatic parameters as well as for screening of inhibitors or natural substrates. However, it is important to keep in mind that the presence of the dye can modify the binding properties of the nucleotide, and displacement experiments of labeled nucleotides using unlabeled nucleotides are essential to validate the approach. For example,
Figure 4.6 Fluorescently labeled nucleotide analog. (A) Most common dyes used on fluorescently labeled nucleotides on the ribose, the base, and the phosphate moieties. (B) Amino-hexyl-modified nucleotides for custom-made labeling with a large panel of dyes.
the binding of Mant-ATP to the cell cycle protein kinase cyclin–dependent kinase (Cdk2) results in a 2.5–fold increase in fluorescence (Fig. 4.7) and allows for the determination of the dissociation constant at the steady-state level using either hyperbolic or quadratic equations, which take into account the concentration of the enzyme and probe:27,28

\[
F = F_{\text{ini}} - (\Delta F) \left\{ \left( E_t + L + K_d \right) - \left[ \left( E_t + L + K_d \right)^2 - 4E_tL \right]^{1/2} \right\} / 2E_t \tag{4.3}
\]

where \( F \) is the observed relative fluorescence intensity, \( F_{\text{ini}} \) is the fluorescence intensity at the start of the titration, \( \Delta F \) is the variation of the fluorescence intensity between the initial value and at a saturating concentration of substrate, \( E_t \) is the total concentration of enzyme, \( L \) is the total concentration of ligand, and \( K_d \) is the dissociation constant of the enzyme–ligand complex. Bisubstrate nucleotides (Ap5A and Ap4A) have been designed to characterize enzymes harboring multiple nucleotide binding sites. An interesting system was described to measure the affinities of nucleotide kinases (NDP-, ADP-, AMP-, or TMP-kinases) for their substrates and inhibitors, based on a fluorescent analog of the bisubstrate inhibitor diadenosine pentaphosphate (AP5A): \( \alpha,\gamma\text{-di[}3'\text{- or } 2'\text{-}O-(N\text{-methylanthraniloyl)}\text{] adenosine-5'}\text{] pentaphosphate (mAP5Am).}^{29–31} \)
2.2. Protein/nucleic acid interactions

Several technologies have been developed to evaluate protein/nucleic acid interactions.\textsuperscript{32} As for nucleotides, new chemistry has been proposed for labeling of nucleic acids, and a large panel of dyes have been attached to DNA or RNA molecules (Fluorescein, Cyanine, Alexa, ATTO, etc.). One of the major breakthroughs in the design of tools for measuring protein/nucleic acid interactions is the development of chemistry for accurate high-throughput DNA sequencing by a synthetic approach. DNA sequencing by synthesis is based on the extension of a primer hybridized to its target sequence by DNA polymerase with a reversible fluorescent chain terminator 2′-deoxynucleotide.\textsuperscript{33,34} Fluorescently labeled reversible chain terminator nucleotides are stable during the polymerase-mediated extension step, and their structure (geometry and size) and the location of the dye within the 2′-deoxynucleotide moiety do not prevent their recognition by standard DNA polymerases.\textsuperscript{35} Oligonucleotide can be easily labeled at the 5′-position through the introduction of a primary amine (NH\textsubscript{2}) at the 5′-position to functionalize the corresponding terminus of the nucleotide for conjugation with an activated N-Hydroxysuccinimide (NHS) ester or isothiocyanate fluorescent label. Several hydrophobic spacers have been proposed of 3, 6, or 12 methylene (CH\textsubscript{2}) groups between the terminal phosphate and the amino part. Amino-modified nucleotides have been used to incorporate a dye in post-coupling reactions on the base, with the main advantage of providing labeling anywhere in the oligonucleotide sequence without altering the 5′-position for elongation. Finally, modifications on the deoxyribose have also been proposed to add more than one dye anywhere in the sequence or on either terminus after post-coupling reactions between the amine group and an activated label. In most cases, the dye is linked to the deoxyribose via a six-carbon-atom spacer, which reduces steric hindrance (Fig. 4.8). As most of the dyes and probes are largely hydrophobic, the risk of nonspecific association with the protein is present and it is essential to confirm interactions with displacement experiments using unlabeled oligonucleotides and other approaches to confirm the affinity values. As for fluorescently labeled nucleotides, it remains essential to take into account the impact of the probe on the interaction and binding parameters.

