1 Once upon a (length and) time (scale)...

Truly it has been said, that to a clear eye the smallest fact is a window through which the Infinite may be seen. (T. H. HUXLEY, *THE STUDY OF ZOOLOGY*, 1861)

GENERAL IDEA

Here we discuss the conceptual foundations of single-molecule biophysics in the context of cellular biology. We provide an overview of existing ensemble average techniques used to study biological processes and consider the importance of single-molecule biology experiments.

1.1 Introduction

Some of the most talented physicists in modern science history have been led ultimately to address challenging questions of biology. This is exemplified in Erwin Schrödinger's essay 'What is Life?' (see Schrödinger, 1944). It starts with the question 'How can the events in space and time which take place within the spatial boundary of a living organism be accounted for by physics and chemistry?' In other words, can we address the big questions of the life sciences from the standpoint of the physical sciences. The ~60 years following the publication of this seminal work has seen a vast increase in our understanding of biology at the molecular scale, and the physical sciences have played a key role in resolving many central problems. The biological problems have not been made easier by the absence of a compelling and consistent definition of 'life' – writing from the context for a meaning of 'artificial life', the American journalist Steven Levy noted 48 examples of definitions of life from eminent scientists, no two of which were the same (Levy, 1993). From a physical perspective, life is a means of trapping free energy (ultimately from the sun or, more rarely, geological thermal vents) into units of increased local order, which in effect locally decrease entropy, while the units maintain their status in situations which are generally far from thermal equilibrium.

This notion of a *life unit* is robust since it links a physical concept to one of the greatest discoveries in biology, made in the nineteenth century, that living organisms were composed of cells. Modern biochemistry as we know it today is largely about trying to understand the molecular mechanisms that make cells do what they do. The very first cellular structures were observed as far back as the seventeenth century when Robert Hooke published the first images from a functional microscope in *Micrographia*. He observed the porous microstructure of cork and first coined the term *cell* as the basic unit of life. Thus, even from the very conception of cell biology, our understanding of the nature of living organisms has progressed hand in hand with many groundbreaking technological developments of the day in physics – one only has to look at the pioneering studies of nerve and muscle tissue, as well as the earliest developments at the molecular level in our knowledge of the structures that drive biology, such as proteins and DNA.

Biological physics, or *biophysics*, is still a comparatively young but fearlessly ambitious discipline which has grown from its childhood, essentially involving biological

investigations using physical instruments, to being now a handsome and youthful adult with much freedom of thought as to its purpose and future. It is a subject which can apply concepts from all the physical and mathematical sciences towards understanding how living matter really functions. Physicists often struggle with the language of biology, and biologists often toil with the mathematics that physicists use to describe the natural world. The meeting of these two worlds can often be uncomfortable at first, and sometimes stormy, but the key to understanding and enjoying biophysics is for physicists to make the effort to learn at least the basics of core biological concepts, while biologists try to come to grips with the intellectual concepts of physical models even if some of the mathematics may seem intractable. There are now several university level courses emerging across the globe which attempt to integrate life and physical sciences.

Modern experimental single-molecule biology research essentially combines many examples of cutting-edge biological approaches with state-of-the-art physics technology and physical insight, and is arguably the most active area of biophysics research at present. The purpose of this handbook is to guide both biologists and physicists, with relatively little or no experience of single-molecule approaches, through the real, practical methods in use today for investigating molecular level biological questions, and to do so in a manner accessible to people from both physical and life sciences backgrounds. In this first chapter you will find an overview of existing single-molecule approaches as well as relevant ensemble (i.e. many molecules) level techniques. There is also an 'orientation' chapter to follow which is especially important for readers from a background more rooted in the physical sciences, which describes some core biological concepts. The subsequent three chapters are devoted to a description of the biological single-molecule techniques in current use. In its most reduced form, a single-molecule method will tell us something about either *position* or *force*.

The ways in which positional information is obtained from single biological molecules can be sensibly divided into methods which primarily utilize *visible light*, and those which do not. A separate chapter is devoted to each, as is the discussion of techniques which allow us either to *measure* molecular forces or to use force to *manipulate* single molecules controllably. The rest of the book is arranged broadly by themes along the lines of different biological processes in the living cell, with descriptions of real experimental single-molecule approaches in use from a variety of both recent and seminal investigations.

