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Introduction to Bioimaging

Bioimaging can be defined as visualization of a biological object. The most basic bioimaging may be just "seeing" the living object using our own eyes. This function is called "vision" and the procedure is mediated by visible light. The visible light is a part of electromagnetic wave in the wavelength range of 400–700 nm, and the image information is generated by the interaction between light and object, such as reflection, scattering, and diffraction. The generated information-rich light package travels and reaches our eyes. The focused light through lens would be projected to the screen as in a camera. The retina in our eye is the screen of the image, which is composed of the two-dimensional array of optic nerves. The photon in light signal (containing the information of the object) reaches the retina and activates optical neurons, and the signal is transferred to the brain and is reconstructed into the image of the object by neuronal processing. Even though the screen is two-dimensional, the processed images via two retinas provide three-dimensional information about the shape and distance of the object. Visible light travels at a so-called speed of light (3×10^8 m/s), so the information transfer in the vision could be almost instantaneous. If there is a possible delay, it may be from the signal transition step from the optical nerve to the brain and the information processing time in the brain.

Bats live in the dark environment without enough environmental light for vision. Instead, they use ultrasound for bioimaging platform. If other conditions are same, the light vision could be million times faster than ultrasonic sensing (340 m/s) (Figure 1.1). Among all the sensors, light vision is the fastest and most information-rich system. Therefore, the invention of eye (in more general term, photoreceptor) is one of the most dramatic events in the evolution of life. Due to the high quality and also huge quantity of information, vision is the most important sense, easily accounting for more than 90% of information we receive through all other senses, including hearing, taste, smell, and touch.

1.1 Color

Visible light sensing not only generates black-and-white images, but also can provide color information. The visible light is composed of a spectrum of electromagnetic wave in the range of 400–700 nm. Human eye has three color photoreceptors,

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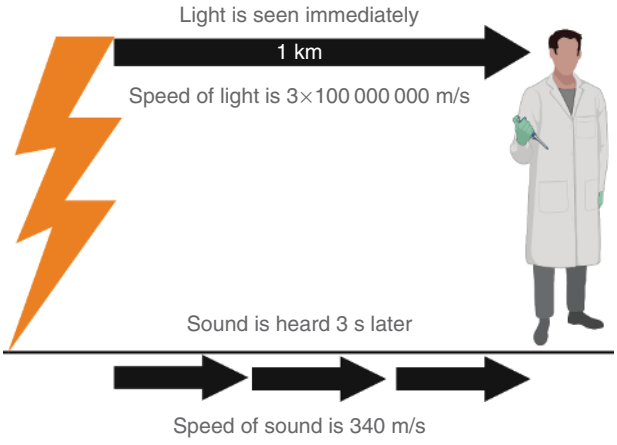


Figure 1.1 Vision through light and sound in different speed.

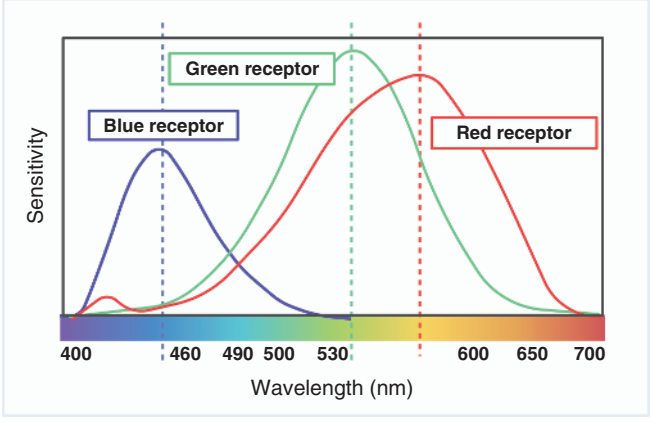


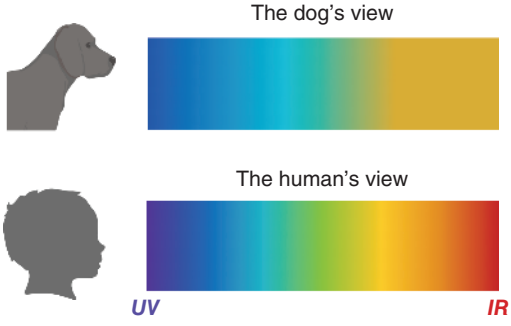
Figure 1.2 Three color receptors and their sensitivity to different wavelengths.

of which the maximum sensitivity is for blue (445 nm), green (535 nm), and red (575 nm) (Figure 1.2). For example, when we receive 445 nm light, we sense it as a blue color, and 575 nm light as a red color. Therefore, color recognition is the ability of sensing different wavelengths of light. And, the term spectroscopy is derived from spectrum, i.e. spectroscopy is the study of the wavelength-dependent interaction between the light and the object.