Although RNA and DNA polymerases are known to share the same general catalytic mechanism, they are all unique in their structural dynamics and constitute a major challenge for the enzymologist.\textsuperscript{36} RNA and DNA
Figure 4.8 Fluorescently labeled oligonucleotide. Oligonucleotide labeling can be performed by adding an amine group (NH$_2$) for post-coupling reactions with an activated dye. Labeling is performed at the 5' (A) and 3' (B) ends by the introduction of primary amine and variable spacers on the base with different chemistry and linkers (C) and on the deoxyribose, where the dye is attached to the deoxyribose via a six-carbon-atom spacer (D).
polymerases are very dynamic enzymes that need to control simultaneously parameters such as specificity, fidelity, and efficacy together with interactions with different types of substrates and partners. Fluorescently labeled oligonucleotides were used to investigate the mechanism of several DNA or RNA polymerases as well as to monitor the binding of regulatory proteins. Fluorescently labeled oligonucleotides were used to investigate the mechanism of several DNA or RNA polymerases as well as to monitor the binding of regulatory proteins.37 RNA or DNA polymerase activity has been probed by measuring changes in extrinsic fluorescence at the steady-state and kinetic levels as well as by fluorescence anisotropy as the size of the binder is significantly smaller than that of the protein.18

The reverse transcriptase (RT) of HIV (human immunodeficiency virus) is a key enzyme in the virus replication cycle and catalyzes a chain of reactions to convert the single-stranded HIV RNA genome into a double-stranded DNA for further integration in the cell host genome. This requires that RT is able to discriminate between different nucleic acids and to place them correctly in one of the three catalytic sites for RNA-dependent DNA synthesis, DNA-dependent DNA synthesis, and RNase-H. RT structure and mechanism have been investigated in detail by combining steady-state transient kinetic, and FRET single-molecule assay using dyes attached to the enzyme or to its different partners: tRNA, viral-RNA, and DNA primer/template.38–41 These investigations have shown that catalysis of RT is dependent on the binding orientation of the substrate, which adopts opposite conformations for DNA and RNA duplexes (Fig. 4.9). The large change in dye fluorescence upon binding of primer/template oligonucleotide to RT was used to follow this interaction at both the steady-state and pre-steady-state levels (Fig. 4.9). Stopped-flow technology has been developed for real-time fluorescence kinetic analysis.42–44 A stopped-flow apparatus is a rapid mixing device used to study the chemical kinetics of a reaction in solution, and stopped-flow measurements involve the rapid mixing of two or more solutions (Fig. 4.9). The dead time, corresponding to the time between the end of mixing and the beginning of the observed kinetics of the reaction, ranges between 0.5 and 2 ms. In most cases, protein/partner interactions cannot be analyzed as a simple first-order or second-order reaction, as they often combine binding and conformational events that can be discriminated depending on the rate of each step. In the case of HIV RT, experimental use of fluorescently labeled double-stranded oligonucleotide for rapid kinetics studies has allowed for a detailed characterization of the mechanism and structure of the enzyme during initiation of replication. Kinetic analysis of the Fluorescein phosphoramidite (FAM) – labeled
Figure 4.9 Stopped-flow analysis of reverse transcriptase (RT)/primer:template binding. (A) Scheme of a stopped-flow apparatus with two driving syringes and a single mixing chamber. (B, C) Kinetics of binding of a fluorescently labeled p/t to RTs. Typical stopped-flow time course is shown, where 20 nM of 3′-FAM-labeled p/t was rapidly mixed with 100 nM of RT. Excitation was at 492 nm and emission was detected through a 530-nm cutoff filter. The kinetics were fitted using a two-exponential equation. (C) Secondary plot of the dependence of the fitted pseudo first-order rate constants for the first phase on RT concentrations. The on rate constant of the first phase ($k_{+1}$), corresponding to the formation of an RT–p/t collision complex, is dependent on the concentration of RT and was extrapolated from the slope of the secondary plot of $k_{obs1}$ versus RT concentration. The off rate $k_{-1}$ was estimated on the same plot from the intercept on the $k_{obs}$ axis. The kinetics of p/t binding onto RT followed a two-step mechanism (D) including a rapid diffusion-controlled second-order step leading to the formation of the RT–p/t collision complex, followed by one slow, concentration-independent conformational change from the closed to the open conformation of the polymerase (adapted from Divita et al. $^{38}$ and Agopian et al.). $^{41}$
primer/template (p/t) binding to RT reveals two-exponential kinetics, corresponding to a two-step mechanism (Fig. 4.9). The first phase corresponds to the formation of a RT–p/t collisional complex inducing a conformational transition of RT from the closed- to the open-polymerase typical right-hand organization. The on rate \( (k_{+1}) \) of the first phase is dependent on the concentration of RT and is extrapolated from the slope of the secondary plot of \( k_{\text{obs1}} \) versus enzyme concentration. The off rate \( (k_{-1}) \) can be estimated from the same plot from the intercept on the \( k_{\text{obs}} \) axis. This first step is followed by conformational changes of the preformed RT–p/t complex, which correctly places the nucleic acid in the appropriate binding site for catalysis. The \( k_2 \) \( (k_{+2} + k_{-2}) \) rate constant of the second phase is independent of the enzyme concentration and corresponds to the second exponential term of the kinetics. \(^{40,41,45}\)

