CHAPTER 1

INTRODUCTION TO MOLECULAR IMAGING

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Molecular imaging may be defined as the imaging of molecules either delivered to the body or already present in the body. Generally, this refers to *in vivo* imaging, that is, imaging within a living multicellular organism. The process requires an imaging instrument, a subject, and, commonly, an imaging agent. These tools enable longitudinal assessment of delivered materials, specific targets, mechanisms of action, and biological processes. Molecular imaging has already found utility in the clinic.

An example of a clinically useful molecular imaging system is positron emission tomography (PET) imaging using ^{18}F-fluorodeoxyglucose (^{18}F-FDG). The agent is a glucose analogue labeled with a radioactive substance, ^{18}F, with known decay characteristics. Glucose is the primary source of energy in animals. Most foods that we ingest are broken down to or converted to glucose. This sugar enters the bloodstream and then cells via one of several specific transporters, known as GLUT transporters. Once inside the cell, glucose is phosphorylated by hexokinase into glucose-6-phosphate. This is then isomerized by phosphoglucose isomerase to fructose-6-phosphate and continues along the path for energy generation. Fluorodeoxyglucose (FDG) mimics glucose and also enters the cell via GLUT transporters and is phosphorylated by hexokinase; but once inside the cell, it is a poor substrate for phosphoglucose isomerase and for glucose-6-phosphatase and remains phosphorylated. This adds a negative charge to ^{18}F-FDG, which prevents the molecule from crossing the cell membrane and entraps it inside the cell.

Cells with greater metabolic demand require more glucose and therefore entrap more ^{18}F-FDG. Cells can use glucose to generate more ATP, usable energy within the cell, using oxidative phosphorylation rather than using anaerobic respiration. Because cancer cells are constantly reproducing, they have high energy demand; however, they are also inefficient at energy production and behave as though they are in an oxygen-poor environment. They tend to use the less efficient anaerobic pathway, and thus, require more glucose. In turn, in the presence of the glucose analogue, cancer cells tend to accumulate more ^{18}F-FDG than normal cells.

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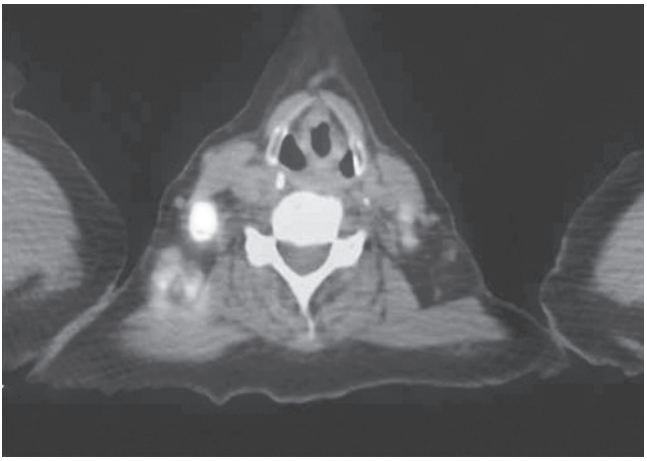

FIGURE 1.1 ^{18}F-FDG PET/CT. Axial view of the neck demonstrates increased uptake (orange) of ^{18}F-FDG in lymph nodes signifying involvement by lymphoma. For color details, please see color plate section.

In this imaging agent, the FDG provides specificity by mimicking glucose rather than another sugar such as sucrose. ^{18}F enables imaging. The known decay of ^{18}F can be imaged using a PET camera sensitive to the positron decay that is employed by ^{18}F to come to a more stable atomic state. ^{18}F-FDG enables one to study a basic biological process, cellular metabolism, that is used to identify and characterize disease. Clinically, ^{18}F-FDG imaging (Fig. 1.1) is used to identify, localize, and stage many types of cancer, such as breast cancer and lymphoma. It is also used to assess response to therapy. More recently, it has been used to predict response to certain therapies and to assess durability of response at the end of therapy [1, 2]. It has also had a significant impact in cardiology, primarily for assessing ischemia and infarction, and in neurology for locating a seizure focus.

