Light as a Tool for Biologists: Recent Developments

5

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5.1 Introduction

Light has, of course, been exploited by scientists as far back as there has been any science. Until recently, almost everything we knew about the universe beyond the lower terrestrial atmosphere was information carried by light. Since Bunsen's time, optical spectroscopy has provided information about things as small as atoms and as large as galaxies. Spectrometry (absorption or fluorescence) or optical microscopy, or both, has been important for most biologists. The following account will therefore not attempt to be comprehensive but will focus on a few recent developments of interest for biologists. A good guide to much of the pre-2003 literature is provided by *Methods in Enzymology* (Elsevier) Vol. 360 and 361 (2003).

5.2 Optical Tweezers and Related Techniques

When we hear the word tool, we probably first see in our minds something we can hold in the hand, like a hammer, pliers, or tweezers. We use tweezers to handle things that are too small for our fingers to manipulate. But mechanical tweezers, and even traditional micromanipulators, are too large for particles smaller than single cells. Such objects can be handled with optical tweezers, invented by Ashkin (1970).

The description in Fig. 5.1 applies to light beams that are most intense in the center and with irradiance tapering off towards the periphery approximately as a Gauss function (as may be recalled from elementary statistical theory) does. But another type of beam has also been tried for optical forceps, a so-called Bessel beam. This beam has a complicated cross section. True Bessel beams cannot be produced, but an approximation can be made by focusing an ordinary beam from a laser (approximate Gauss beam), not with an ordinary lens, but with a conical lens. A Bessel beam has the advantage that the irradiance is kept high over a longer distance,

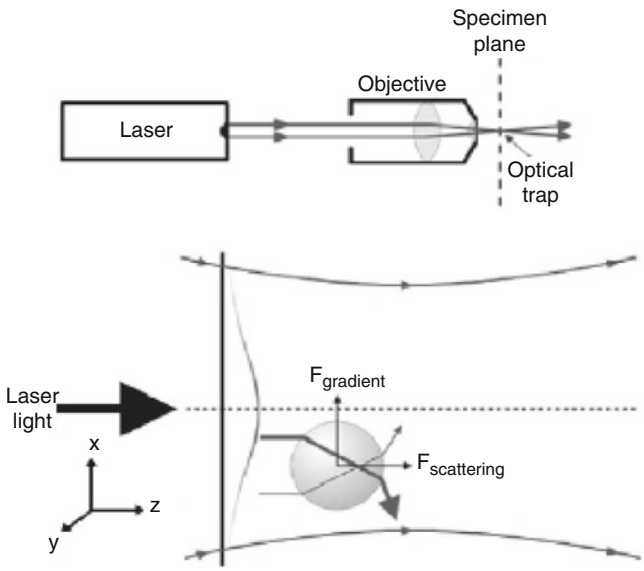

Fig. 5.1 A simple form of optical tweezers. A particle is trapped in the focus of a laser beam. The picture shows how a strong ray of light is bent downwards by a transparent (or at least semitransparent, as all sufficiently small biological objects are) sphere. This gives an upward force acting on the sphere. If the sphere from the beginning is a little off-center in the beam, the corresponding ray giving a downward force is weaker, and the resulting force will be towards the beam center. Bending of both rays will also produce a force on the sphere towards the right, since they both decrease the rightward momentum of the light and this must be compensated for by the sphere's momentum. Light absorption as well as back reflection will give a force in the direction of the light, but for small objects, this will be a small effect. Light refraction will be more important for the forces along the light beam. There is a gradient in light refraction, and if the particle starts to move past the focus, a restoring force is created (see Fig. 5.2) (Slightly modified from Stanford University's URL http://www.stanford.edu/group/blocklab/Optical%20Tweezers%20Introduction.htm)

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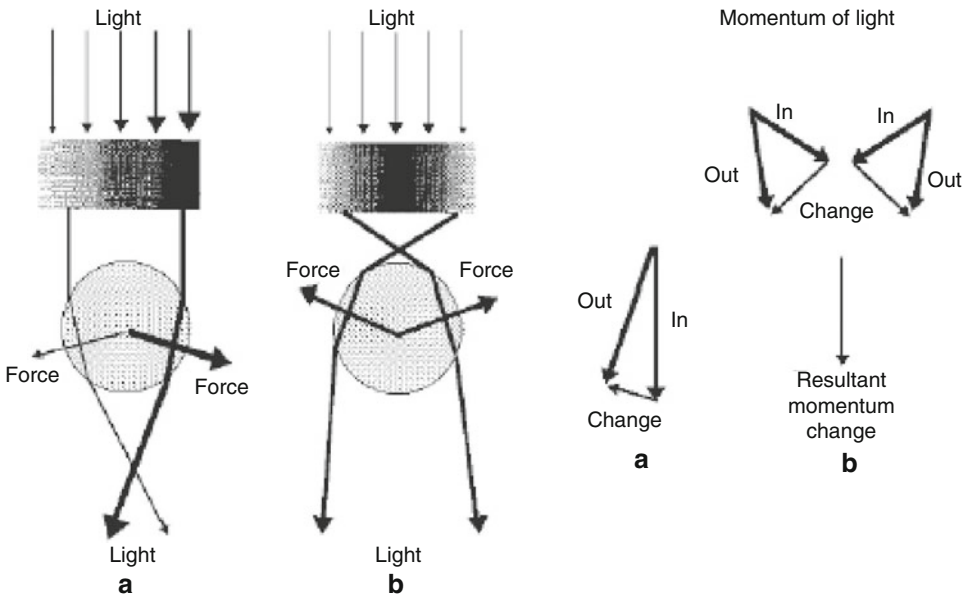


Fig. 5.2 Diagrams showing the refraction of light in a transparent sphere (*left*) and the resulting change in momentum of the light (*right*). The forces on and movements of the sphere are such that they compensate for the change in momentum of the photons. Surprisingly, the amount of momentum transfer has even recently been a matter of much discussion among physicists (e.g., Loudon and Barnett 2006). The diagrams to the right show how the momentum change can be computed graphically by subtraction and addition of vectors

not only in a focal spot. Therefore, it can be used to manipulate particles in directions perpendicular to the beam, while they can move freely along the beam.

In another method, instead of "clamping" particles in a spot (as with Gaussian beams) or along a Bessel beam, a kind of "optical washboard" or "corrugated surface" is produced, and this can be "shaken" so that particles move along the surface, with different speeds depending on size (Paterson et al. 2005). For this, a Bessel beam is used.

Using holograms, any light distribution can be created and used for manipulating objects (Grier and Roichman 2006), and light in combination with sheets of certain polymers can be used to transport materials over microscopic distances (Stiller et al. 2004). Dorman (2007) exemplifies the use of optical tweezers in biology.

5.3 Use of Lasers for Ablation, Desorption, Ionization, and Dissection

The reader has probably already heard or read about the "light scalpel" that doctors use for some kinds of surgery. Laser beams can be used for killing cancer cells, for fixing retinae that have come loose, and for several other medical purposes.

Lasers also have many uses related to this in nonmedical biology. One of the most important applications is to make large molecules, such as protein molecules, available for mass spectrometry, for determination of molecular mass and content of, e.g., phosphate groups (Krüger et al. 2006; Marshall et al. 2002).

The methods of evaporating the samples are referred to as laser ablation (any removal of material from a solid or liquid surface) and laser desorption (Peterson 2007). In addition, molecules and molecular fragments can be ionized by the laser beam, and the resulting electric charge is, of course, an important factor in mass spectrometry. A new method combining the use of laser and electrospray ionization (ESI) is electrospray-assisted laser desorption ionization (ELDI) (Huang et al. 2006).

Laser beams can also be used for microdissection of biological specimens (Nelson et al. 2006). There are two main methods for this: "laser capture microdissection" (LCM) and "laser cutting." Several commercial instruments are marketed for each of these. In the former method, a film of ethyl–vinyl–acetate is locally heated by an infrared laser beam, melted, and glued to the structure one wishes to pull away from the rest of the sample. In laser cutting, an ultraviolet laser beam (a 337-nm nitrogen laser is suitable) is used to cut out the desired structure.

5.4 Fluorescent Labeling

In classical light microscopy, biological structures in most cases had to be stained with various pigments to become clearly visible. Gradually, methods became more sophisticated, so that it became possible to determine the chemical makeup of various cell structures by staining them with specific colored compounds ("cytochemistry"). A great leap forward was taken when pigments were combined with antibodies, which make very specific associations with cell structures and particularly with proteins. In the microscopy methods to be described in the following sections, labeling with fluorescent pigments plays an important role, and these pigments are commonly combined with antibodies to make the labeling of cell constituents specific. Antibodies are best for rather large molecules, while for small metabolites,

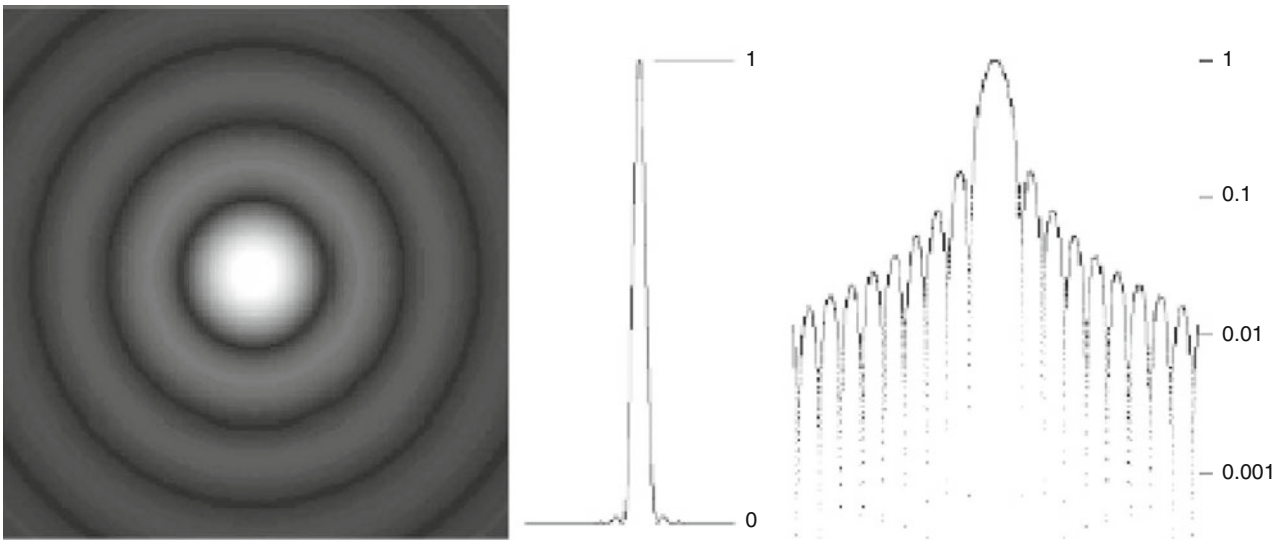


Fig. 5.3 The Airy disk: the real appearance and graphs of the radiance distribution on logarithmic and linear scales

specific RNA species can be used in combination with fluorescent molecules (Paige et al. 2012).