By combining FRET and single-molecule fluorescence, Patel and colleagues have elucidated the initiation of transcription catalyzed by RNA polymerase\(^ {46,47} \) using two double-stranded DNA strands corresponding to the upstream promoters and downstream template DNA, labeled at different positions with two different dyes. The donor dye is associated to the upstream promoter and the acceptor to the template strand. Essential changes in DNA and polymerase conformation associated with initiation and abortive RNA synthesis have been identified, and a mechanism involving DNA scrunching following by its rotation has been proposed for early initiation catalyzed by T7 RNA polymerase. Similarly, combining single- and double-stranded labeled DNA offers a powerful sensing system to monitor the open/closed conformational transition of the RNA or DNA polymerase during initiation as well as nucleotide incorporation in real time and the walking of the polymerase along the DNA or RNA during elongation, as reported for mitochondria RNA polymerase\(^ {48} \) and for HVC polymerase (NS5B).\(^ {49} \)

### 2.3. Peptide/nucleic acids

During the last two decades, understanding peptide/nucleic acids interactions has become a major challenge in the field of drug delivery. Small peptides called cell-penetrating peptides (CPPs) have been developed to improve the cellular internalization of a wide range of nucleic acids (from
plasmid DNA, siRNA, and single-stranded antisense molecule). The main development of CPPs has focused on noncovalent delivery involving formation of mixed complexes between peptides and nucleic acids, which improve the cellular internalization of oligonucleotides. In this case, the ability of peptides to form stable interactions with nucleic acids constitutes a key parameter for the selection of potent carriers, and both extrinsic and intrinsic fluorescence have been used for screening peptides and for a better understanding of peptide/nucleic acid complex formation.

Analysis of peptide/nucleic acid interactions can be carried out by monitoring intrinsic peptide fluorescence as well as specific labeling of the cargo. It has been demonstrated for several CPPs that the fluorescence emission maximum of their tryptophan residues is affected by the presence of increasing amounts of nucleic acids and that the fluorescence emission maximum of a labeled oligonucleotide varies with increasing concentrations of the carrier peptide. Thus by monitoring both the intrinsic tryptophan fluorescence of peptides and the extrinsic fluorescence of a labeled cargo, it is possible to investigate the stability of CPP/nucleic acid interactions and thereby characterize complex formation. The intrinsic fluorescence of tryptophans within peptides usually exhibits a strong quenching in the presence of nucleic acids. This quenching might be attributed to both direct peptide/nucleic acid interactions and peptide/peptide interactions that occur when forming complexes with nucleic acids. Although positively charged residues of peptide are able to carry out electrostatic interactions with negative charges of the phosphate groups of nucleic acids, changes in tryptophan fluorescence intensity also suggest aromatic stacking effects. It has been demonstrated that short peptides or protein domains are able to undergo tryptophan fluorescence quenching when interacting with single-stranded nucleic acids as well as DNA duplex and triplex. For example, strong binding of the KWGK peptide to a 21-mer duplex involves intercalation and stacking interactions of the tryptophan with GC regions of the oligonucleotide. The quenching of fluorescence of the tryptophan has been ascribed to an electron transfer from indole of the tryptophan side chain (in the excited state) to purine and pyrimidine bases.