KEY POINT

A single-molecule method will either (1) give us information on the position of a molecule in space at a given time, in other words it has the ability to detect the presence of a single molecule at a specific location, or (2) allow the measurement and/or control of forces exerted by/on the molecule – or sometimes both.

1.2 There are already many informative 'multi-molecule' methods

There exist many reliable, standard biophysical experimental approaches for investigating biological processes at the level of typically many thousands of molecules, so-called *bulk ensemble* techniques. Although a full discussion of these approaches is beyond the scope of this handbook (for a more comprehensive description see Van Holde

et al., 2005 and Nölting, 2009), it is first important to understand the basics of what these approaches are, and why they are useful in complementing single-molecule investigations.

1.2.1 Calorimetry

Changes in a variety of *thermodynamic potentials*, for example in the *Gibbs free energy G*, *enthalpy H* and *entropy S*, can be measured directly and/or deduced by carefully measuring temperature changes using a specifically calibrated sample chamber in which the sample is undergoing some chemical or physical transition of interest. One of the most useful forms of this technique is *isothermal titration calorimetry* (ITC) which is often used to study binding affinity and stoichiometry, as well as changes in thermodynamic potentials, of small ligand molecules binding to larger biopolymers such as proteins and DNA (see Chapter 2 for a full explanation of the various different types of biological molecules).

Being able to measure thermodynamic changes is enormously important; they allow us to predict the likelihood of processes occurring under given environmental conditions, and also may indirectly give us insight into the underlying mechanisms of these processes.

PHYSICS-EXTRA
Classical thermodynamics uses ensemble parameters, assuming a system with many, many particles (for example, a single 'microlitre' of water contains $\sim 10^{19}$ molecules). To apply the same concepts to a single molecule requires the **egodic hypothesis** – that all accessible microstates are equally probable over a long time, i.e. that the average properties determined over many molecules ('classical thermodynamics') are equal to the properties of any given single molecule averaged over a long time ('single-molecule thermodynamics'). In other words, the ensemble state equals the time-averaged state, i.e. if one allows the system to evolve in time indefinitely, the system will eventually pass through all possible states.

1.2.2 Chromatography and dialysis

A general chromatography device is used to separate different molecular components present in an heterogeneous sample. There is a wide range of different techniques such as *gas chromatography*, *high-performance liquid chromatography* (HPLC) and standard solid–liquid phase methods such as *gel filtration*, *thin-layer chromatography* (TLC) and even simple paper chromatography. Each molecular component in the sample will bind with some characteristic affinity to an immobile substrate in the given chromatography device, to form a *stationary phase*. The binding lasts for a characteristic *dwell time*, dependent upon factors such as the physical and chemical nature of the immobile substrate and the surrounding molecules.

Once unbound, a particular molecular component will enter the *mobile phase*, and thus can move through the chromatography device either by diffusion or by being driven in a given direction via, typically, a pressure gradient. This process of binding and unbinding can occur through the physical extent of the chromatography device, until the given molecular component emerges at the exit of the device, where it can be detected, typically using some form of optical absorption technique, and collected. The end result is the separation of different molecular components on the basis of their relative binding strengths to the immobile substrate and of their mean speeds of translocation through the chromatography device.