Many fluorescent compounds have been used in this way. One particular compound has become particularly important—the so-called green fluorescent protein (GFP). It is produced by the jellyfish *Aequorea victoria* (see Chap. 26). It has a major excitation peak at 395 nm and a minor excitation peak at 475 nm. The emission peak is in the green region at 509 nm, which makes it very suitable for visual detection of fluorescence. Another somewhat similar protein is produced by the sea pansy *Renilla reniformis.*

The advantage with a protein is that it can be used as a reporter for gene expression. For this purpose, the gene for GFP is fused with a regulator gene to be studied and inserted into the genome of the organism under study. The first publication (Chalfie et al. 1994) introducing this technique has, at the time of this writing, been cited 2,532 times. Tsien (1998) has written an early review of the subject, which is also well cited.

Fluorescent proteins with other emission spectra can be obtained in different ways: (1) by extracting and cloning proteins from other organisms, such as the red-emitting pigment from the railroad worm (Viviani 1999) or various proteins from corals (Verkhusha and Lukyanov 2004); (2) by changing amino acids in the protein by genetic engineering or chemically modifying the chromophore (Heim 1994; Shkrob et al. 2005); (3) by photoactivation or phototransformation, i.e., by various photochemical reactions of the protein (see below); and (4) by combining two proteins with different fluorescence properties so that energy absorbed by one can be transferred to the other one. By labeling a sample simultaneously with proteins having different fluorescence properties, regulation of different genes can be studied in the same sample (e.g., Jiang et al. 2006).

Proteins can also be made to change their fluorescence spectra by light treatment (e.g., Ando 2002; Elowitz et al. 1997; Habuchi et al. 2005; Lukyanov et al. 2005). The exact mechanism for this color change varies from case to case. It can be due to a conformational change of the protein or to photochemical change of an amino acid in the protein.

The fluorescence quantum yield of GFP is so high that single molecules can be registered (e.g., Cotlet et al. 2006; Peterman et al. 1999).

A recent comprehensive review (Fricker et al. 2006) describes a number of special methods in quantitative fluorescence microscopy which we shall not recount here. They include quantitative imaging of gene expression, in vivo imaging of mRNA localization and dynamics, methods for protein localization, level and turnover, and protein–protein interactions. One method for judging the proximity of two protein molecules is to study the resonance energy transfer between chromophores attached to them (see Chap. 1). Still another novel microscopy method using fluorescence is "fluorescent speckle microscopy" (Danuser and Waterman-Storer 2006) for the study of macromolecule dynamics (such as cytoskeleton assembly/disassembly) in living cells.

5.5 Abbe's Diffraction Limit to Spatial Resolution in Microscopy

We have seen in Chap. 1 that light is diffracted when passing through an opening. This diffraction limits the resolution of conventional light microscopy. A bright point will not be imaged as a point but as a diffraction pattern, somewhat similar to what we have encountered in the section on diffraction in slits, but with a circular shape, a so-called Airy disk (Fig. 5.3).

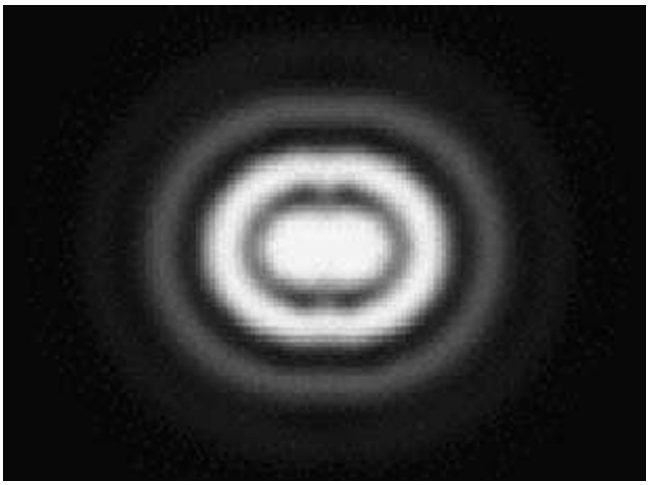


Fig. 5.4 Image of two bright spots at the minimum resolvable distance

More than 130 years ago, Ernst Abbe showed that the smallest distance between two points (Fig. 5.4) that can be resolved by a conventional light microscope is $s = 1.22 \cdot \lambda / (2 \cdot n \cdot \sin\alpha)$, where λ is the wavelength of the light used, n the refractive index of the medium between the objective lens and the object, and α the half-angle of the objective lens subtended at the object plane. The so-called numerical aperture, $n \cdot \sin\alpha$, is often stated on the objective of a microscope, so it is easy to estimate s. With immersion oil as the medium between the objective and the object and a high-quality immersion objective, the numerical aperture can be made as high as 1.4.

With light for which the human eye has a high sensitivity and resolution, 500 nm, we get $s = 1.22 \cdot 500/(2 \cdot 1.4)$ nm = 218 nm = 0.218 μm. The brightness distribution for this case, which is considered to be the limit for resolution of two points, is shown in Fig. 5.4.

Using a longer wavelength, while diminishing the resolution, has the advantage of better penetration and less scattering in most biological specimens.

The resolution can be further improved by using ultraviolet radiation instead of visible light (since the Airy disk will be smaller with a lower wavelength) and still further by using electrons instead of photons (electron microscopes use wavelengths in the nanometer range, but the numerical aperture is less favorable).

An improvement in factual resolution for biological specimens was achieved with the invention of the confocal microscope, but this is merely a way of improving focusing and reducing disturbing scattered light. The ultimate resolution is still set by Abbe's criterion.

More recently, however, Abbe's diffraction limit has been broken through, in more than one way, and resolutions of only a few tens of nanometers achieved using visible light (see below).

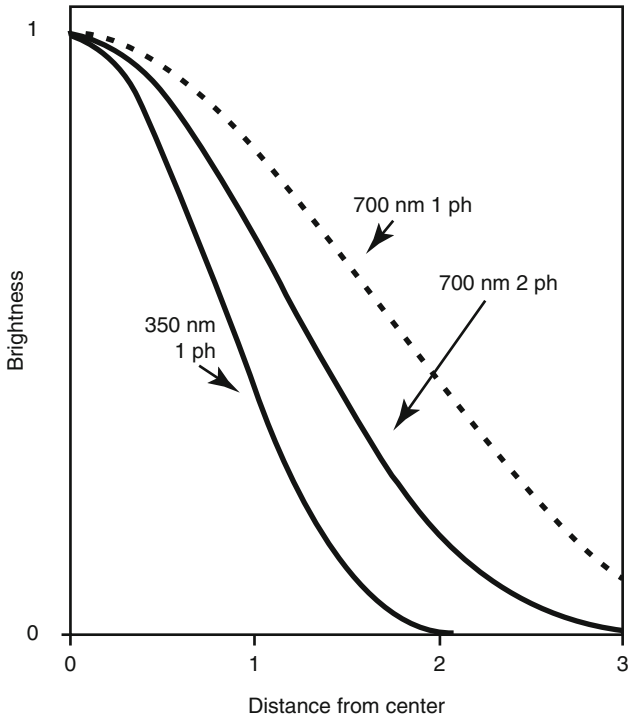
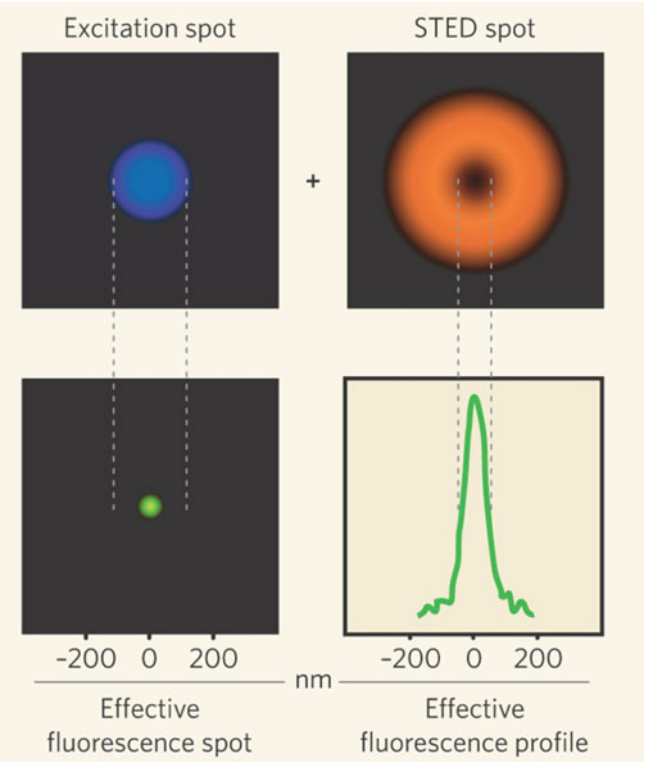


Fig. 5.5 Brightness of a small fluorescing spot as imaged with one-photon fluorescence (350 and 700 nm) and two-photon fluorescence (700 nm)

5.6 Two-Photon Excitation Fluorescence Microscopy

In this technique, the biological specimen is stained with antibodies to which a fluorescent pigment has been attached. The antibodies attach to the structures that one wishes to study, such as a specific protein. The method utilizes the fact that fluorescence can be induced not only by light of a wavelength corresponding to an absorption band of the fluorescent pigment but also by light of twice that wavelength (photons with half the energy of the energy gap in the Jablonski diagram), provided the concentration of photons is high enough that two of them will be absorbed by the same molecule within a short enough time. If the equipment is suitably adjusted, this happens only in the center of the laser beam with which fluorescence is excited. The specimen must be examined point by point by a scanning laser beam. In two-photon microscopy, in order to use the same absorption band of the fluorescent pigment as in one-photon microscopy, the wavelength must be doubled. Doubling of the wavelength alone would worsen the resolution by a factor of two. Since, for two-photon excitation, the fluorescence brightness is proportional to the square rather than the first power of the fluence rate of the exciting light, this disadvantage is only partly compensated for, as shown in Fig. 5.5.