If we have three color receptors, then do we recognize only three colors? No, it is not. At least, we give seven names of color to the rainbow! Our color sensors have the maximum sensitivity to a specific wavelength, but the sensing wavelengths are broad and overlap with each other. If the eye receives 560 nm light, both green and red receptors are activated, and we sense it as a yellow color. The light with 590 nm will more strongly activate red receptors and less strongly green receptors, making the color as orange. That means our color sense is determined by the ratio of the three

Figure 1.3 Comparison between black-and-white picture and colored picture.

Figure 1.4 The color spectrum of dogs and humans.



receptors' activation degree. Using the three-color receptors, the distinguishable colors by human eyes are more than 10,000! With this ability, we can find our food (e.g. red apple) better, also our enemy (e.g. red ant) faster (Figure 1.3). We can imagine how useful this ability is to help us survive better during the evolution process.

Interestingly, this three-color recognition is not common to all animals, even to mammals. Only our very close cousins such as chimpanzees and gorillas have three color receptors, but even then not all the monkeys do. Including our remote cousins, dogs and cows have only two-color receptors. It sounds trivial whether it is two or three. But, with two color receptors, the distinguishable colors are narrowed down only to the level of 100! It is the difference between two- and three-dimensional combination power (Figure 1.4). Therefore, the visions of cows and dogs would be much more boring than the colorful flowers and spectrum of rainbow we see. This is why many sensors are designed for color change to achieve maximum effect to the naked eyes. Our eyes are a wonderful color sensory system!

There is a funny story in the bull fight. The fighters use red cloth to stimulate bulls, as red color may be related to the image of blood. Funny thing here is the bull may see red color more like dark gray rather than bloody red. The red cloth is to stimulate the audience, not the bulls at all!

Color blindness arises when part of the color receptor is defunctionalized. In humans, most common type is green-red blindness, which occurs when either green or red receptor has problem. If you look at the receptor property carefully, you may realize that the maximum wavelengths of green (535 nm) and red (575 nm) receptors are rather closer, compared to that of blue (445 nm). We call the receptors green and red, but they are more like yellow and orange. To maximize the combination power in color contrast, this design may not be the optimum choice. If we design the color pixel of a computer screen, we may choose more even distribution of the colors, such as 465, 525, and 630 nm [1]. Not surprisingly, the green and red receptors are structurally closer to each other, implying that they evolved from the common ancestor. So, we can imagine, a long time ago, we also had two color receptors similar to dogs or bulls (blue and yellow), and the yellow receptor diverged to two receptors, green and red. Without this evolution of color receptors, we might not be able to enjoy the beautiful sunset!

1.2 Colorful Material

The synthetic colorful materials are mainly organic dyes and inorganic pigments. Conventionally, dye is defined as the material that imparts its color to other substances, such as fabric or tissue. Usually, dyes are soluble in solvents, but pigments are insoluble solids. For printing purposes, pigment powder needs to be dispersed into a liquid binder before use.

On the earth, the strongest light source is the sun. To minimize the background of light sensing, our visionary system adjusts our sensors to recognize the sunlight as a background, called “pure white.” White light is not the status of no color, but it is the collection of all the colors included in the sunlight. The colors of the white light can be manually separated into a spectrum by a prism through a process of dispersion, which is the same mechanism of rainbow formation. Therefore, white is the combined color of all the visible light in the rainbow.

The color of the colorful materials is determined by the wavelength of the absorbed light, i.e. leftover reflected color after absorption of white light. Therefore, the appeared color is complementary to the absorbed color. The concept of complementary color has been known for a long time and is widely used in painting art for vivid color contrast. Even though the wavelength of visible light is in linear scale (400–700 nm, violet to red), our color receptors deceive our color recognition due to the tiring of receptors. The relationship of the complementary color in our color sensing system is described in a color wheel (Figure 1.5).

A chromophore is the part of the molecule which is responsible for the color. The chromophore of inorganic pigments is usually is transition metal, which has a visible light range of electron excitation energy. The chromophore of organic dyes

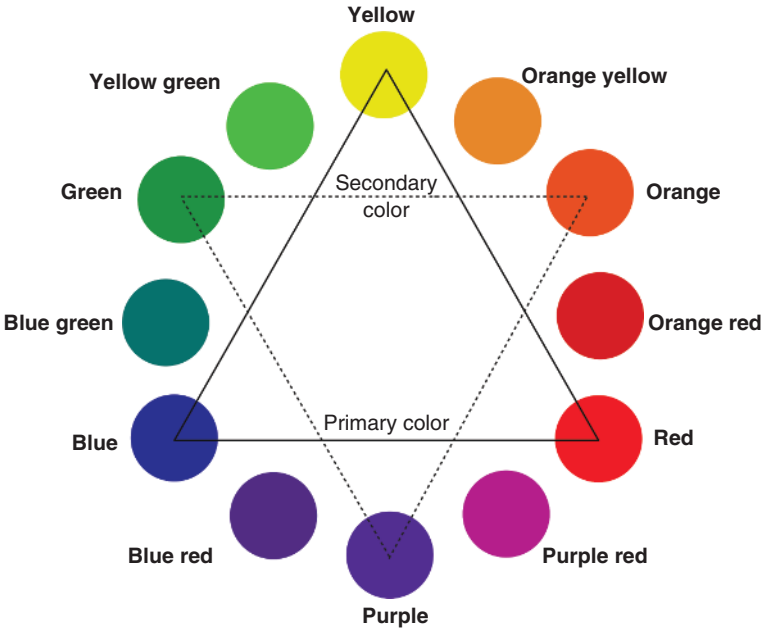


Figure 1.5 Color wheel.