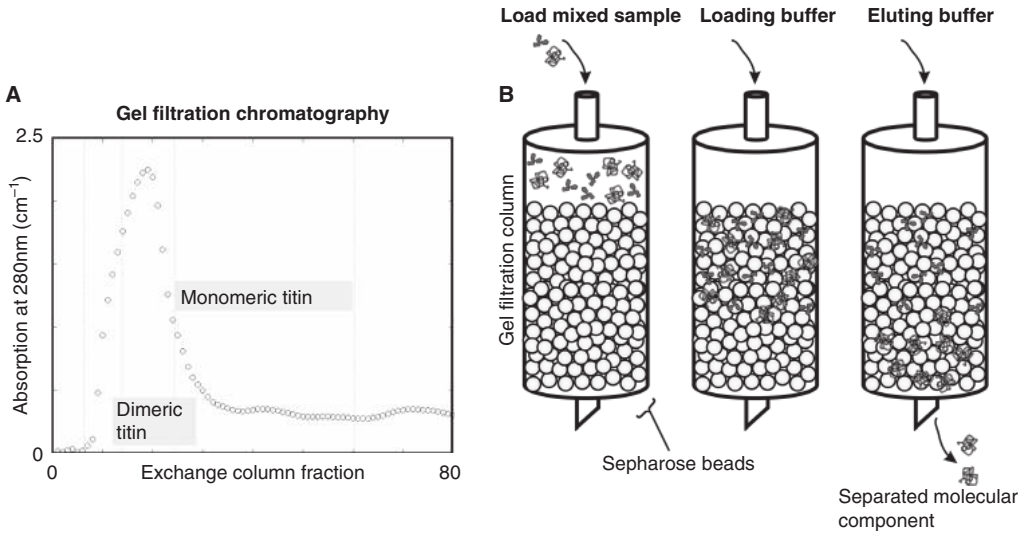

FIGURE 1.1 **(A)** Column-based solid–liquid phase gel filtration chromatography, shown here for a heterogeneous 'oligomer' mix (i.e. different numbers of monomer subunit molecules stuck together) of the muscle protein titin in which the heavier fractions (i.e. dimers and heavier multimers) run off in the first column 'fractions' (a small ~1 ml extracted volume which is then tested for its absorbance at wavelength 280 nm). **(B)** A mixed sample can be separated into different molecular components by eluting the column using different chemical solvent buffers.

The immobile substrate itself often takes the physical form of small tightly packed beads, having a typical diameter in the range 40–400 μm, which is designed to produce optical separation of the given molecular components in the sample. A common substance used for these beads involves a large sugar molecule extracted from seaweed called *sepharose*. These beads are typically packed into a glass column (for *gel filtration* chromatography), or there may be chemical binding, often using specific *antibodies*, to the bead surface of the sample (so-called *affinity* chromatography). In the mobile phase, typically, the sample is solvated and swept through the device in controlled, directed flow, which might at its simplest be gravity driven, or it may involve a pump-controlled pressure gradient. The likelihood for a given molecular component in the sample being in the stationary phase is dictated by issues such as molecular mass, ionic charge, electrical surface features and the presence of exposed chemical groups – for example, *ion-exchange* chromatography is a form of liquid chromatography in which the stationary phase consists of charged beads (Figure 1.1A).

These factors determine the typical dwell time of the substance bound in the immobile stationary phase before unbinding into the mobile phase and being swept along in the flow. Thus the mean *drift speed* through the device of a substance is determined by these factors. Hence this can be used to separate out different substances, either for purification or to measure these factors directly using suitable calibration. Often, since the binding strength to the immobile substrate is dependent on the conditions of the sample solvent, such as ionic strength and pH, different molecular components can be *eluted* selectively through a chromatography column by running through different solvents which are known to reduce selectively the binding between the immobile substrate and a particular molecular component (Figure 1.1B).

Dialysis, or *ultrafiltration*, has similar principles in that the sample mobility is characterized by the same factors as for chromatography, except here the solvated sample sits on one side of a dialysis membrane with a pre-defined pore size. This sets an upper limit on what molecules can pass through, in terms of molecular weight. If the other side of the membrane contains only solvent then, given sufficient time, high mass molecules can be separated from small mass molecules. This process can be speeded up by applying a forced pressure gradient, and by repeated use of different pore sizes a sample can be purified from a mix of different molecules.

1.2.3 Circular dichroism and optical rotation

Optically active samples will rotate the polarization plane of linearly polarized light, and the degree to which this occurs can provide mean average structural information about the sample. A wave of light is really a propagating oscillation of electric and magnetic intensity. These force-fields have directionality, or *polarization*. If this directionality stays constant with respect to the wave itself, polarization is *linear*. If this directionality rotates as the wave propagates, the polarization is *elliptical*; a special case of this is *circular polarization* when the relative components at right angles to each other are equal.

If circularly polarized light is shone through certain optically transparent samples, as is the case for circular dichroism, the *ellipticity* of the polarization may change, typically due to the presence of repeating molecular structures of a given shape. In this way, changes in ellipticity may be indicative of certain structural motifs in the sample. The extent to which fine structural detail can be explored is generally limited, but the technique can often give an indication of the relative proportions of different *secondary structure*.