With regard to extrinsic fluorescence of nucleic acids, the use of different fluorescent probes can show distinct behaviors with peptides. Although most of the peptide/nucleic acid interactions induce fluorescence
quenching, enhancement of the quantum yield has also been observed.\textsuperscript{54,58} However, one has to keep in mind that the hydrophobic part of the fluorescent probe confers a hydrophobic anchor for the peptide/cargo interactions: first through $\pi$-stacking on the probe, then through electrostatic interactions between charged residues and the phosphates. Nevertheless, the extrinsic approach provides alternative information through fluorescence anisotropy/polarization measurements. Indeed, the probe possesses a degree of freedom that may vary in the presence of peptides. The variation of the steric environment of the probe induces a variation in its degree of freedom, resulting in a modification of fluorescence polarization. As shown in Fig. 4.10, in the case of CADY/siRNA complexes, a net fluorescence quenching of an FITC (fluorescein isothiocyanate) labeled siRNA was also associated with a clear increase in fluorescence polarization.\textsuperscript{55–57} These data tend to demonstrate that a strong reduction in the degree of freedom of the FITC conjugated to the siRNA occurs in parallel to fluorescence quenching. Thus both fluorescence intensity and polarization variations support the formation of mixed peptide/siRNA complexes.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4.10.png}
\caption{Fluorescence analysis of peptide/nucleic acid interactions through the formation of CADY/siRNA complexes. While a strong quenching of the fluorescence intensity of a FITC-labeled siRNA is induced by the presence of CADY peptide (solid label), a net increase in the fluorescence polarization is also detected (opened label), suggesting both interactions and formation of a large complex between peptides and the siRNA (adapted from Deshayes et al.).\textsuperscript{57}}
\end{figure}
3. MONITORING PROTEIN/PROTEIN AND POLYPEPTIDE INTERACTIONS

Protein/protein interactions control major pathways in biology and numerous technologies have been developed to better understand these processes. Indeed technologies such as cross-linking, pull-downs, and mass spectrometry require cytosolic extraction by cellular lysis, which may induce a loss of the natural properties, especially in situ interactions. In addition, double or reverse hybrid strategies involve genetic constructs that may interfere with the real function of the targeted proteins and/or induce detection of false positives. In order to optimize sensitivity and specificity and to decrease the invasiveness of other methods, large numbers of both natural and synthetic fluorescent probes have been developed, yielding a toolbox of probes for the characterization of protein/protein interactions. The grafting of fluorescent probes enables the detection and quantification of the interactions between protein and partners. A large number of fluorescence approaches have been developed to improve the knowledge of protein/protein interactions in vitro, from steady-state to kinetic investigations, and a combination of all of these approaches provides a better understanding of protein/protein and peptide/protein interactions and multiprotein complex formation.

3.1. Probing protein/protein interactions

An important requirement in setting up fluorescence experiments to investigate or monitor protein/protein interactions is the selection of an appropriate probe to follow the interaction/dissociation of complexes in a noninvasive manner. Ideally, one should monitor changes in intrinsic protein fluorescence, mainly related to tryptophan. Tryptophan residues constitute sensitive probes that are often located at protein/protein interfaces and/or involved in substrate or ligand binding domains. Extrinsic fluorescence has also been applied using solvatochromic dyes which are conjugated to proteins or dyes compatible for FRET between two partners (protein/protein or protein/ligand). In the latter, it is important to validate that the presence of the dye does not alter protein structure and/or function.