is a long-conjugated double bond system. The light absorption had been modeled in early quantum mechanics era through a particle-in-a-box model, which later led to Schrödinger equation for atomic structure of electrons. Interestingly, the organic conjugation system could be described as a particle-in-a-box model, where the σ bond electrons define the size of the box and π electrons are the particles in the box. As the box size becomes bigger, the wavelength of absorption light gets longer, through the narrowing electron transition gap. When the absorption maximum reaches the boundary of visible light (violet color), the appeared color of the material would be the complementary color of violet, yellow (colors in opposite direction in the color wheel). If the conjugation gets longer, the absorption maximum moves from violet to blue and then green. Accordingly, the appeared color changes from yellow to orange and then brown. You may recall the old books turn into the yellow color first, and then change into reddish tone. This is the result of the extension of conjugation in the lignin component in the paper pulp and is one of the examples of the organic dye model of particle in a box (Figure 1.6).

However, if the conjugation is too long, any oxidation or reduction reaction in the middle of the chromophore will break the conjugation bridge, which is the principle of bleaching agents (either oxidants or reductants). That means the dye with a long conjugation system is weak for the chemical damages, and usually the color of naturally occurring organic dyes easily diminish over time. As a result, the color of simple carbon-conjugated systems is not vivid, as shown in the paper of old books. In the nineteenth century, German organic chemists opened the way to synthetic dyes to replace the natural dyes. Adding electron-donating and -accepting motifs at

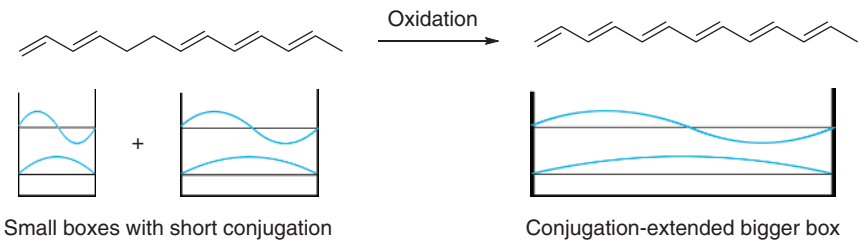


Figure 1.6 Conjugation and the maximum wavelength of the absorption light.

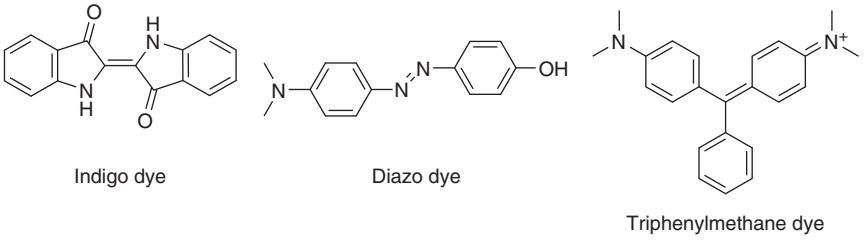


Figure 1.7 Representative structure of dyes.

each end of the conjugation system provides a stronger dipole, which makes the conjugation effect longer and also makes the absorption stronger to give a vivid color. So, most of the synthetic dyes are composed of the conjugation system with electron-donating and -accepting motifs at each end (Figure 1.7). The wavelength of absorption of the organic dyes can be predicted by molecular orbital calculations, and Pariser–Parr–Pople (PPP) method is one of the best-known models [2].

1.3 Light Source of Bioimaging

If bioimaging is visualizing a biological object, which part of the body is the object? If we take the daylight photography as an example, we can use a casual camera catching the visible light to visualize the surface of our body. The surface imaging is easy, and the damage by light exposure to skin is trivial. However, what if we want to visualize the inside of our body? Usually we use catheters composed of a metal tube with a light source and camera on the tip, and insert them through the mouth or anus to visualize the gastrointestinal tract (Figure 1.8). While we call this technique as an endoscopy, is this really inside of our body? Well, compared to the exposed skin surface, the gastrointestinal tract seems more like a hidden part of our body. But, topologically speaking, if we consider our body to be donut shaped, the surface of the gastrointestinal tract should be considered as part of the outside, not the real inside.