As we will see later in Chapter 2, the primary structure of a biological molecule describes how the subunit chemicals forming the molecule are linked directly via strong *covalent* chemical bonds. Secondary structure describes how the primary structure forms a three-dimesnsional shape using weaker non-covalent bonds (typically *hydrogen bonds*). *Beta sheets* and *alpha helices* are the main secondary structures of a protein (see Chapter 2), and circular dichroism can discriminate reasonably well between regions of β sheet, α helix and *random coil* secondary structures in proteins, and also of different helical forms of DNA.

1.2.4 Electron microscopy

Structural features in fixed biological samples may be visualized by introducing some form of heavy-metal contrast agent into the sample, shining a beam of electrons through it (Figure 1.2A) and imaging either the scattered beam (as in *scanning electron microscopy*, or SEM) or the transmitted beam (*transmission electron microscopy*, or TEM). The principle here is that different structures within the sample take up the contrast agent to different extents and so will transmit or scatter different proportions of electrons, thereby allowing different structural features to be seen. *Negative staining* may be used in which the contrast agent (typically a heavy-metal chemical salt, for example uranyl acetate) stains everything apart from the structures of interest (Figure 1.2B), or *positive staining* (typically evaporating a heavy metal such as platinum onto the sample to create 'shadow' features from the local topography) may be employed. Fixation and staining often involve multiple steps and an issue is the prevalence of not uncommon staining/fixing artifacts, in addition to radiation damage to the sample from the electron beam.

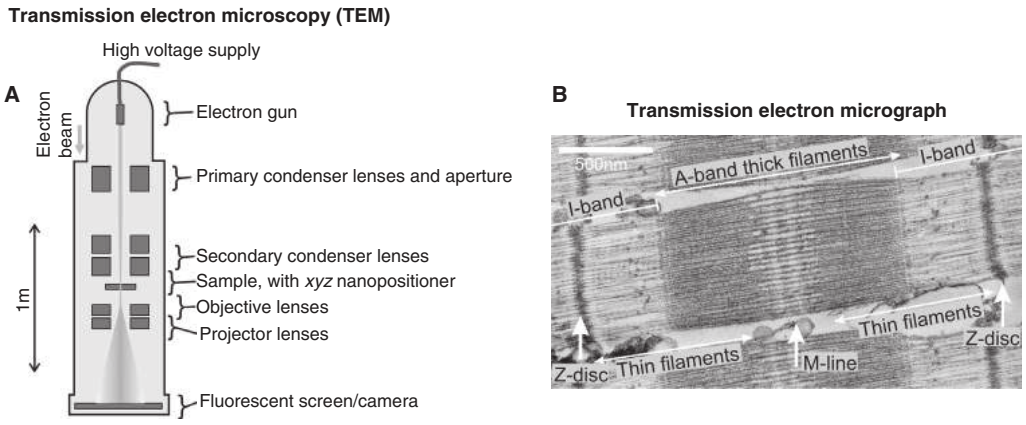

FIGURE 1.2 **(A)** Schematic cross-section through a transmission electron microscope. **(B)** Negatively stained transmission electron micrograph of a thin section of a 'myofibril' found in muscle tissue (see Chapter 4).

Similarly, a significant disadvantage is that the sample is dead, so this approach cannot be used to monitor physiological processes directly (though by careful *snap-freezing* at different time points these can sometimes be inferred indirectly). The technique began as a bulk ensemble method (for example, discriminating different relatively macroscopic length scale tissue features, such as occur in muscle) but with finesse can now provide information at the level of single molecules.

1.2.5 Electrophysiology

Many processes in biology involve the transmission of an electrical current, for example, conduction through nerves, and the flow of ions through channels into and out of cells. These can be monitored using sensitive electrical recording devices either by measuring the changes in voltage inside the cell in question relative to the outside as a function of time, or by measuring the flow of ion current directly. For example, *patch clamping* can be used to extract a patch of several ion channels on the surface of a cell onto the end of a micropipette, and the ion flux through these channels can be monitored directly. Traditionally this has involved monitoring the flow through multiple ion channels simultaneously, but recent improvements now allow the ultrasensitive measurement of single ion-channel currents, and so at the cutting-edge this can be used as a single-molecule technique (see Chapter 4).