Protein complex association/dissociation and reversible unfolding can be followed by size exclusion chromatography, circular dichroïsm, analytical centrifugation, and fluorescence spectroscopy. The sensitivity of fluorescence to its environment has been largely employed to understand
transition states involved in protein complex formation/dissociation and protein/polypeptide reversible unfolding by both steady-state and rapid kinetics, and anisotropy analysis.\textsuperscript{65} Investigation of protein/protein association by mixing the two purified partners in solution and following their association in a time-dependent manner is limited to only few examples.\textsuperscript{37} Usually, solvent (acetonitrile, dimethyl formamide, isopropanol, etc.) or chaotropic agents (guanidinium chloride, urea, etc.) are used to promote complex dissociation and protein unfolding. Fluorescence changes can be transformed to yield the relative fraction of unfolded/monomeric protein in order to determine the thermodynamic parameters of protein complex stability.\textsuperscript{66} In most cases, fluorescence follows a sigmoidal transition according to a one-step/two-state model:

$$D^2 \leftrightarrow 2U/M_t$$ \hfill [4.4]

The process can be described by the following equations in which the folded dimer (D) is at equilibrium with the unfolded monomer (U/M$_t$). The total concentration of monomers (M$_t$) at any concentration of solvent can be defined in terms of the fraction of monomeric protein (M$_m$) and K$_d$ can be expressed in terms of measurable values M$_t$ and M$_m$.

$$K_d = 2M_t(M_m)^2/(1 - M_m)$$ \hfill [4.5]

$$K_d = [U]^2/[N_2] = 2P_t[f_u^2/(1 - f_u)]$$ \hfill [4.6]

The free energy of unfolding/dissociation for a two-state model is defined as a linear function of the concentration of the unfolding/dissociating agent.

$$\Delta G_d = \Delta G^{H_2O} + m[\text{solvent}] = -RT\ln K_d$$ \hfill [4.7]

where $m$ corresponds to the slope of the plot of $\Delta G_d$ versus [solvent]. [Solvent] is the concentration of solvent, and $R$ and $T$ are the gas constant and absolute temperature, respectively. $\Delta G_d$ was calculated via $K_d$ at the corresponding concentrations of solvent used. $P_t$ corresponds to the total protein concentration and $f_u$ is the fraction of unfolded/monomeric protein. $\Delta G^{H_2O}$ is the extrapolated free energy of unfolding in the absence of any unfolding agent.

Steady-state fluorescence measurements have been combined with lifetime anisotropy to sense protein complex dissociation,\textsuperscript{67} as described for the small protein Dim2, a regulatory component of the splicesome machinery. Analysis of Dim2 dissociation using guanidinium chloride by steady fluorescence and lifetime anisotropy (Fig. 4.11) has revealed that the small Dim2 protein exists in two states, monomer and dimer, with a dissociation constant in the nanomolar
Noncovalently linked external probes can also be applied to monitor changes in protein/protein interactions. 1-Anilinonaphthalene-8-sulfonate (ANS) or bis-ANS interacts with hydrophobic pockets that are accessible at the surface of proteins and has been used to follow transition states upon dissociation and unfolding processes. Moreover, spectral properties of ANS (emission 490 nm and excitation 340 nm) are compatible with tryptophan for FRET measurements. ANS and bis-ANS were used to monitor the dissociation of heterodimeric RT. The interface between the two subunits (p66 and p51) involves large hydrophobic patches and the dissociation of RT results in a large increase in the fluorescence of the probe due to noncovalent interactions of ANS to exposed surface hydrophobic motifs on the subunits, thereby providing a good signal for following RT dissociation in a time-dependent manner (Fig. 4.12)\textsuperscript{69}

Another interesting alternative to monitor protein/protein interactions is the use of fluorescently labeled substrates, which, upon binding, reflect the formation of stable or/and active enzymatic complexes. The major advantage of this approach lies in the ability it provides to discriminate between active and inactive forms of the protein/enzyme. Two scenarios can be observed:

range, in contrast to Dim1 homolog, and that Dim2 dimerization regulates its association with the splicesome machinery.\textsuperscript{68}
or the protein/protein interaction results in conformational changes within the substrate binding site located on one of the subunits. The substrate binding site is located at the interface or formed by the association of the two subunits as in the case of the dimeric polymerase RT, Mdm2/P53 interfaces, or protease. Peptide beacon/biosensor (HIV and caspase biosensors) or fluorescently labeled nucleic acid has been used to monitor protease or RNA or DNA polymerase activation as discussed in Section 3.