In contrast, if the camera visualizes the beneath skin area, we may consider it as a real inner part of our body imaging. For this real endoscopy, one possible way may be to put the camera penetrating the skin to reach the target tissue. However,

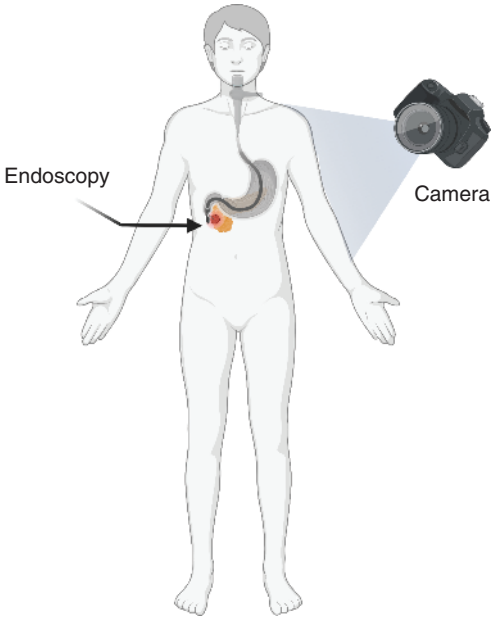


Figure 1.8 Regular camera and endoscopy.

if it is not really necessary for treatment purposes, such an invasive approach may not be desirable for humans or any live animals. If it is not physical penetration of the camera, how about light penetration? If light penetrates the skin reaching the inner space and returns back with the information of the target area, it would be much less damaging than physical insertion of the camera. In this case, the penetration depth of light would be an important factor. If our skin is like a transparent jelly fish, the inner world imaging may be straightforward. The term "transparency" itself implies free penetration of visible light. Unfortunately, our body skin is not so transparent, and most visible light can penetrate at most several millimeters of depth under the skin. Then, how can we increase the penetration depth of light through the tissue?

Visible light lies in the 400–700 nm range, and there are other light sources outside of visible light. The shorter wavelength of visible light comprises ultraviolet (UV), X-ray, and γ-ray, etc. The longer wavelength makes infrared (IR), terahertz light, microwave, and radio wave. Coming back to tissue imaging, the penetration of electromagnetic waves is diminished by mainly scattering and absorption of the light source in the tissue. Absorption wavelength is dependent on the tissue composition, but the scattering is usually higher in shorter wavelength light. So, longer wavelength light tends to penetrate better than the shorter wavelength light, by reduced loss by scattering. For this reason, near-infrared (NIR: longer than 700 nm light up to 1000 nm) light is a popular optical imaging source for noninvasive tissue imaging or whole-body imaging of small animals such as mice. Recently, even longer wavelength of 1000–1700 nm is popularly used for bioimaging as the second NIR window or NIR-II [3]. With further reduced scattering and negligible autofluorescence, NIR-II may provide a higher signal-to-noise ratio and deeper tissue

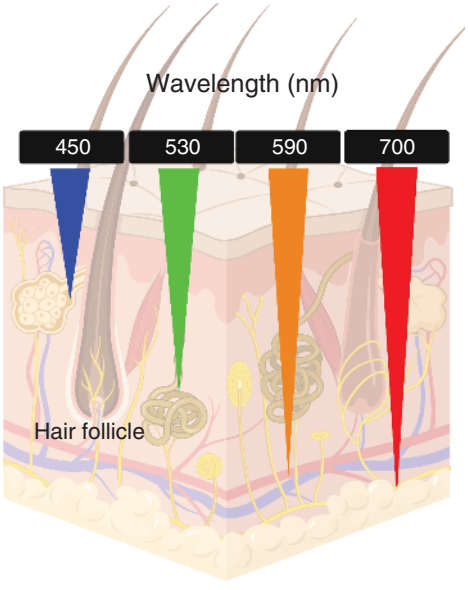


Figure 1.9 Penetration of light into the skin.

penetration than the conventional NIR imaging (Figure 1.9). As our eyes cannot sense NIR directly, the detected NIR should be converted to artificial visual light image as in the night vision goggle of battle field. The green color in the night vision goggle image is a processed artificial color. Green is the usual choice of color due to its best sensitivity to our eyes. NIR is better than visible light for the penetration depth, but still it is difficult to proceed further than a centimeter into the tissue.

The even longer wavelength light sources, such as microwaves or radio waves, have better penetration through the whole body and have been used in magnetic resonance imaging (MRI). MRI requires a high magnetic field to separate the nuclear spin energy status of protons in the body. The separated energy gap of nucleus absorbs microwaves and MRI detects the signal of the relaxation of the absorbed microwave light. Protons in different environments (such as water or lipid) generate distinguishable signals and through a computed tomography (CT), three-dimensional sectional images could be constructed. MRI is a noninvasive CT technique, especially useful for soft tissue (which contains protons) imaging.

If we go to the other direction of shorter wavelength light, there are still possibly different applications in bioimaging. In the X-ray range, the wavelength of light is a hundred times shorter than visible light, and the photon of X-ray would be small and rigid. If visible light photon is like a tennis ball, X-ray is like a needle and can easily penetrate soft matter. Thus, X-ray images mainly show the rigid bone structure, through which X-ray cannot penetrate (Figure 1.10). By adopting CT techniques, X-ray three-dimensional imaging has been well developed even before MRI is introduced. Due to the order of historical development, conventionally the term "CT" is used for "X-ray CT", unless other description is provided.