1.2.6 Fluorimetry

Here a cuvette of a sample is excited into fluorescence using typically a broadband light source such as a mercury or xenon arc lamp, with individual wavelengths of the fluorescence often measured at 90° to the light source to minimize unwanted detection of non-fluorescence light. The emission spectrum as a function of excitation wavelength is an indication of electronic energy level transitions within the sample, and thus is a characteristic of the underlying molecular structure as well as the physical and chemical environment. This has particular use for natural or engineered fluorescent dye molecules. *Tryptophan fluorescence* involves measuring the native fluorescence of the aromatic amino acid tryptophan.

Tryptophan is a very *hydrophobic* molecule – this means that it has no significant electrical polarity and so will not attract polar solvent water molecules. As a result, tryptophan is generally found buried at the centre of folded proteins so it will not be exposed to surrounding water. Its fluorescence changes upon exposure to water, and so this may be used to indicate whether a protein is in a folded or unfolded state.

A further extension to fluorescence involves *Förster energy resonance transfer* (FRET). This is a non-radiative energy transfer between a donor and an acceptor molecule, which often but not always are fluorescent molecules, whose electronic energy levels overlap significantly. This process only occurs efficiently over length scales less than typically a few nanometres, which is of the order of the length scale of small molecular machines and protein complexes, and so FRET measurements may be used to indicate co-localization/molecular interaction between regions of one or more such molecules tagged with a suitable donor and acceptor molecule.

Also, in *fluorescence anisotropy*, the polarization of the emission signal from a fluorescently labelled sample changes with time if the molecules are free to rotate, and so this technique has been used to estimate the rough shape of large molecules as well as to determine binding constants in the case of interacting molecules.

1.2.7 Gel eletrophoresis

Here, a sample, often containing a mixture of different biological molecules, is injected into a semi-porous gel and exposed to an electric field gradient. The gel is typically either *polyacrylamide* (for protein samples) or *agarose* (for samples containing nucleic acids such as DNA or RNA). The sample can be denatured first by heating and combined with a charged surfactant such as SDS, or it can be run in a non-denatured native state. The mobility of the sample is a function of its molecular weight as well as its shape and (if run in the native state) the presence of charged residues on the surface. Thus, a mixture of different biological molecules can be separated by running the gel for a sufficient time. The positions of the different molecules appear as bands at different distances from the start position (Figure 1.3A), which can be visualized using appropriate staining, either directly in visible light (for example, *Coomassie Blue* is a standard stain of choice for proteins, though *silver staining* may also be applied if greater sensitivity is required) or using fluorescence via excitation of a stain from ultraviolet light (for example, *ethydium bromide stain*, used for nucleic acids). Each band may also be carefully extracted to reconstitute the original, now purified, sample. Thus this technique may be used both for purification and for characterization (for example, to estimate the molecular weight of a sample by interpolation of the band positions from a reference calibration sample).

1.2.8 Mass spectrometry

For the analysis of bulk ensemble average biophysical properties, mass spectrometry is one of the most quantitatively analytical techniques. Here, a small quantity of sample (typically $\sim 10^{-15}$ kg) is injected into an ionizer, and ionized (note though that although this quantity appears meagre this could still equate to typically millions of molecules, so this is far from a single-molecule method). This ionization process generates fragments of whole molecules with different masses and charges. The simplest machine is the *sector* mass spectrometer which accelerates these ion fragments in a vacuum using an electric field sector, and deflects them using a magnetic