Monitoring protein/protein interactions does not necessarily require an interface substrate binding site. Several studies have shown that protein/protein interactions induce a marked conformational change in the closed environment of the catalytic site. As reported in Fig. 4.13, binding of cyclin A to Cdk2 results in an important fluorescence change in Mant-ATP bound to the Cdk moiety. This change in fluorescence has been used to probe cyclin/Cdk interactions at the steady-state and pre-steady-state levels and to discriminate between the different Cyclin partners (Fig. 4.13). Similar studies were also performed to understand the interaction of the ras oncogene with the GAP exchange factor.

Figure 4.12 Monitoring acetonitrile-mediated RT dissociation kinetics using a noncovalent external probe and the implication for inhibitor sensing. Heterodiomeric form or RT (0.5 μM) can be dissociated using acetonitrile and dissociation kinetics followed in the presence of 0.8 μM bis-ANS. The kinetics of dissociation was monitored by following the fluorescence resonance energy transfer between tryptophan of RT and bis-ANS. Tryptophan excitation was performed at 290 nm, and the increase of bis-ANS fluorescence emission at 490 nm was detected through a 420 nm cutoff filter, by adding 10% acetonitrile in the absence (black line) or presence of a dimerization inhibitor (gray line). Data were fitted according to a single-exponential equation (adapted from Agopian et al.).
3.2. Probing peptide/protein interactions

Protein/protein interaction studies provide a better understanding of the different parameters that rule the association of protein complexes. Analyses of these parameters enable the development of shorter peptides with similar interaction properties. These peptides may then associate with complexes in the same fashion as the protein partners and induce activation and/or inhibition of the whole complexes. This approach has been extensively developed to design novel inhibitors of specific protein/protein interactions as well as biosensors of protein complexes. 75–78 In parallel to the inhibitor, activator, or biosensor approaches, short peptides have also been developed for drug delivery. From the fluorescence point of view, as for protein/protein interactions, the intrinsic tryptophan fluorescence cannot be systematically used to monitor association since the cargo protein has to be devoid of tryptophan residues. In this case, the approach allows for the recording of intrinsic tryptophan fluorescence spectra of peptides in the presence of increasing amounts of cargo protein. Thus interactions may induce
variations in the fluorescence intensity, which can be correlated with partner association. However, if cargo proteins possess one or several tryptophan residues, the intrinsic fluorescence approach can no longer be applicable. In this case, an extrinsic probe is clearly required to determine peptide/protein affinity and characterize the formation of carrier/cargo complexes. Whatever be the location of the probe, on the cargo protein/peptide or on the carrier, its fluorescence emission can be monitored to gain insight into carrier/cargo interactions. As shown for several carrier peptides and various cargoes, this fluorescence approach allows for the comparison of the affinity between different carrier/cargo combinations.

4. FLUORESCENCE FOR PROTEIN/MEMBRANE INTERACTIONS

Biological membranes constitute important components of living cells. Constituting a physical barrier between the cytosol and the extracellular environment, lipid membranes also compose the architecture of several intracellular compartments such as the Golgi, the endoplasmic reticulum, mitochondria, or the different types of endosomal/lysosomal vesicles involved in intracellular traffic. Membrane studies are often associated with analyses of specific proteins or peptides that are able to interact with, or are especially localized through, the lipid bilayer or the surface of membranes. From the role of membrane proteins to the translocating properties of some peptides, the protein/membrane interactions have been widely investigated. Among the different strategies for deciphering the nature and strength of protein/membrane interactions, the use of fluorescent probes as well as the involvement of new fluorescence technologies provides a better insight into protein/lipid affinity. In this section, the focus is on fluorescence approaches for protein or peptide/membrane interactions, from lipid bilayer insertion to direct phospholipid interactions.