Although most of the X-ray light does not interact with soft part of the body, in a molecular level, there could be a small amount, but strong damage to

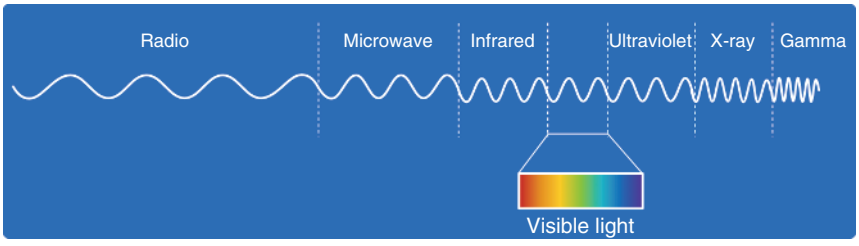


Figure 1.10 Electromagnetic waves as the light source of bioimaging.

the biomolecules can occur by breaking the covalent bonds or ionization. The accumulated damages in DNA can cause mutations of cells, resulting in cancer in somatic cells and mutagenesis in fetus. So, excess amount of exposure to X-ray is not recommended due to health concerns, especially for pregnant women.

γ-Ray has a shorter wavelength than X-ray and the higher energy allows its penetration even through bones, the hardest part of our body. The intensive γ-ray can be used for tumor treatment, which is called as γ-knife technique. To minimize the damage to normal tissue, multiple sources of γ-ray are used from different directions, and only the tumor site is focused to accumulate a high density of γ-ray. In principle, γ-ray also can be used for bioimaging in a similar way of X-ray imaging, but it is not common in practice. Instead, γ-ray-generating radioactive materials are used as imaging agents. In this case, the γ-ray is not provided from outside as in the X-ray method, but is irradiated from inside of the body, through an administered imaging probe into the target site of the body. The position of the isotope could be imaged through a γ-camera similar to an X-ray film. When CT technique is combined with γ-ray-generating radioactive isotope, a three-dimensional single-photon emission computed tomography (SEPCT) imaging is also possible. A similar, but higher performance technique is positron emission tomography (PET). In PET, instead of a direct γ-ray-generating isotope, a positron-generating isotope is used. Positron is a positively changed electron, a kind of anti-particle of electron. When a positron meets an electron, they are annihilated, generating one pair of γ-ray photons. As the two γ-ray photons travel in direct 180° providing richer information for the original position of positron, usually the spatial resolution of PET is better than SPECT. It is noteworthy that X-ray uses an external light source for the imaging, but SPECT and PET use endogenous γ-ray generated from an isotope-labeled probe (Figure 1.11). That is why SPECT and PET are called molecular imaging techniques, in contrast to X-ray imaging.

As shown earlier, electromagnetic waves with different wavelengths from visible light also can be used as the light source of various imaging techniques, when coupled with a proper detector or camera system. The different wavelengths of light render different modes of interaction with matters, and each can generate unique information for the target object. Therefore, unexplored areas of electromagnetic waves would provide novel chance of new imaging technology or modality. Terahertz light is such an emerging new source of light.

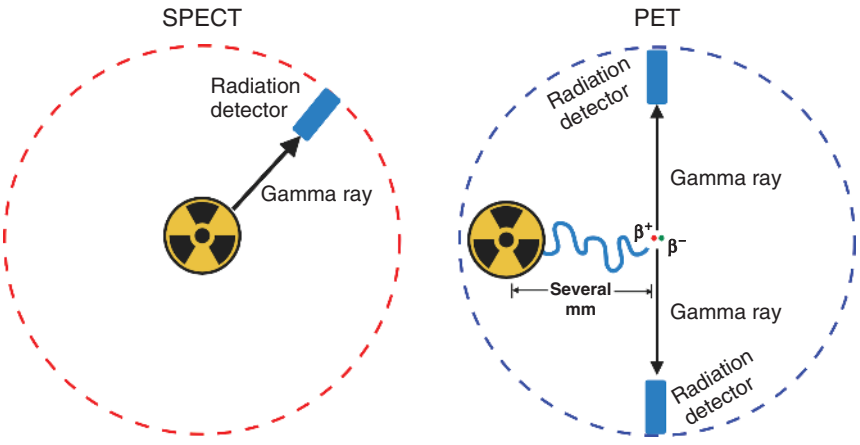


Figure 1.11 Endogenous γ-ray imaging in SPECT and PET.