An important advantage with two-photon excitation is that less bleaching occurs than with one-photon excitation.



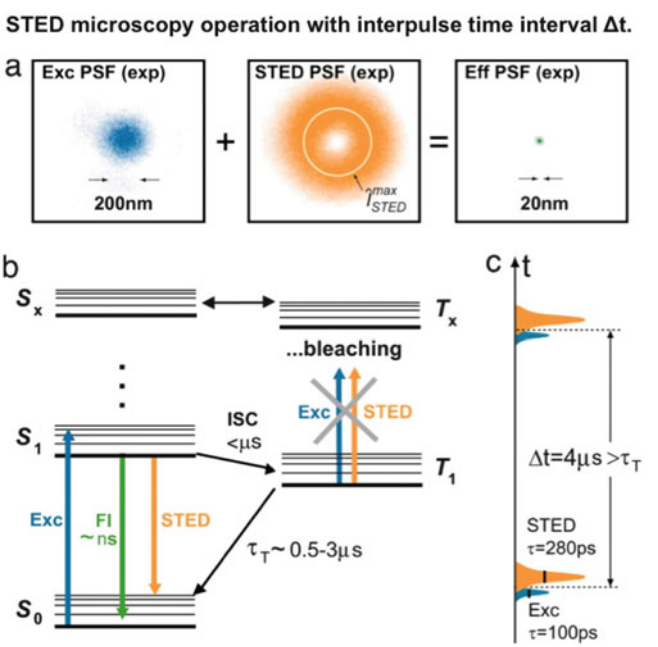


Fig. 5.6 The principle for STED (*Left picture* reprinted by permission from Macmillan Publishers Ltd: Simpson (2006), Copyright 2006 STED microscopy operation with interpulse time interval ?t. (**a** *Left* and *Center*) (**a**). Measured spots for excitation (*blue*) and STED (*orange*) pulses resulting in 22-nm STED spot. (**b**). Conversions between pigment energy levels. If time between *blue* and *orange* pulses is too short, excitation of triplet pigment causes bleaching. (**c**). With pulse interval long enough the triplet state has decayed and bleaching is prevented. *Right* STED microscopy operation with interpulse time interval Δt. (**a**). Measured spots for excitation (*blue*) and STED (*orange*) pulses resulting in 22-nm STED spot. (**b**). Conversions between pigment energy levels. If time between *blue* and *orange* pulses is too short, excitation of triplet pigment causes bleaching. (**c**). With pulse interval long enough the triplet state has decayed and bleaching is prevented. (Reprinted, with permission, from Donnert et al. (2006))

5.7 Stimulated Emission Depletion

Another method, stimulated emission depletion microscopy (STED microscopy or STEM), also uses fluorescent labeling of the specimen in the same way but a different method for exciting the fluorescence. In this method, too, the specimen is scanned spot by spot by a laser beam, which excites fluorescence (Fig. 5.6). Diffraction of the beam causes the illuminated spot to have a minimum diameter of about 200 nm if blue light of 470-nm wavelength is used. Immediately after the pulse of blue light and before any appreciable amount of fluorescence light has had time to be emitted, a second pulse, now of a higher wavelength, hits the sample. The time between the pulses is long enough for the excited pigment to decay to the lowest vibrational level of the first excited singlet state. The wavelength of the second pulse is such that it causes the pigment to return to the ground level via stimulated emission (the same phenomenon that takes place in a laser). The second pulse has a ring-shaped cross section, i.e., the intensity in the center is almost zero. Thus, the depletion of the excited state will take place around the circumference of the excited spot, and fluorescence will be emitted only from the most central part.

A problem with this method is that since very strong light has to be used, and thus a high concentration of excited state, a triplet state is easily formed, resulting in chemical reactions that destroy the pigment (bleaching). This problem can be circumvented by suitable choice of pigment and irradiation protocol.

5.8 Near-Field Microscopy

A completely different approach for breaking the diffraction limit in microscopy was suggested long ago (Synge 1928), but was first implemented in a practically useful way by Betzig and Chichester (1992). The principle is to either collect or deliver the viewing light so close to the specimen that the light has had no space to spread by diffraction (Figs. 5.7 and 5.8).

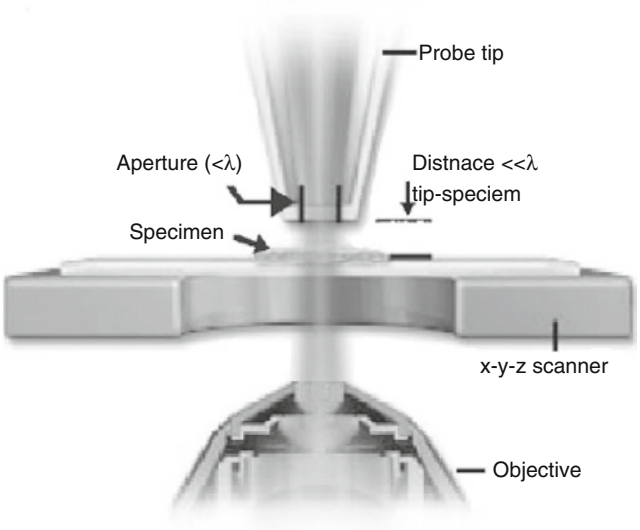


Fig. 5.7 Near-field microscopy (Slightly modified from http://www.olympusmicro.com/primer/tecniques/nearfieldintro.html. Courtesy of Dr. Michael W. Davidson)

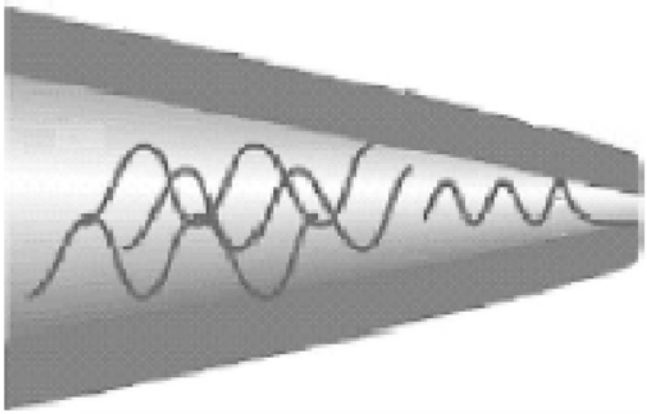


Fig. 5.8 Tapered and aluminum-coated glass fiber for near-field microscopy, schematic. Light waves are squeezed together until there is no room for them to propagate further. But a so-called evanescent electromagnetic field still protrudes a short distance beyond the tip of the fiber, and if a scattering medium is brought into this region, the light energy can propagate into the "far field" and be registered with a photomultiplier. The same device can work in the opposite direction, and sample photons from the evanescent field of the sample if this is illuminated (Modified from http://www.azonano.com/details.asp?ArticleID=1205)

Betzig and Chichester (1992) introduced the use of a very thin light-conducting fiber for this purpose. The light aperture at the end of the fiber is much less than the wavelength of light, about 25–100 nm in diameter, and the distance between the fiber tip and the sample often even less, 5–50 nm. Also, in this method, the specimen has to be probed point by point, and an image is obtained by scanning (Fig. 5.9). The method is sometimes referred to as near-field scanning optical microscopy (NSOM). Because the probe is so close to the sample, with a few modifications the setup can also be used for atomic (shear) force microscopy.

5.9 Optoacoustic Tomography and Microscopy

Both light and sound can be used for imaging. Light has an advantage over sound in that it can give more specific contrast, perceived by us as color. For scientific purposes, one can use light of specific wavelength for rather specific imaging of chemical compounds, such as hemoglobin, oxyhemoglobin, or nucleic acid. One drawback of light for imaging of biological objects is that it is almost completely scattered within a short distance (less than 1 mm) in biological tissue, and some light does not even penetrate very deep.

Sound also has advantages over light for biological imaging. Ultrasonic imaging for medical purposes was pioneered by Hellmuth Hertz at Lund University (http://en.wikipedia.org/wiki/Carl_Helmut_Hertz) and has been extensively used for imaging of hearts, bowels, nasal sinuses, livers, and fetuses. Many animals, such as dolphins and bats, have of course used sound for imaging long before that. Sound penetrates much deeper than light in biological tissue, and the scattering is a thousandfold lower than that of light. Another advantage is that it travels more slowly, and one can therefore more easily determine the distance to a reflecting structure by measuring the time of travel from source to receiver and thus map organs in three dimensions. The wavelength of the sound waves cannot be made as short as of light, and therefore the spatial resolution is limited, and the contrast is not as great and in particular not as discriminatory (colorful) as that of light.

The optoacoustic technique combines the advantages of optical and acoustical imaging. In this technique, the object is illuminated with light, but it is an acoustic signal that forms the image.