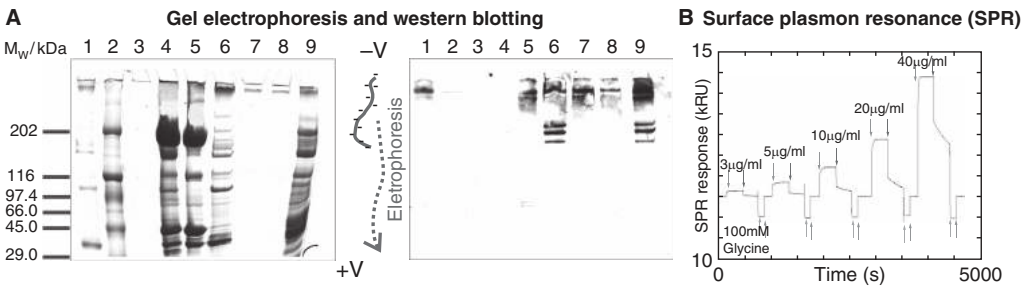

FIGURE 1.3 **(A)** Polyacrylamide SDS gel electrophoresis of a mix of different muscle proteins stained with Coomassie Blue (left panel) with molecular weight markers indicated (lane 1) and lanes 2–9 corresponding to sample extract taken at different stages of a titin preparation from raw muscle tissue; the right panel shows the corresponding western blot probed with an anti-titin antibody. The protein molecules run in a denatured state from cathode to anode, covered with negative surface SDS charges (inset), so lighter proteins will be found further down the gel in general, but there is also a mobility effect related to molecular shape. **(B)** Surface plasmon resonance showing the effect of adding increasing quantities of an anti-titin antibody ligand which binds to titin molecules that have been immobilized on the surface of the sample flow-cell; the binding can be disrupted via addition of the amino acid glycine.

field sector at right angles to this so that the ions follow a roughly circular path. The radius of this circle is a function of the *mass-to-charge ratio* of the particles in the beam. Different ionized molecular fragments can be collected and analysed depending upon the location of the detector in relation to the circular ion path, generating a *mass spectrum* which yields detailed information concerning the relative proportions of different ionic species in the sample.

Other similar types of machine include the *Fourier transform* mass spectrometer (ions are injected into a cyclotron cell and resonated into orbit using an oscillating electric field; this generates a radio-frequency signal from the ions which is detected and subsequently Fourier transformed to yield the mass spectrum), *ion trap* mass spectrometer (injected ions are trapped in a cavity using electric fields and are ejected and subsequently analysed on the basis of their mass-to-charge ratio), *time-of-flight* mass spectrometer (an ion vapour pulse is created, typically using a high-energy laser, and the ions are accelerated using an electric field with the time taken to travel a given distance for this cloud of ions measured; this can then be related back to the mass-to-charge ratio) and *quadrupole* mass spectrometer (the accelerated ion beam is passed between four metal rods to which DC and AC potentials are applied; this causes resonance to the ion beam such that only ions with a narrow range of mass-to-charge ratio will pass through the rod cavity into the detector unit, so by methodical variation of the electric potentials applied to the rods a mass spectrum can be generated).

The biophysical applications of these instruments are significant and include highly sensitive detection/biosensing of biological particles (for example, although the sensitivity is not strictly speaking at the single-molecule level, the minimum concentration level is only around one particle per litre, which is very sensitive relative to other bulk ensemble average techniques, which can be detected in around a few minutes), quality control of sample purity, detecting relatively subtle mutations in nucleic acids such as RNA and DNA, protein conformation and folding studies and proteomics experiments investigating protein–protein interactions. In fact, when NASA's robotic space rover *Curiosity* touched down in Gale Crater on the surface of Mars on 6 August, 2012, one of its key onboard devices was a portable mass spectrometer for the detection of biological-related material.

1.2.9 NMR and ESR spectroscopy

Nuclear magnetic resonance (NMR) is a powerful technique utilizing the principle that magnetic atomic nuclei will undergo resonance by absorbing and emitting electromagnetic radiation in the presence of a strong external magnetic field. A magnetic nucleus implies a non-zero spin angular momentum, which is the case for all stable *isotopes* which have an odd number of protons and/or neutrons. The most common isotopes used for biological samples are ^{1}H (the most sensitive stable isotope) and ^{13}C (relatively low natural abundance compared to the non-magnetic ^{12}C and also a low sensitivity but used since carbon is the key component of all organic compounds). Other lesser used isotopes include ^{15}N (low sensitivity but used since nitrogen is a key component in proteins and nucleic acids), ^{19}F (rarely present in most natural organic compounds so in general needs to be chemically bound into the sample first, but has a high sensitivity) and ^{31}P (moderate sensitivity and phosphorus is a key element of many biological chemicals).

In the presence of an external magnetic field, the different spin states of the nuclei have a different energy; by absorbing or emitting electromagnetic radiation of the right frequency the nucleus can resonate between the different spin energy states. However, not all atomic nuclei in a sample will have exactly the same differences in spin energy states because there is a small shielding effect from the surrounding electrons which causes subtle differences in the absolute level of the external magnetic field sensed in the nucleus. These differences are related to the physical distribution of the local electron cloud, which in turn is a manifestation of the local chemical environment. In other words, this shift in the resonant frequency, sometimes referred to as the *chemical shift*, can be used to deduce the chemical structure of the sample.