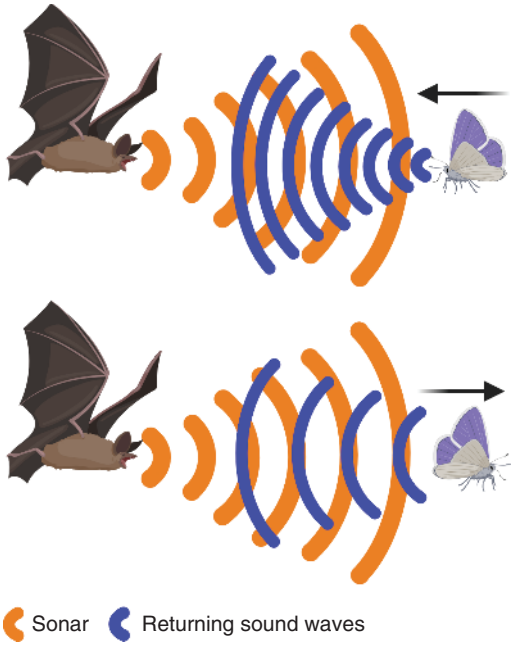


Figure 1.12 Sound imaging of bats.

Not only electromagnetic waves, sound waves or seismic waves also can generate processed images through interaction with matters. The bat's vision through ultrasonic waves would be a good example. Combining electromagnetic waves and sound waves for improved or unique imaging technique, such as photoacoustic imaging, is also a powerful visualization technique (Figure 1.12).

Electron beam is another source to provide ultrahigh-resolution imaging of materials. There are several modes of electron microscopy, such as transmission electron microscopy (TEM) and scanning electron microscopy (SEM). In TEM, an ultrathin sample is irradiated with an electron beam and the transmitted electrons

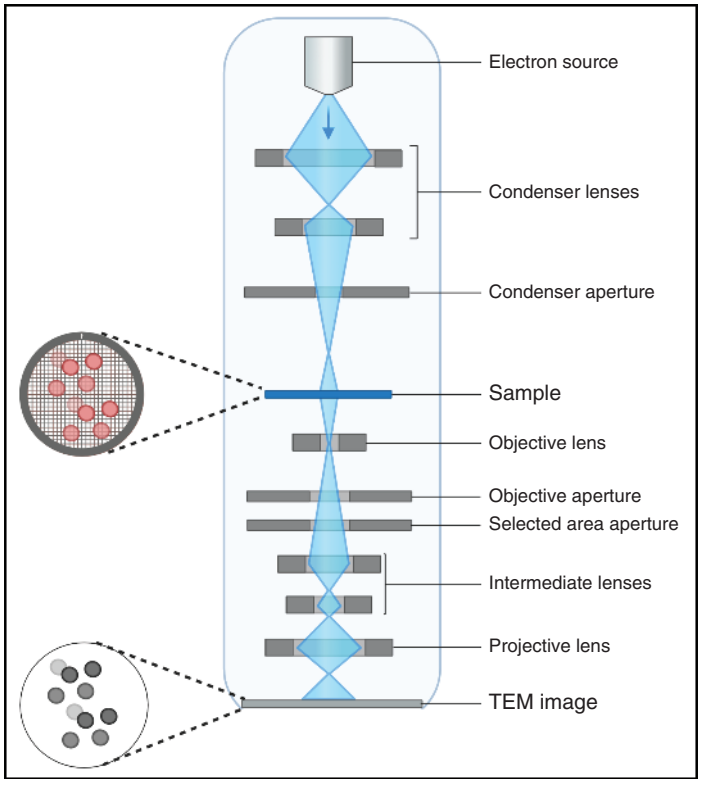


Figure 1.13 Transmission electron microscopy.

are used for the two-dimensional image construction, which is similar to X-ray imaging. In the sample area with a high electron density, the input electron beam would be scattered and may not transmit, generating the dark contrast in TEM image. Therefore, heavy metals are absorbed to the sample to enhance the contrast, and the procedure is called electron staining. The electron staining can also be achieved by an organic dye. After diaminobenzidine (DAB) is stained, through photooxidation, an electron-dense precipitate can be formed to increase the TEM contrast, which is similar to dye staining in the optical imaging.

In SEM, the incident electron beam interacts with the surface atoms of the sample and generates back scattered electrons or secondary electrons. The incident beam is focused on a sample spot and scan the surface, and the detectors are located in the same side of the input beam. As a result, SEM image shows the surface morphology with three-dimensional information especially provided by secondary electrons. The resolution of SEM image is in nanometer range, and usually TEM has higher resolution than SEM. While optical imaging suffers from the diffraction limit in sub-micrometer range, electron microscopy provides much higher resolution. By the imaging resolution scale, electron microscopy could be called as "nanoscopy," rather than microscopy. Both techniques require vacuum condition for the imaging due to the electron beam usage (Figure 1.13).

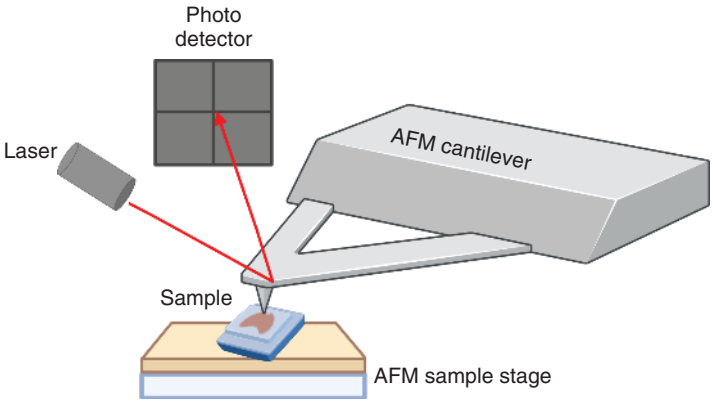


Figure 1.14 Atomic force microscopy.