4.1. Probing membrane interactions with tryptophan solvatochromism

Membrane insertion of a molecule may be easily correlated with its ability to be partially or fully embedded through fatty acyl chains that maintain phospholipid bilayer integrity. The development of membrane models has led to different systems that can mimic the natural membrane. Indeed, biological membranes are usually composed of numerous compounds. From phospholipids to membrane proteins, there are clearly too many components to study
in a single fashion. Moreover, with regard to biological diversity, cellular membrane composition depends on the cellular type and can vary with the environment or with the physiological state. Conception of a universal cellular membrane model is consequently too complicated. In order to circumvent this limitation, step-by-step approaches have been developed with artificial membranes. Liposomes, that is, small, large, and giant unilamellar vesicles (SUV, LUV, and GUV), have been developed to provide reliable models. The combination of these models with fluorescent probes enables accurate monitoring of molecule/membrane interactions.

The first basic approach to investigate membrane insertion by fluorescence consists in monitoring the intrinsic tryptophan fluorescence of peptides or proteins in the presence of liposomes. The effect of membranes on the fluorescence emission of tryptophan provides information on the protein or peptide localization in the phospholipid bilayer by sensing environmental changes. Among the water-soluble membrane-active proteins, the channel-forming family of Colicins has been largely studied. These proteins are able to spontaneously insert into negatively charged membranes from aqueous media at low pH. A lack of change in the tryptophan fluorescence spectrum upon insertion indicates that their hydrophobic environment is preserved while becoming accessible to lipids, suggesting that membrane insertion only induces modifications in the relative positioning of the helices. In contrast, monitoring fluorescence of four tryptophans of phospholipases indicates a change in the environment of one or more tryptophan residues, suggesting enzyme/membrane interactions. However, the solvatochromism of tryptophan was mainly studied for membrane-active peptides. For example, comparison of the membrane insertion of two analogous carrier peptides enabled the identification of slightly different behaviors (Fig. 4.14). The liposome effect on tryptophan fluorescence of the CPP MPG-α and MPG-β was compared. Depending on the nature of the phospholipid, headgroups, that is, neutral or negatively charged, different results were found. Addition of negatively charged phospholipids to a solution of MPG-β promotes a blue shift with maximum fluorescence (from 348 to 328 nm) associated with an enhancement of fluorescence intensity (about twofold). This behavior is characteristic of a tryptophan moving from a polar to a nonpolar environment. On the contrary, neutral lipids containing vesicles do not induce any modification of the fluorescence spectrum, suggesting no insertion of peptides into neutral phospholipid bilayers. For MPG-α, while negatively charged vesicles induce a similar blue shift, neutral lipids promote
a small but significant shift, indicating that MPG–α also inserts in neutral bilayers with a positioning of the tryptophan closer to the lipid/water interface (Fig. 4.14). This kind of behavior was also observed for other peptides. Further analyses also enabled the determination of the proportion of bound versus free peptides through the plot of binding isotherms. Thus the insertion of a single tryptophan residue in the sequence of several carrier peptides led to comparative membrane insertion analyses.

### 4.2. Probing membrane insertion by tryptophan quenchers

Although the solvatochromism approach can provide information on protein/membrane interactions, the possibility of insertion through the lipid bilayer or at the interface is usually ruled out by quenching measurements and FRET investigations. Indeed, the combination of tryptophan fluorescence with both improvements in vesicle formation and phospholipid chemistry enabled the development of useful methods to determine the depth of insertion of molecules through a lipid bilayer. Acrylamide and potassium iodide (KI) are well known to interact with tryptophan and to induce a specific quenching of fluorescence intensity. In fact, by combining these tryptophan quenchers with LUVs, it is possible to sense the accessibility of the indole in phospholipid bilayers and then the penetration of the protein or peptide in a
membrane. Basically, the observed enhancement of intensity in the fluorescence of tryptophan in the presence of LUVs is usually titrated with increasing concentration of quenchers. The resulting data are then analyzed by using the Stern–Volmer equation:  

\[ \frac{F_0}{F} = 1 + K_{SV}[Q] \]  

where \( F_0 \) and \( F \) are the fluorescence intensities in the absence and in the presence of quencher, respectively, \([Q]\) is the molar concentration of quencher, and \( K_{SV} \) is the Stern–Volmer quenching constant. A strong decrease of the \( K_{SV} \) value in the presence of LUV is indicative of a loss of accessibility of the tryptophan to the quencher, suggesting thus a deeper insertion of molecule in the lipid bilayer.  