If a light-absorbing object is exposed to a very short (nanosecond range) pulse of light (a laser is used for this), its temperature is transiently slightly increased, and it undergoes a rapid, small expansion. This creates a pressure pulse, in other words a sound wave, that travels out from the object. If we have pressure receivers ("microphones") placed at four points around the object, we can calculate the position of the object after recording the time of arrival of the pressure pulse to the four receivers. This is the basic principle of optoacoustic imaging. In case we are pursuing photoacoustic computed tomography (see below), it does not matter if the light is diffuse when it reaches the object to be imaged, since the imaging is not done by the light, but by the sound. To image an extended three-dimensional object is, of course, a bit more complicated and involves a lot of sophisticated mathematics and

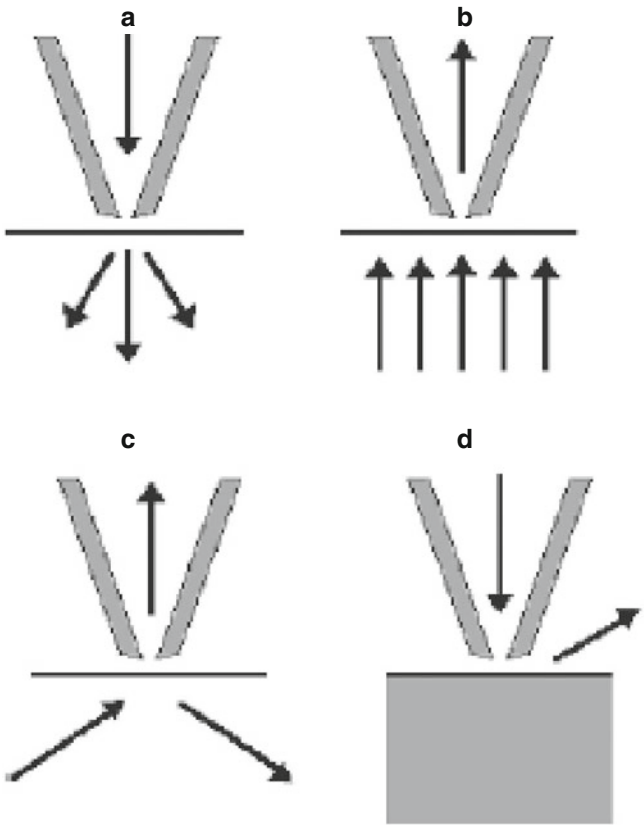


Fig. 5.9 Four methods for scanning near-field microscopy. (**a**, **b**) Light is either delivered (**a**) from the tapered fiber or collected (**b**) by it. (**c**) How "frustrated" total reflection is used. All light coming from below undergoes total reflection except where the evanescent field from structures in the sample comes sufficiently close to the fiber. (**d**) Light delivered from the fiber is scattered from structures in the sample only if they come within reach of the evanescent field, and then the scattered light can be picked up by a photomultiplier or photodiode

equipment. There are three main branches of photoacoustic imaging techniques: photoacoustic microscopy (PAM), photoacoustic computed tomography (PACT), and photoacoustic endoscopy (PAE).

For photoacoustic microscopy, a laser pulse is focused onto a spot not very far from the surface of a tissue, and a sound receiver is focused onto the same spot, either from the same or the opposite side, so it is a kind of confocal microscopy. The specimen is then scanned by moving the focusing spot in a raster-like way, repeating the laser pulses to cover the whole area of interest.

For photoacoustic computed tomography, the laser pulses are not focused but are distributed across the whole specimen, and the receiver is moved around to receive sound pulses from all sides. A computer program transforms the received pressure signals to a three-dimensional image of the object. Both methods are described in more detail by Wang (2009) and Wang and Hu (2012), who also provide many references.

5.10 Further Methods for "Super-Resolution Microscopy"

A large number of other methods for "super-resolution microscopy" have recently been developed and are being developed. A good overview is available on the Internet at http://en.wikipedia.org/wiki/Super_resolution_microscopy (accessed on August 24, 2013).

5.11 Optical and Photoacoustic Detection of Malaria Infection via Vapor Nanobubbles

Over 200 million people are infected by malaria every year, and about 600,000 die. The sooner infection is detected, the greater the chance of survival, but until now, diagnosis can be made only from blood samples. Recently, Lukianova-Hleb et al. (2013) have described a new noninvasive and rapid method for the first transdermal detection of malaria infection as low as 0.00034 %, so far tested on animals (and on infected humans and human blood cells in vitro). It depends on the photothermal properties of an excretion product from the malaria parasite, *Plasmodium falciparum*.

Plasmodium develops inside red blood cells, which contain mainly hemoglobin. The parasite metabolizes the protein part of the molecule, but the heme content far exceeds what the organism needs and must be disposed of. This results in formation of nanoparticles of modified heme, so-called hemozoin. These particles have a well-ordered, almost crystalline structure and, for this reason, have an absorption spectrum that differs from that of hemoglobin or of amorphous or dissolved heme. This allows to efficiently generate a vapor nanobubble around hemozoin with a laser pulse. Furthermore, when a short laser pulse is applied, a vapor nanobubble spectral narrowing takes place, similar to that which has been observed for gold nanoparticles: a short and intense laser pulse applied at the absorption maximum creates a gas bubble around the nanoparticle (Lukianova-Hleb et al. 2013). This event can be recorded either as a pressure pulse (photoacoustic method) or as increased light scattering, using a second (probe) laser pulse applied a short time after the first (actinic) pulse. It is likely that the photoacoustic method will prove to be the more useful for noninvasive detection of malaria infection in the human body.

Figure 5.10 shows that the action spectrum for the vapor nanobubble signal from hemozoin is quite different from that of the absorption spectrum of healthy blood. By choosing 672 nm as the wavelength for the laser pulse, one can avoid any interference from undamaged hemoglobin. No photoacoustic signal is obtained for any wavelength if hemozoin is not present, as is shown by the green curve. Figure 5.11 shows the kind of images that are obtainable with this technique and

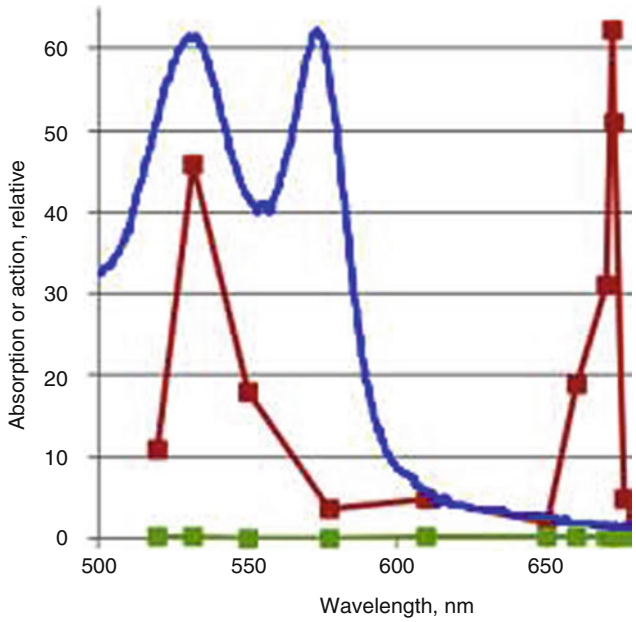


Fig. 5.10 Photoacoustic signal from an infected red blood cell containing hemozoin (*red*) and a healthy cell (*green*). The *blue curve* shows the absorption spectrum of an oxygenated blood, corrected for scattering and normalized to the same maximum as the *red curve* (Data from Lukianova-Hleb et al. (2014), redrawn)

how the hemozoin-containing cells can be destroyed by a laser pulse.

5.12 Quantum Dots

"Quantum dots" is a rapidly expanding field. A search for this term on the Web of Science in September 2014 gave 1,55,500 hits, the first two from 1986. Although the term quantum dot had not yet been coined, a few earlier publications, such as Brus (1984) and Rosetti et al. (1982), do in fact deal with them. During the past decade, each year an average of 926 more papers on the subject have been published than in the preceding year. The main application in biology is for fluorescent labeling of cell constituents. Overviews of biological applications are provided by Michalet et al. (2005), Du et al. (2006), Klostranec and Chan (2006), and Xu et al. (2006). Before going further into biological applications, it is necessary to begin with a general introduction to quantum dots.

A quantum dot is a semiconductor (or sometimes a metal) nanostructure that confines mobile charges, negative electrons, and/or positive holes, in all directions. In this respect, the concept resembles the somewhat older ones, quantum well and quantum wire. However, a quantum well confines the charges in just one dimension and allows them to move freely in the other two, while a quantum wire allows the charges to move freely in one direction. The restriction can have various causes, such as electric potential gradients or interfaces between different materials. Quantum dots usually consist of 100–100,000 atoms. The smallest ones are slightly less than 2 nm across and the largest ones of interest to biologists about 15 nm. Some of them share many properties with atoms, such as having discrete energy levels. Because the charges are spatially confined, quantum dots have optical properties which differ from the properties of larger objects of the same materials. The same principles apply here as for the double bond systems with π electrons described in the chapter on spectral tuning in biology. Thus, smaller size corresponds to a smaller optical wavelength, and just by choosing size, a quantum dot can be tuned, so it absorbs or emits light of a certain wavelength, without adjustment of chemical composition.

Although many scientists regard quantum dots to be semiconductors by definition, structures that behave in much the same way may also be constructed of some metals (Zheng et al. 2007), and therefore, it may also be acceptable to speak about metal quantum dots.

The most popular quantum dot material so far for basic and applied work has been cadmium selenide, CdSe. In biological contexts, the main disadvantage of this material is its toxicity due to release of cadmium ions, but with appropriate coating (cladding), this disadvantage can be decreased (Bakalova et al. 2005; Derfus et al. 2004; Hardman 2006). There are also other reasons to provide the quantum dot core with a cladding. It increases the quantum yield for emission and may modify the wavelength. Quantum dots with CdSe cores are usually coated with ZnS and outside this a shell of something else Bakalova et al. (2006). The outermost layer can be configured with ligands to biological structures, for instance, polynucleotides or antibodies.

The photon energy ($E=h\nu$) and thus the wavelength ($\lambda=c/\nu$) of the light emitted from a quantum dot depend mainly on two quantities, the energy gap E_{bulk} in the bulk material of which the (core of) quantum dot is made and the extra energy arising from confinement of the mobile charge, E_{conf}: $hc/\lambda=E=E_{bulk}+E_{conf}$.

A somewhat simplified derivation from the time-independent Schrödinger equation of a formula for the emission wavelength of a spherical quantum dot with mobile electrons and holes, as, for instance, one made of CdSe, leads to $E_{conf}=h^2/(6.513\bullet 8m_eR^2)$, where m_e is the electron mass and R the radius of the quantum dot. The value of E_{bulk} varies, of course, with the material, and for CdSe, it is $3.85\cdot10^{-19}$ J. Using this relationship, we can produce the solid curve in Fig. 5.12.