Atomic force microscopy (AFM) is another nanoscopy technique. Using the physical contact force sensing, the physical probe scans the sample surface, providing the information of surface morphology. The result is similar to SEM images, but with a much higher spatial resolution. It is interesting to compare AFM with SEM as AFM does not require a light or electron beam source. Also, AFM does not require the electron stain, which may change the surface landscape. However, the physical contact of the probe with the sample surface may partially damage the sample, especially during the close-contact mode process. A modified AFM technique also allows liquid environment in addition to the vacuum condition for the imaging, and more biologically relevant samples could be imaged, such as live-cell surface imaging (Figure 1.14).

1.4 Subcellular Imaging

When the object of visualization is too far from our eyes, we use a telescope. If the object is too small, we use a microscope. Superficially, they may seem to use opposite principles, but actually they are similar in a sense that they "magnify" the "too small images" to a sensible size for naked eyes. One is for too small images due to the long distance of the object and the other is for nearby, but physically too small object. If they are similar, can we use telescope instead of microscope for the small object or vice versa? No, we cannot. What is the difference, then? The difference lies in focal distances depending on the position of the object. In a telescope, the focus is on the long distance, and in a microscope, the focus is on the sample slide right under the lens.

Now, let's focus on the microscope for visualizing small objects in biological systems. The basic unit of life is cell. For unicellular organisms, a single cell is an individual or entity. In multicellular organisms, cells gather together to make tissue, and tissues make organ, and organs assemble to make an individual body. The reason why a cell is the basic unit of life is that each cell contains the whole

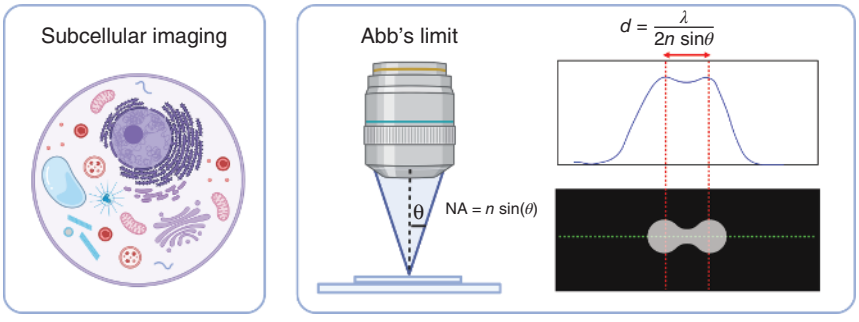


Figure 1.15 Subcellular imaging and Abbe's limit.

genomic information of the individual. In other words, starting from any single cell, in principle, we can reconstruct the whole body.

The usual size of cells in animals or plants is around 10 μm, and unicellular bacteria are about 1 μm in size. While bacteria cell structure is relatively simple, animal or plant cells have complex intracellular structure, called organelles, such as nucleus, mitochondria, lysosomes, Golgi body, and endoplasmic reticulum (ERs). The intracellular organelles are usually about 1 μm or smaller size. When light encounters an object with a similar size to the wavelength, the light path is altered by diffraction. The visible light is in 0.4–0.7 μm (400–700 nm) and if the object is about half micrometer or smaller, the image become blur. This is known as Abbe diffraction limit, named after Ernst Abbe who found it in 1873, and is considered as the physical limit of the optical resolution (Figure 1.15). Therefore, the physical size limit of a microscopic image is about ~ μm range.

To overcome the size limit, several optical and mathematical tricks were developed into "super-resolution" techniques or so-called nanoscopy, which means nanometer-resolution imaging. In addition to the size limit, organelles are usually transparent, so the optical visualization is further challenging, as it is difficult to distinguish different organelles. That is why organelle-selective dyes are widely used for vivid subcellular organelle visualization. In other words, bioimaging is a process of visualizing a biological object, otherwise invisible. Most of the cell images we have in our mind are "stained" images rather than natural cell images. For example, chromosome, as condensed form of DNA, means "color body (chromo-some)" as it is easily stained by dyes. You may have seen the change of the chromosome during the cell division, such as condensation, alignment, and division of DNA. It implies that most of the chromosome images are also obtained from DNA-stained cells rather than intact natural cells. By the same token, if there is a selective dye for each organelle, it would be possible to see specific organelle standing out from a transparent background. These selective dyes are called organelle-selective probes, and if the dyes change their colors upon binding to the target, they can be called as sensors. Therefore, the definition of probes embraces sensors. In other words, sensors are special type of probes in bioimaging, providing the information of change of the target.