Typically, the resonance will be in the radio-frequency range (of the order of 100 MHz) for the high fields of ~10 Tesla used (almost a million times the Earth's magnetic field) with the detected chemical shift being typically in range of ~10 parts per million (ppm) of the non-shifted value. This technique has been used to great effect in obtaining atomic level structures of several important biological molecules, especially of membrane proteins. In general these proteins are very difficult to crystallize which is a requirement of the competing atomic level structural determination technique of *x-ray crystallography*. The concentration of the sample for NMR needs to be relatively high to obtain good signals for structural studies, and necessarily therefore this is an in vitro technique. However, at a lower spatial resolution it can be used in vivo in, for example, *magnetic resonance imaging* (MRI), which is widely used in medical physics but also utilized in biophysical applications to follow in vivo biochemistry. The spatial resolution is typically of the order of millimetres at best.

Electron spin resonance (ESR), also known as *electron paramagnetic resonance* (EPR), relies on similar principles to NMR but here the resonance is from the absorption and emission of electromagnetic radiation due to transitions in the spin states of the electrons. This only occurs for unpaired electrons, since paired electrons have a net spin (and hence a magnetic strength) of zero. Unpaired electrons are unstable and associated with highly reactive species, such as free-radicals. These reactive species are short lived, which limits the application of ESR, though this can be used to advantage in that standard solvents do not produce a measurable ESR signal and so the relative strength of the signal from the actual sample above this background *solvent noise* can be very high.

1.2.10 Optical interferometry

There are two principal multi-molecule optical interferometry techniques: *dual polarization interferometry* (DPI) and *surface plasmon resonance* (SPR). In DPI, a reference laser beam is guided through an optically transparent sample support, while a sensing laser beam is directed similarly through the support but at a very oblique angle to the surface. At this steep angle the laser beam is totally internally reflected away from the surface, but with the by-product of generating an *evanescent field* into the sample, generally solvated by water for the case of biophysical investigations, with a characteristic depth of penetration of ~100 nm. Small quantities of material from the sample which bind to the surface have subtle but measureable effects upon the polarization in this evanescent field. These can be detected very sensitively by measuring the interference pattern produced between the sensing and reference beams. DPI gives information on the thickness of the surfaced-adsorbed material, and its refractive index.

SPR works in a similar manner in that an evanescent field is generated, but here a thin layer of metal, ~10 nm thick, is first deposited onto the outside surface. At a certain angle of incidence to the surface the sensing beam reflects slightly less back into the sample. This is due to a resonance effect via the generation of resonant oscillations in the electrons at the metal surface interface, so-called *surface plasmons*. This angle is a function of the absolute amount of the adsorbed material on the metal surface from the sample, and so DPI and SPR are essentially complementary techniques. Both ultimately can yield information on the stoichiometry and kinetics of binding of biological materials. This can be used to understand how cell receptors work, for example. If the surface is first coated with purified receptor proteins and the sample chamber contains a ligand thought to bind to the receptor, then both DPI and SPR can be used to measure the strength of this binding, and subsequently unbinding, and to estimate the relative numbers of ligands that bind for every receptor protein (Figure 1.3B).

1.2.11 Optical microscopy

Here, visible light is either transmitted from, or scattered by, a sample. Different features within a biological sample, for example in a cell, will absorb different relative proportions of light and so the intensity of the image is some indication of different underlying structural features (so-called *brightfield* microscopy). However, the inside of most biological samples is generally mostly composed of water, which is the same as the surrounding solution, and so the imaging contrast on the basis of transmitted intensity alone is often poor.

To improve the contrast several methods have been employed. Optical interference enhancement methods include *phase contrast* (increases the sensitivity to changes in refractive index in the sample) and *differential interference contrast* or DIC (the image is enhanced for areas of high spatial gradients in the refractive index, for example at the perimeters of cells or organelles inside the cells). *Darkfield* microscopy provides enhancement to image contrast by imaging the scattered features from the sample (so that the non-sample regions appear dark). This is useful for investigating surface features of cells and tissues, and also finds application in single-molecule methods.

Staining methods are also employed to success. These include the relatively crude methods involving coloured organic stains which bind differentially to different biological material (so they can be used for example to differentiate cell and/or tissue types, but with a disadvantage that the sample is killed in the process, and non-specific binding can reduce the ultimate contrast), and also include those methods which use fluorescence