As seen in Fig. 5.12, with increasing quantum dot size, the emission wavelength approaches a limiting value. Emission wavelengths up to 700 nm can be obtained with CdSe as core material (Wang and Seo 2006), and other types of quantum dots can be produced that emit above 1,000 nm (see Michalet et al. 2005 for examples). Because, in practice,

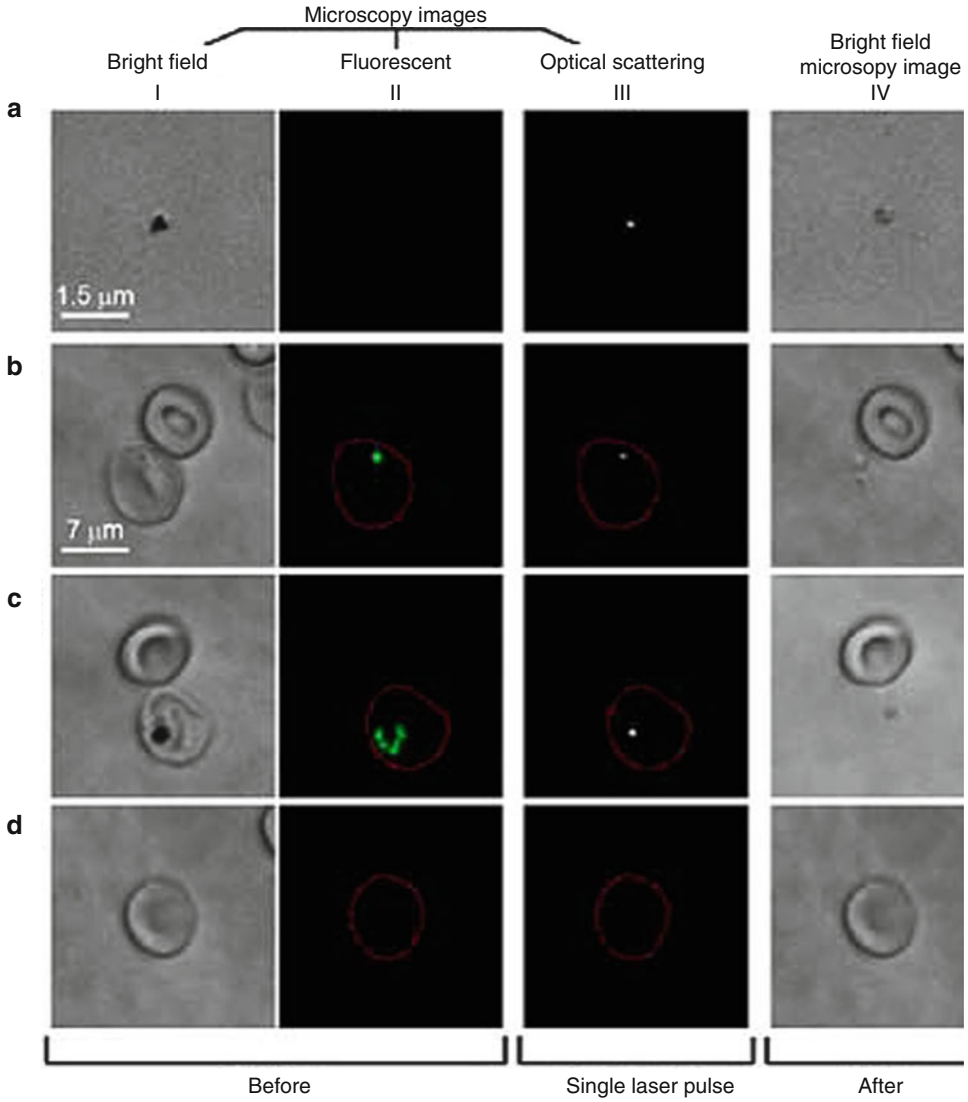


Fig. 5.11 Pulsed laser (532 nm, 40 mJ·cm^{-2}) exposure of isolated hemozoin and cultured human blood cells results in hemozoin-dependent nanobubble generation, which is detectable optically and acoustically and results in infected cell destruction. (**a**) Hemozoin nanoparticles in water. (**b**) Uninfected (*top*) and *P. falciparum* early ring stage–infected (*bottom*) human red blood cells. (**c**) Uninfected (*top*) and *P. falciparum* mature schizont stage–infected (*bottom*) human red blood cells. (**d**) Uninfected human RBC. (*I*) Bright field image shows cells before laser pulse. (*II*) SYBR green I fluorescence image reveals parasite presence before laser pulse. (*III*) Time-resolved optical scattering images of nanobubbles. (*IV*) Bright field images after laser pulse (From Lukianova-Hleb et al. (2014))

all quantum dots in a preparation do not have exactly the same dimensions, the emission is spread out over a spectral band of finite width. However, quantum dots have emission bands more narrow than most fluorescent molecules. The absorption spectrum is, on the other hand, quite wide (Fig. 5.13), so different types of quantum dots can be excited with the same kind of light, which is an advantage when one wishes to image quantum dots emitting different colors at the same time in the same sample.

The power dependence with the quantum dot radius raised to −2 based on the simplified theory is not universal, and Brus (1984) and more recently Wang and Seo (2006) and Cademartiri et al. (2006) derived more accurate relationships. One of these (more relevant for CdSe quantum dots) is shown in Fig. 5.10 (dashed curve).

The main advantages of using quantum dots rather than molecular fluorescent labels are (1) greater stability against bleaching, (2) higher fluorescence quantum yield and therefore stronger fluorescence, (3) more narrow emission bands, and (4) the possibility of continuously tuning of the emission wavelength and choosing any desired value. The first two properties make it possible to follow single-labeled macromolecules as they move around in a cell, are taken up, or are excreted. The second two properties make it possible to label several different kinds of molecule in different colors in the same preparation.

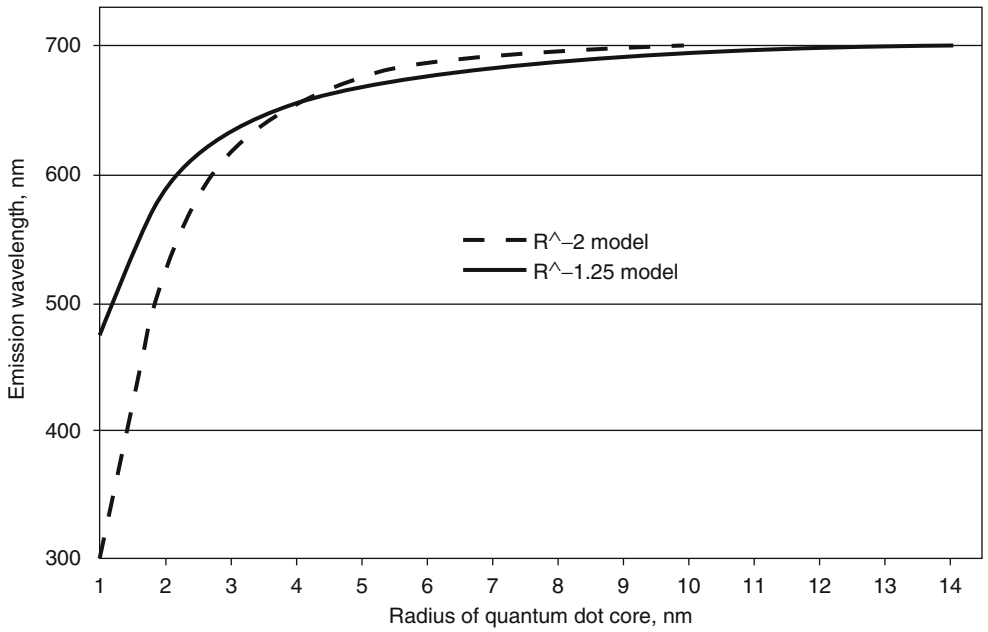


Fig. 5.12 Emission wavelength versus quantum dot radius for spherical CdSe quantum dots without cladding, computed as described in the text. The *solid line* is for the empirical model with exponent −1.25, the *dashed line* for the theoretical model with exponent −2. An E_{bulk} value of $2.78781 \cdot 10^{-19}$ J is used here

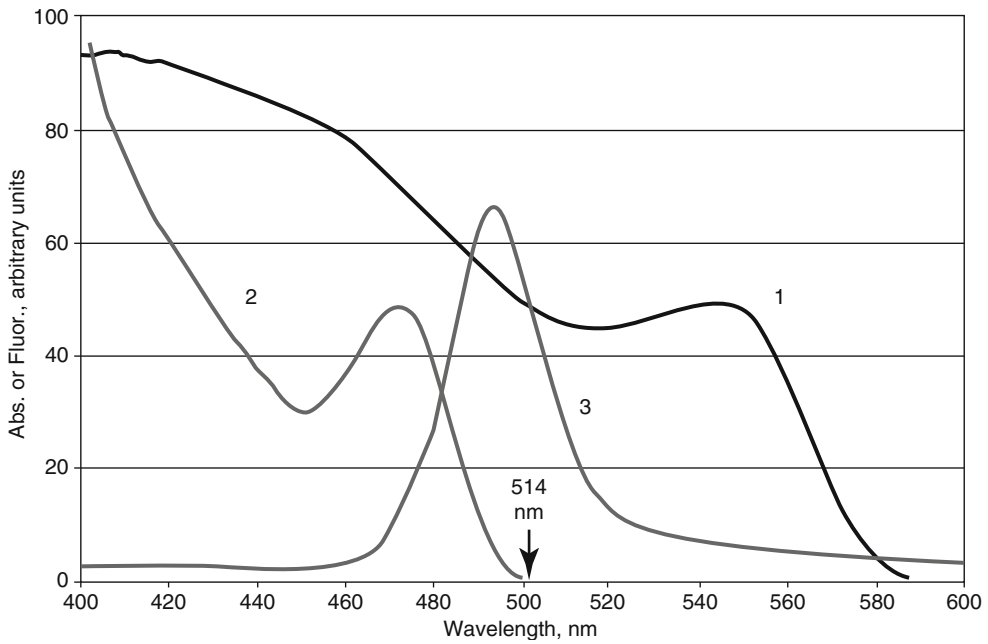


Fig. 5.13 Phototuning of CdSe quantum dots with SiO_2 shells. (*1*) Absorption spectrum of the original quantum dots. The vertical axis does not show absorbance in this diagram, but absorption coefficient divided by scattering coefficient, but this does not make much difference in shape of the spectrum. These original quantum dots have an absorption maximum at 553 nm and an emission maximum at about 572 nm. After photoetching for 20 h with 514-nm light, the absorption spectrum changes to *curve 2* and the emission spectrum to *curve 3*. Quantum dots with other emission maxima can be obtained by photoetching with light of other wavelengths. In every case, the absorption spectrum changes so that absorption becomes practically zero at the etching wavelength (Redrawn and simplified, after Torimoto et al. (2006))

The fluorescence yield of small CdSe quantum dots can be increased to >0.5 by careful control of temperature, Cd/Se ratio, and other factors during production (Donega et al. 2003) and following treatment with ultraviolet radiation (Bakalova et al. 2005). McBride et al. (2006) show that structural relations between the CdSe core and the ZnS shell are important. The quantum yields decrease with increasing quantum dot size (increasing wavelength), which is attributed to decreasing overlap between the wave functions for electrons and holes. Larger quantum dots, on the other hand, absorb more excitation light. Quantum dot size and thus emission wavelength can be varied by varying the duration of crystal growth time at high temperature and also by adjusting the temperature (190–270 °C) for crystal growth (Wang and Seo 2006). A particularly elegant method to tune cadmium selenide quantum dots with SiO_2 shells has been devised by Torimoto et al. (2006), who use monochromatic light of selected wavelengths to etch the quantum dots (by photochemical oxidation of the selenium) until they stop absorbing the etching light. The peak emission will then be at about 20 nm lower wavelength than the etching light. This procedure should also help to decrease the size variation between the particles and thus result in a narrower emission spectrum. The SiO_2 shell serves to prevent the particles from coalescing.