1.5 Cell-Selective Imaging

In a multicellular organism or mixed bacteria community, distinctive visualization of different cells or bacteria would be critical for the study of intercellular interaction. If the different cells have unique shapes and sizes, it would be easy to discriminate them. However, in many cases, distinction of one type of cell from others is generally difficult due to their similar appearance under bright-field microscope. Even the same kind of cells may have different stages of development or death process, showing off different morphology. Considering the fact that all the cells in the same body contain exactly the same genetic information, the discrimination of their phenotypic difference is the key for the study.

To overcome the problem, cell-selective probes have been explored for a long time. Antibodies have been the most common probes for the cell distinction and are widely used. Hundreds of antibodies have been developed and validated for cell discrimination and imaging. While useful, due to their high molecular weight of 150 kDa, their access to the intracellular target in live status is intrinsically limited. Even though the binding target of antibodies is on the cell surface, they are usually functionally important enzymes or receptors. As a result, antibodies often induce functional influence in the treated cells, which is not desirable for normal cell study. Alternative solution may be a smart small molecule probe, which may complement the antibodies' weak points, especially for the intracellular target.

Not only for our own cells, we also need to distinguish and visualize foreign life forms, as our body is always interacting with them. For example, our body hosts huge numbers of bacteria as guests in similar or even higher number than our own cells, which is called the microbiome. The bacteria in the microbiome established symbiotic relationships with our body and majority of them are not harmful to us. But, if we get pathogenic bacterial infection, figuring out the identity of the bacteria would be urgent and important for making decision of the proper treatment. The morphological difference may not be informative enough to get a good discriminating information. Media-selective culturing is a standard test for the identification, but the process takes days of time, and also the identification is limited only to the known strains for their culture condition. While polymerase chain reaction (PCR)-based genetic analysis is getting more and more popular for high accuracy and sensitivity, the need for an in-site imaging probe increases for faster analysis and functional monitoring through the visual images. So far, such an efficient and practical cell-selective probe is yet to be developed.

1.6 Tissue and Organ Imaging

When cells gather to make tissues and organs, a tangible physical structure emerges, and macroscopic imaging technique is required. For diagnosis of diseases, often a biopsy (tissue sampling from live body) procedure is required for tissue imaging or biochemical testing. Usually, the tissues are stained with dyes and imaged to determine the disease status. As the test is performed outside of the body, the procedure

is called ex vivo imaging. For example, from a surgery for cancer, the excised tissue (suspected as a tumor) is processed through cryo-section or paraffin treatment, and then stained with dyes for visualizing the tumor and healthy tissue. Most likely, the sample is sent to a pathologist who has long-term training and experience to make the call if the tissue is indeed cancer or not. The procedure takes easily an hour or longer, and it is quite difficult to get the results back before the surgery procedure is over. If the sample preparation procedure becomes simpler and faster, and also a user-friendly probe is available, which does not require a pathologist for reading, it would be possible to get the results within the surgery procedure. Not only for tumors, any kind of disease symptoms such as inflammation or infection could benefit by the selective probes.

1.7 Whole-Body Imaging

If the tissue imaging can be performed without removing the tissue from the body, it would be even better. Such an optical imaging in the live body is called intravital microscopy, as a kind of in vivo imaging. The imaging for blood cell flow or extravasation is an example, and unlike the ex vivo imaging, the intravital microscopy allows repeated measurement with minimal invasiveness for long-term monitoring of diseases. Some of the imaging could be achieved from the natural tissue itself, but sometimes it is necessary to use probes to get a clear contrast.

For example, in cancer surgery, it is often difficult to discriminate the exact boundary between the tumor and normal tissue. If there is a selective probe for a tumor to show a clear boundary, it would greatly help the surgeon to decide the excision line for saving maximum healthy tissue for the patient. If the dye was colorless before binding to the tumor, but generate a strong color in the tumor, the probe could also be a sensor for the tumor and carries low background in the normal tissue. The imaging technique used in operation is called intraoperative imaging.

The eventual goal of bioimaging would be a noninvasive (without an open-up surgery) whole-body imaging without a biopsy sampling (for ex vivo imaging). The ideal probe could act as a diagnostic tool to detect disease occurrence with precise position and size information of the target. The probe should not be toxic and also could be used for body response to drug treatment as a prognostic procedure. There is huge room for improvement in the current in vivo imaging with smart probes and improved image process/analysis method.

1.8 Probes in Bioimaging

Probes help to visualize target organelles, cells, tissues, and organs with an outstanding contrast. Sensors are part of probes, and respond to the analyte or environment by changing the color or intensity. Most of the biological images are physically stained images or artificially drawn pictures, which reflect the practical importance of probes in the field. In this book, the history of probe development,

their applications in different levels of body, i.e. intracellular organelles, different cells, tissues, and whole body. In later chapters, the probe application in biological environmental changes and diseases, and various imaging techniques both for nonoptical imaging and fluorescence will be described. In perspective, design or discovery of selective probes and the future direction will be suggested.

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