By specifically labeling fatty acyl chains or polar heads, quenching as well as enhancement of fluorescence can provide a more accurate mapping of the bilayer location of a protein. A large number of different labeled phospholipids have also been developed to enable direct FRET on the surface membrane or inside the bilayer. Although more difficult to use, this approach is more accurate. For example, brominated phospholipids were engineered by the addition of bromide at specific positions of the acyl chains of phospholipids: that is, position (6, 7), (9, 10), and (11, 12) of a stearoyl fatty acid chain. From the hydrophobic tail of the acyl chain to the polar head of phospholipids, the use of distinct positions of bromide enables the mapping of the insertion of peptide in a lipid bilayer by bromide quenching of the tryptophan intrinsic fluorescence. Thus the resulting depth-dependent fluorescence quenching profiles enables comparison between different membrane-active peptides.

### 4.3. Probing membrane interactions with specific probes

Based on the principle of solvatochromism, several different fluorescent probes were developed for specifically sensing membrane environments and membrane domains. Indeed, membranes cannot be considered simply as single phospholipid bilayers with a hydrophobic core and a polar surface. Involving different types of domains with various physical states, the “fluid mosaic model” of the structure of cell membranes is clearly in agreement with the dynamics of membrane components throughout the phospholipid bilayer. Like the physical state of phospholipids, the polarity of the lipid bilayer, the membrane potential, and the hydration of membrane are parameters that contribute to the integrity of the membrane. Studies of protein/
membrane interactions generally require the use of specific probes. In this context, several probes have been designed and synthesized in order to confer specific environmental sensitivity.

In contrast to the monitoring of the intrinsic fluorescence of tryptophan, extrinsic labeling of proteins and peptides usually involves the covalent attachment of a fluorophore to a single site on the target molecule, generally via the thiol function of a cysteine residue. Indeed, cysteines are of low abundance in proteins and peptides, and their modification chemistry proceeds under conditions that do not compromise protein structure or function. There are many different probes that can be associated with cysteines. Among them, Acrylodan and Badan are two derivatives of the environment-sensitive dye Prodan, which can be used to label peptides or proteins to investigate their interactions with lipid membranes. However, their poor solubility in water may induce their own burial in a lipid bilayer or in the hydrophobic pocket of proteins. Thus polarity-sensitive fluorophores that are more water soluble, such as NBD, are generally used. NBD is one of the most famous membrane-sensitive probes and has been used as a reporter group in many studies, including investigations of cotranslational protein translocation and integration at the Endoplasmic reticulum (ER) membrane and of toxin insertion into bilayers.

Another approach consists in the use of specifically labeled phospholipids to measure FRET with membrane-active peptides and proteins. For example, Laurdan can act as a FRET acceptor of tryptophan emission and can be used to study the physical state of lipids within Förster distance from donor tryptophan residues in integral membrane protein. However, some probes are more sensitive to the environment than others. Thus several technologies have been developed to insert specific donor/acceptor couples to induce membrane FRET through the lipid bilayer or at the surface of a model or natural membrane. For example, NBD-labeled phosphatidylethanolamine can be combined with rhodamine-labeled proteins to investigate FRET between membranes and proteins.

5. CONCLUSIONS AND PERSPECTIVES

During the last 10 years, fluorescence technology has been used by scientists from many disciplines and for a large panel of applications. Fluorescence constitutes one of the most powerful methods to monitor interactions in a biological context and to answer a wide range of biological and chemical questions. This technology is very advantageous in understanding
protein/protein, protein/ligand, and protein/membrane interactions in real time and in a noninvasive manner. The development of accurate equipment to measure FRET, single molecule, rapid kinetics, fluorescence anisotropy, and fluorescence polarization together with the access to new chemistry for fluorescent probes has opened new routes to identify critical protein conformational changes involved in the regulation of major signaling pathways, as well as aberrant cell behavior and pathological disorders such as cancer. Therefore, fluorescence technology constitutes a major piece of the puzzle for the development of future medicine both at the therapeutic and diagnostic levels.

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REFERENCES


49. Fourar M, Divita G. Fluorescence-based methods to monitor the real-time kinetics of nucleotide incorporation by N55B, RNA polymerase (submitted).


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