Because of the extremely low detection limits of quantum dot fluorescence labels, they can be employed for various forensic purposes, such as detection of anthrax spores (Park et al. 2006), specific DNA traces (Raymond et al. 2005), or fingerprints (Bouldin et al. 2000; Menzel et al. 2000). Specificity and suppression of background fluorescence interference can be improved by monitoring the fluorescence lifetime (Bouldin et al. 2000).

Among applications on the medical side, early cancer detection (Nida et al. 2005; Chu et al. 2006; Hu et al. 2006; Li et al. 2006) deserves special mention.

One of the most interesting properties discovered during the study of single quantum dots is that even if they are steadily illuminated, they usually do not fluoresce continuously, but intermittently. In other words, they "turn on and off," a phenomenon known as blinking. Although of great theoretical interest, we shall not expand on the reasons for blinking here. In some cases, blinking may be a disadvantage, for instance, if quantum dots are used for tracking molecules which move rapidly in unpredictable ways, but in most cases, it is not a problem because of the high speed of on and off switching. In other cases, blinking can be taken advantage of. For instance, blinking makes it possible to determine whether light comes from a single or two quantum dots (by determining whether the blinks have just one light level or two) and to determine the distance between two quantum dots, even if they are too close to be resolved in the microscope (Lagerholm et al. 2006). Blinking can be suppressed by covering the quantum dots in a special way (Hammer et al. 2006).

For some applications, it may be of advantage to make the quantum dots emit light without being themselves illuminated, and this is possible to achieve by supplying energy in some other way (So et al. 2006). If the quantum dots are tuned to emit very long-wavelength radiation, they may be visible (by instruments) even if buried deep in the body, something that may be of great value for tracing cancers.

Because of the possibility of seeing individual quantum dots, many different molecules have been individually tagged with them, for instance, DNA (Crut et al. 2005) and RNA (Chan et al. 2005) molecules and ion channels (O'Connell et al. 2006; Nechyporuk-Zloy et al. 2006). Since quantum dots can be counted, they have also been used for counting molecules. For such a count to be reliable, however, one has to be able to ascertain that all molecules become labeled, and O'Connell et al. (2006) have worked out a procedure to check if this is the case, using two labels with different colors and seeing how many two-color labeled points there are in relation to those labeled with one color only. In their particular case, they determined that a membrane channel protein could be labeled with a maximum of two quantum dots.

By conjugating quantum dots to signal peptides (used by cells to target proteins to correct organelles), the quantum dots can be directed to specific organelles (Hoshino et al. 2004). Many other examples of adaptations of quantum dot surfaces for different cellular targets are described by Medintz et al. (2005). Mutations in DNA can be detected with a method employing quantum dots (Yeh et al. 2006).

Many different types of quantum dots are now commercially available. Some of them are so easy to make that several student experiments for this have been worked out, both for CdS and for CdSe quantum dots (Fig. 5.14) (Kippeny et al. 2002; Boatman et al. 2005; Winkler et al. 2005). Traditionally, CdSe quantum dots have been manufactured at high temperature using poisonous organic solvents, but Deng et al. (2006) show that it is also possible to work below 100 °C and use water as solvent. In addition to production by these "wet" methods, there exists a "dry" method for manufacture of another kind of quantum dots for the electronics industry.

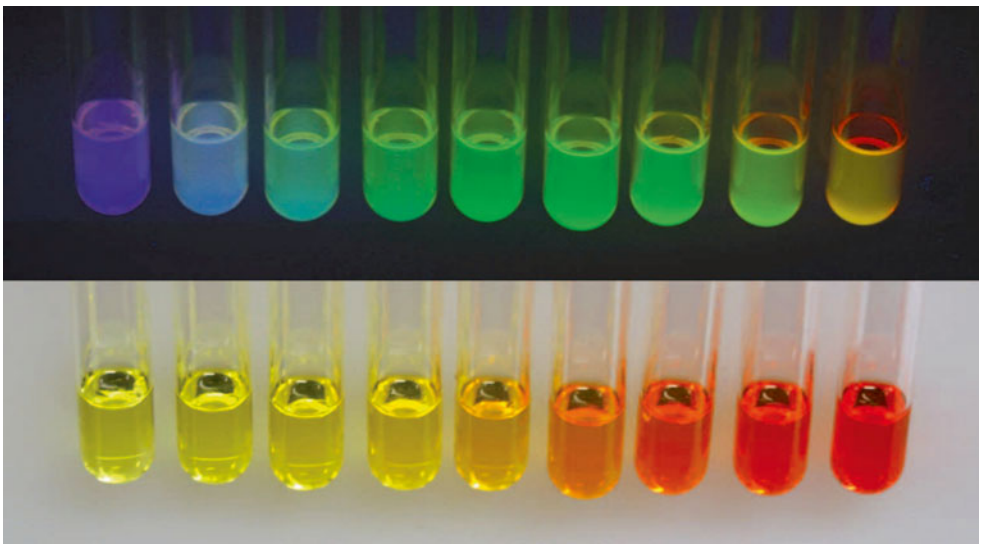


Fig. 5.14 CdSe quantum dots prepared in a student experiment. The *top picture* shows fluorescent colors with UV-A excitation, the *bottom* one the absorptive colors under white light. CdSe quantum dots can be prepared emitting fluorescence at longer wavelength, up to 700 nm (From Boatman et al. 2005 with permission from *J Chem Educ*. Vol. 82, No. 11, pp. 1697–1699; copyright ©2005, Division of Chemical Education, Inc.)

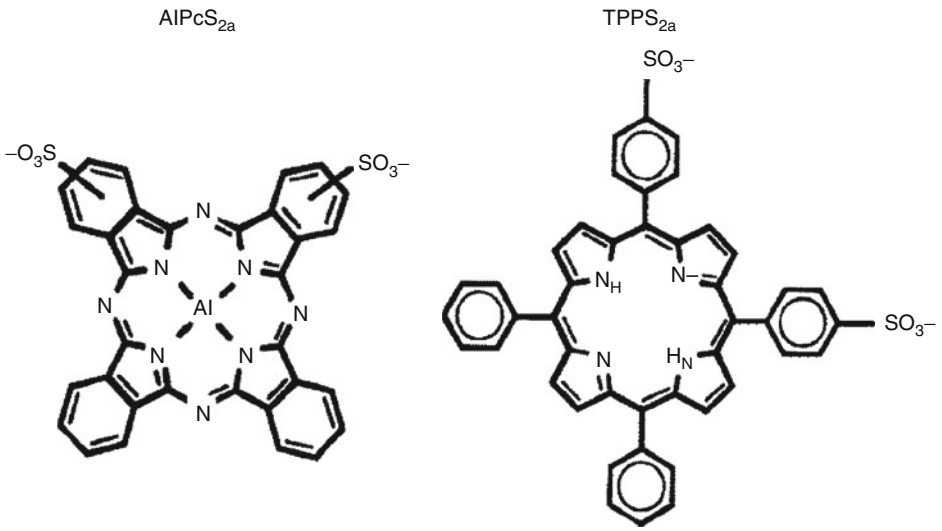


Fig. 5.15 Two photosensitizers that become specifically localized to endocytic vesicle membranes after being taken up into cells. TPPS2a is *meso*-tetraphenylporphine with two sulfonate groups on adjacent phenyl rings, and AlPcS2a is aluminum phthalocyanine with two sulfonate groups on adjacent rings (From Høgset et al. (2004))

5.13 Photochemical Internalization

Photochemical internalization is a technique for delivering macromolecules or other particles to the cytoplasm of cells. Molecules that would otherwise be excluded from the cell can be made to pass through the cell by being combined with a suitable molecule or atom group that causes it to be taken up into a vesicle by endocytosis. A problem with this is that the material taken up in a cell remains in membrane vesicles and may eventually end up in lysosomes and be broken down there. The enclosing membrane can, however, be punctured by photochemical production of singlet oxygen (Berg et al. 1999). Singlet oxygen is very short lived in vivo (Moan and Berg 1991) and therefore does not diffuse more than a few nanometers (20 nm at most) before being destroyed. Therefore, the photodynamic attack on the vesicle membrane is most efficient if the sensitizing pigment is incorporated into the vesicle membrane. Some photosensitizers have, in fact, been found which after uptake in cells become specifically localized to lysosome membranes (Fig. 5.15). The photosensitizer may be administered together with the

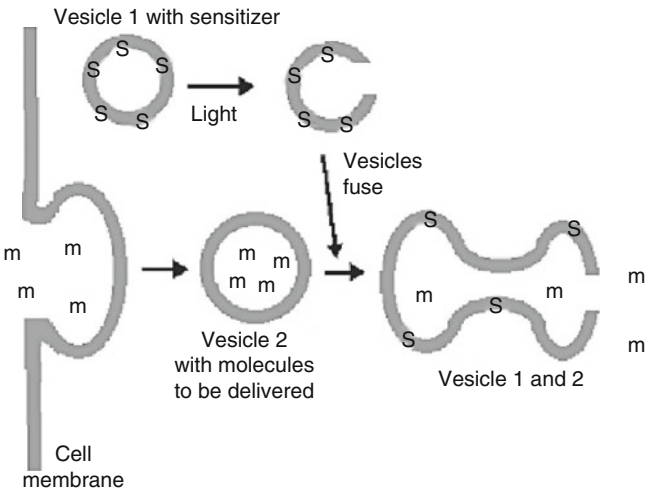


Fig. 5.16 Photochemical internalization using preloaded sensitizer. The cell is first treated with sensitizer(*s*) and the vesicles containing sensitizer punctured with light. When then the molecules (*m*) to be delivered to the cytosol are taken up by endocytosis, the endocytosis vesicles fuse with the already punctured vesicles, and the molecules are released to the cytosol (Redrawn and modified from Høgset et al. (2004))

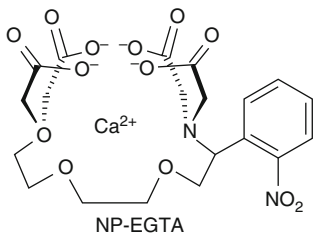


Fig. 5.17 Nitrophenyl ethylene glycol-bis(2-aminoethyl) *N*,*N*,*N*′,*N*′-tetraacetic acid, an example of a substance that can be used to deliver caged calcium ions

substance to be taken up. Alternatively, as shown in Fig. 5.16, the cell may be preloaded with the sensitizer and lysosomes punctured with light.

When afterwards the substance is taken up by endocytosis, the endocytic vesicles fuse with the punctured lysosomes and the substance is released to the cytosol.

An alternative to generation of singlet oxygen for puncturing liposomes is to use photocleavable lipids for constructing vesicle membranes (Chandra et al. 2006).

Many different kinds of molecule and molecular aggregates can be delivered to cells with liposomes in this way, and there are several variants of the method. One is to combine the molecule to be delivered in a liposome with a cationic peptide or cationic polyethyleneimine–amino acid combination. In this way, a faulty (mutated) gene can be replaced with a correct one and find its proper place in the nucleus (Ndoye et al. 2006).

An advantage of photochemical internalization is that it can be very specifically applied to individual cells or groups of cells, for instance, cancers, while a drug remains membrane enclosed and inactive in surrounding cells. Such specificity is achieved both by directing the membrane-puncturing light and by choosing sensitizers which are targeted to, for instance, cancer cells.

The other method for getting molecules where you want to have them in the cell is to attach them to a suitable carrier molecule. We have already mentioned the cell membrane-penetrating cationic peptides, but other ligands can be used for specific purposes. This so-called caged compound can then be set free by breaking the bond to the carrier molecule with light. An example is the delivery of oligonucleotide to the cell nucleus described by Ghosn et al. (2005). They attached the oligonucleotide to 1-(4,5-dimethoxy-2-nitrophenyl) ethyl ester (DMNPE). The DMNPE alters the charge and makes the molecule more lipophilic, so it can pass the membranes into the nucleus. As long as the oligonucleotide is attached to DMNPE, replication is blocked, but after breaking the attachment by irradiation with 365-nm UV-A radiation, the oligonucleotide becomes integrated into the genome. The authors used an optical technique (a so-called molecular beacon) to check whether hybridization took place or not. Alternatives to DMNPE have been investigated by Kim and Diamond (2006). Marriott et al. (2003) describe how proteins can be caged by use of bromomethyl-3,4-dimethoxy-2-nitrobenzene (BMDNB) attached to cysteine residues in the protein. An alternative approach for caging proteins is the introduction of unnatural amino acids through mutagenesis (Petersson et al. 2003).

Calcium ion is an important cellular messenger. The Ca^2+ concentration in the cytosol is usually near 0.1 μmolar. When it is caused to rise, a number of processes, dependent on the type of cell, are initiated. The Ca^2+ is released from organelles, the endoplasmic reticulum, or, in plants, the cell wall. To study these signaling pathways, it is desirable to be able to initiate experimentally a rise in cytosolic calcium concentration without having to activate the initial part of the signaling pathway. This can be done by supplying the cell with caged calcium ions, i.e., calcium ions in a cage of a chelator, which can be photochemically decomposed. Bacchi et al. (2003) describe a number of chelators that can be used in this way, nitrophenyl ethylene glycol-bis(2-aminoethyl) *N*,*N*,*N*′,*N*′-tetraacetic acid (NP-EGTA) being one example (Fig. 5.17).

5.14 Photogating of Membrane Channels and Related Techniques

For a long time, investigators of the nervous system have used electrodes to stimulate nerve cells and find out what kind of signals they conduct and what the result of their activity is. To this, new methods have now been added: photostimulation or photoinhibition.

There are basically three ways of making it possible for light to affect nerves:

1. Photodelivery of caged neurotransmitters. An example of this was provided by Callaway and Katz (1993). They bathed brain slices in a solution containing L-glutamic acid a-(4,5-dimethoxy-2-nitrobenzyl)ester. This compound in itself is inactive. When a brief (1 ms) pulse of ultraviolet radiation is delivered to the preparation, the neurotransmitter L-glutamic acid is released, and a spike of inward current can be recorded. The procedure can be repeated at least 30 times. Photorelease of caged compounds is treated more generally in the previous section.
2. Use of rhodopsins. Natural photogating of membrane channels takes place in our eyes with the help of rhodopsin in the membranes of outer segments of rods and cones. Also, in some microorganisms similar events occur, mediated by chromoproteins similar to our rhodopsins. Both vertebrate rhodopsin and so-called channelrhodopsin from a green alga have been used for artificial gating of other channels than their natural ones (Boyden et al. 2005; Li et al. 2005; Zhang et al. 2006). Genetic engineering is used to express the proteins in the desired tissues and couple them to the signaling pathways for the desired membrane channels. This is one aspect of "optogenetics." The definition of optogenetics varies, but according to Deisseroth (2011), it is "the combination of genetic and optical methods to achieve gain or loss of function of well-defined events in specific cells of living tissue." A general recent overview is provided by Packer et al. (2013).
3. Use of artificial pigments to regulate the channel. These methods can, in turn, be divided into two categories. A molecule that changes conformation upon illumination can be attached directly to the channel protein so that a part of it either blocks the channel or not, depending on the shape of the pigment molecule, or the pigment can be incorporated into the lipid of the membrane and change its conformation. The first method was pioneered by Lester et al. (1980), who chemically modified the nicotinic acetyl choline receptor to make it light sensitive. Banghart et al. (2004) used the method to regulate a potassium channel. They used a compound consisting of maleimide–azo group–quaternary amine. The maleimide reacted chemically with a cysteine sulfhydryl group in the channel protein, the azo group undergoes *cis–trans* isomerization upon absorption of light, and the nitrogen in the amine carries a positive charge, which, in the correct position, can prevent potassium ions from passing the channel. The arrangement is shown in Fig. 5.18. The *cis–trans* isomerization of the compound is photoreversible, and the photoequilibrium spectra (of same shape as action spectra) are shown in Fig. 5.19.

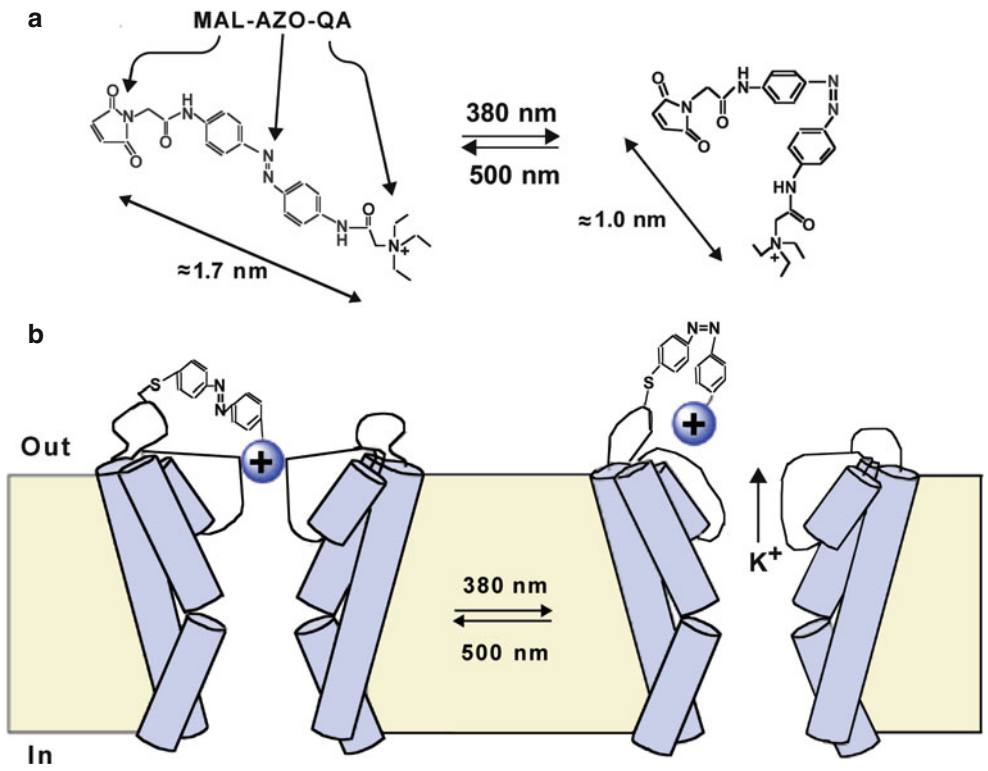


Fig. 5.18 (**a**) The reversibly photochromic chromophore consisting of a maleimide group (MAL), an azo group (AZO), and a quaternary ammonium nitrogen (QA). The latter is, in turn, attached to the potassium channel protein. When the chromophore absorbs 380-nm radiation, the distance between its ends decreases by 0.7 nm (7 Å). (**b**) The potassium channel is reversibly blocked by the positive charge on the chromophore by 500-nm light and opened again by 380-nm UV-A radiation (Reprinted by permission from Macmillan Publishers Ltd: *Nature Neurosci.* 12, 1381–1386 (Banghart et al. (2004), Copyright 2004)

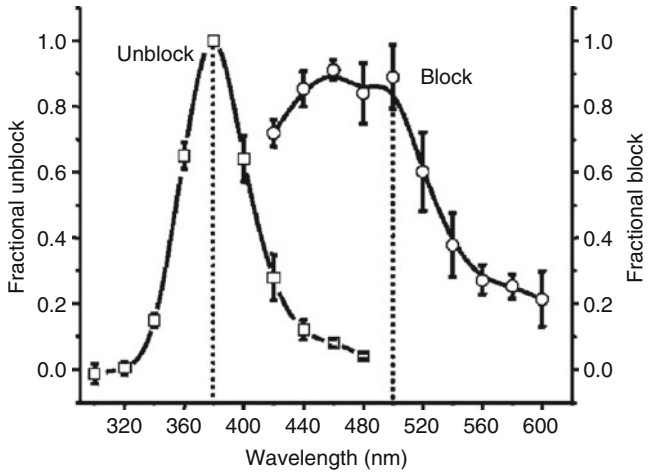


Fig. 5.19 Steady-state spectra for open and closed states of the potassium channel in Fig. 5.16. Steady-state spectra are action spectra normalized so that the sum of the ordinates of the curves is unity in the overlap region (From Banghart et al. (2004))

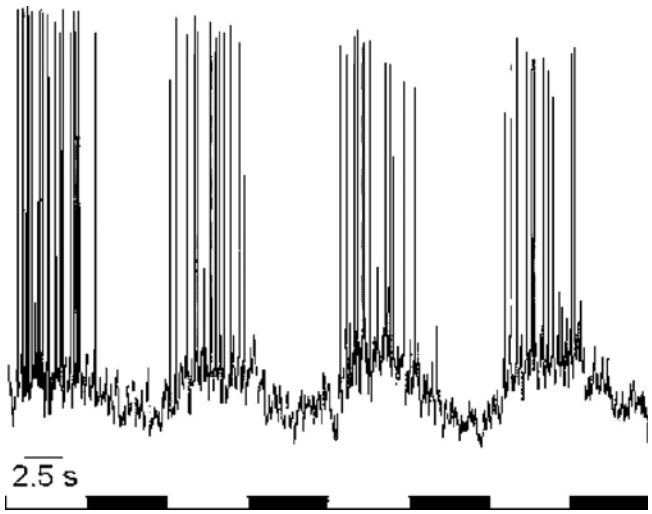


Fig. 5.20 The action potentials in a nerve cell are rapidly switched on by 390-nm radiation (*white bars*) acting on a modified membrane channel and equally rapidly switched off by 505-nm light (*black bars*) (From Chambers et al. (2006))

Membrane channel proteins can be stimulated electrically and in many cases also chemically. So what are the advantages and disadvantages of photoregulation? A particular cell can be probed with either an electrode or a light beam, while you can only use light to choose a particular kind of (modified) channel. The disadvantage with light is that you must modify the channel to make it light sensitive. With chemical stimulation, you can reach a particular channel type, but both spatial resolution and temporal resolution are extremely bad, especially compared to what can be achieved with light. As shown in Fig. 5.20, the switching of a channel on and off with light can be done very rapidly—within milliseconds.

Many of the experiments with photoregulation of membrane channels have been performed on nerve cells, and the results can be quite dramatic. Schroll et al. (2006) show how the behavior of fly larvae, in which neural cation channels have been amended with channel rhodopsin, can be altered just by illuminating the animals with blue light. Depending on what particular channel type has been modified, different behavioral changes result. Here we see another advantage of light stimulation over electrical stimulation: the animals can be completely unattached and free moving, and all the channels of a particular type can be immediately activated at once.

Folgering et al. (2004) show how a mechanosensitive channel can be regulated by light by incorporating a *cis–trans* photoreversible phospholipid-mimicking molecule into the lipid bilayer, so that the membrane can be photoreversibly distorted. So far, this has only been done in vitro in patch-clamp experiments, but it does not appear impossible to use a similar procedure with living organisms. Koçer et al. (2005) also made a mechanosensitive channel light switchable, but by a quite different method. By genetic engineering, they replaced a glycine residue in the channel protein by a cysteine residue. The sulfhydryl group of this cysteine, which was the only one in the protein, could then be reacted with a photochromic compound, by which the channel could be reversibly blocked by irradiation with visible light (λ>460 nm) and opened again by irradiation with UV radiation (366 nm).

Eisenman et al. (2007) have constructed a photoactivatable neurosteroid by which $GABA_A$ receptor function can be light regulated. It can be used for blocking neuron firing by 480-nm light.

Promising animal experiments for restoring vision in blind individuals have been done by many groups, including Doroudchi et al. (2011). These as several other researchers, used virus to introduce channelrhodopsin 2 into the defunct receptor cells. Channelrhodopsin 2 has the advantage of producing the required transport of sodium ions resulting in fast (within 50 ms) depolarization of the membrane in a more direct way than the natural system. The light effect is directly on the ion channel, while the natural system employs intermediate messengers. As long as only the natural channelrhodopsin 2 is used, vision is monochromatic with maximum sensitivity in the violet region. A recent thorough description of this field is provided by Natasha et al. (2013).

Chen et al. (2013) modified the plant UV-B receptor to create a system by which protein secretion can be controlled by UV-B and which can be used for studying protein trafficking in neurons.

5.15 Photocrosslinking and Photolabeling

It is of interest to know which molecules make contact with one another in the cells without forming strong and stable bonds. The three kinds of problem most often encountered

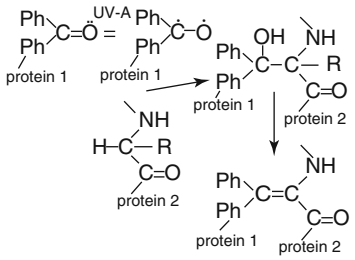


Fig. 5.21 Photocrosslinking two proteins using a protein (protein 1 in the figure) in which p-benzoylphenylalanine (pBpa) has been inserted. Ph stands for the phenyl group. Under irradiation with UV-A, the pBpa is converted to a diradical, which cross-links to an amino acid in protein 2. If R in this amino acid is hydrogen, dehydration takes place, resulting in the structure at the lower right. A more detailed diagram, showing intermediates and alternative reactions, is provided by Dormán and Prestwich (1994)

are elucidation of protein–protein contacts, protein–DNA contacts, and hormone–receptor contacts. Photocrosslinking and photolabeling are methods that have been used for this purpose for four decades. The older literature is reviewed by Brunner (1993), and emphasis here will be placed on specific recent developments.

For a couple of decades, it has been possible to incorporate unnatural amino acids into proteins in a site-specific way in vitro (Bain et al. 1989). A great leap was taken when Wang et al. (2001) designed a method for doing this in vivo. Soon thereafter (Chin et al. 2002), the method started to be used for incorporating photoreactive amino acids, which upon exposure to ultraviolet radiation form bonds to nearby protein molecules. In this way, the in vivo contact points between proteins can be determined. It would carry too far to describe the details of the method here, but up-to-date accounts are given by Xie and Schultz (2005, 2006). In brief, the function of the "amber" codon (UAG) is modified and its natural function suppressed and, using molecular components from Archaea, a translation system is engineered, which uses this codon to insert the desired amino acid. Later, the "opal" codon (UGA) was used for the same purpose. The first experiments dealt with insertion of the engineered systems into bacteria, but by now, researchers have succeeded in doing the same also with eukaryotic (including mammalian) cells. The types of unnatural amino acids that have been inserted now number over 30, and among them are three photoreactive ones, *p*-azidophenylalanine, *p-benzoylphenylalanine* (pBpa) (Fig. 5.21), and *p*-(3-trifluoromethyl-3H-diazirin-3-yl)-phenylalanine. Among these derivatives, pBpa is particularly useful, because it reacts to UV-A radiation (about 360-nm wavelength), which is not very destructive to cellular components and also gives greater penetration depth than the shorter-wavelength radiation necessary for the other photoreactive amino acids. A slight disadvantage to pBpa is that it has a preference for reacting with methionine. Therefore, it does not necessarily indicate the closest neighbor in an adjacent protein but one up to eight amino acid units away from it (Wittelsberger et al. 2006).

Results obtained by this method include elucidation of the contact between ATPase and a transport protein (Mori and Ito 2006), contacts between the components in dimeric enzymes (Blanck and Mehl 2006), and contacts between proteins in a signaling pathway (Hino et al. 2005).

When cells are irradiated with UV-C radiation, cross-links can develop between nucleic acids and adjacent proteins without any prior modification of either. These cross-links, however, are not very informative since they do not necessarily reflect closest neighbor relations. The use of UV-C also has drawbacks because of its destructive action and poor penetration in living matter. Therefore, Geyer et al. (2004) and others have developed more specific methods, which involve the addition of specific photoreactive groups that respond to UV-A radiation.

Also, some hormones can be photocrosslinked to their receptors without artificial photoreactants. Thus, the first photocrosslinking of a plant hormone, abscisic acid, was performed without prior modification (Hornberg and Weiler 1984). Also, steroid hormones containing unsaturated ketone groups can be photocrosslinked in the natural state (e.g., Gronemeyer and Pongs 1980; Schaltmann and Pongs 1982). If the hormone carries a label, such as a radioactive carbon atom, the hormone receptor is said to be *photoaffinity labeled.* But such experiments have later been complemented using hormones linked to artificial photocrosslinking groups (e.g., Todoroki et al. 2001), and for many other hormones, artificial photocrosslinkers are required (e.g., Beale et al. 1992; Swamy et al. 2000). To determine how RNA molecules contact ribosomal proteins, Demeshkina et al. (2003) used mRNA analogs containing a perfluoroarylazido group. Arylazido derivatives are highly reactive and can therefore be expected to react with and indicate close neighbors (not only amino acids in proteins but also bases in nucleic acids). The perfluoro group increases the lifetime of the excited singlet state and therefore also the reaction quantum yield.

5.16 Fluorescence-Aided DNA Sequencing

The Sanger method for DNA sequencing has enjoyed a remarkably long life in the rapidly developing field of molecular biology. However, the strong interest in DNA sequencing for phylogenetic research, forensic investigations, and other purposes has stimulated the search for cheaper and faster methods and methods allowing sequencing of single molecules. In the past few years, several methods using fluorescence techniques have been invented (Metzker 2005). Most of them are based either on the capillary electrophoresis introduced by Jorgenson and Lukacs (1981), or on

immobilizing DNA molecules on a substrate (Seo et al. 2005; Krieg et al. 2006), or on immobilizing an enzyme. Some of them can be used for sequencing single DNA molecules (Werner et al. 2003; Ramanathan et al. 2004; Bayley 2006). There are methods for labeling the bases in DNA with fluorophores with either absorption or emission spectra sufficiently different so that the bases can be distinguished by fluorescence (e.g., Lewis et al. 2005; Seo et al. 2005).

Another fluorescence method important in genomic research is fluorescence in situ hybridization (FISH) (see Jiang and Gill 2006 for a review on the application of the method to plants and references to other literature). In this method, a sequence-specific probe that is made to fluoresce is attached directly to DNA while it is still in the chromosome.

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