1.1 ASSESSING THE TARGET DIRECTLY VERSUS DOWNSTREAM EFFECTS

Systems may be designed to image the target itself versus downstream effects. For example, one may image a receptor or the downstream effect of signaling pathways elicited by the receptor. Practically, one may first want to know if the target is present and how it is distributed within a site of disease and within normal tissues. If the target is present, the drug created against it may be effective. On the other hand, if the target is not present, the targeted therapy is not likely to be effective by the expected mechanism of action. Clinically, although target presence may be evaluated by biopsy, it is not without risk and patient compliance issues may arise if biopsies need to be performed in multiple locations and/or longitudinally. Biopsy samples a small portion of the tissue of interest. By imaging, heterogeneity of target expression may be assessed, for example, within a tumor. Without bystander effect, areas of tumor without target expression may not respond to the targeted

drug. Likewise, heterogeneity of target expression in different metastases may be assessed. If expression is present in some metastases, but not others, there may be a mixed response to the therapy. Longitudinal imaging may be used to assess change in target expression, which may change secondary to the targeted or other therapy that is given to the subject. Reduction in target expression may make the therapeutic less effective.

With targeted imaging used in conjunction with targeted therapy, careful interpretation is necessary. The timing of delivering the imaging agent versus the therapeutic is important since the two may compete. This may be advantageous because one can use it to assess if the therapeutic can displace binding of the imaging agent to the target. On the other hand, the imaging agent may bind to a site separate to that bound by the therapeutic agent; therefore, the imaging agent may not reflect the binding site of the therapeutic agent. Moreover, the imaging agent may bind, but not inhibit function, such as that of a tyrosine kinase domain, whereas the therapeutic agent needs to bind and inhibit function in order to be effective. A caveat for delivery is that to be effective, a therapeutic agent may not need as favorable a biodistribution in terms of signal to background noise as is needed for imaging. Another potential outcome is that the imaging identifies the target and the drug inhibits target function, yet the tumor grows, that is, the drug is not efficacious. This may be because the primary growth/maintenance signaling pathway for the tumor was not targeted or was not adequately suppressed, or secondary/redundant pathways supported growth.

One example of targeted imaging is using ^{111}In-octreotide to image somatostatin receptors. Identifying the presence of this receptor predicts response to octreotide therapy for suppressing carcinoid syndrome, which is associated with neuroendocrine tumors [3]. Another example is ^{18}F-fluoroestradiol (^{18}F-FES). PET imaging with this agent correlates with estrogen receptor (ER) expression and predicts response to tamoxifen [4]. With targeted imaging, the imaging system commonly requires development of a particular contrast agent/radiopharmaceutical for each target; this provides specificity but may limit its utility to a few relevant applications/diseases.

Signaling induced by receptors may activate a variety of downstream pathways that regulate a few critical cellular functions such as growth and metabolism. Processes at the tissue level also represent central processes that may play a key role in normal physiology and diseases. These may be less specific, but because many interesting targets affect them, these downstream effects represent an opportunity for understanding a variety of normal and pathological processes. For example, one may image alterations in glucose metabolism by ^{18}F-FDG PET or changes in the function of the vasculature by dynamic contrast-enhanced magnetic resonance (MR) imaging [5–9] by a number of drugs. Because the process being imaged may be affected by various pathways, a variety of targeted therapeutic agents may be tested using a similar readout. Care must be taken since the downstream readout may not be due to the predicted mechanism of action of the targeted therapeutic. Even so, such a readout may prove practically useful and hypothesis generating. Imaging of downstream effects has the potential for wider applicability than specifically targeted agents, which would be an advantage for clinical translation.

1.2 IMAGING METHODS

Physical properties such as radioactive decay; absorbance or reflectance of light, sound, or X-rays; and behavior in a magnetic field are used to generate images. Machines sensitive to such physical changes are used to create images (Table 1.1). These require appropriate tissue and spatial resolution. If the imaging is performed from one angle, a two-dimensional (2D) image is generated. If the imaging is performed at multiple angles, with appropriate mathematical algorithms and the speed of modern computers, three-dimensional (3D) images can be created.

In vivo imaging may consist of superficial imaging such as that of skin and lumens like the epithelium of the bowel. In such situations, light-based imaging may be applied since one is not as limited by depth of penetration. Due to scatter, light-based imaging is currently limited to a few millimeters or centimeters, thus is not applicable to percutaneous imaging of deep structures. However, for small animals, this is less of an issue since their entire width may fall within this range. Light may be generated from within the animal itself, for example, using an enzyme such as luciferase that converts a substrate such as luciferin into light. Fluorescence may also be exploited in which a certain wavelength of light is input and output of a second wavelength is separated from scatter using a filter and then measured by a camera. Currently, most cameras are cooled charge-coupled device (CCD) chips of the type found in digital cameras.

Due to high sensitivity, light-based imaging is commonly used in small animal imaging, particularly of mice. Luciferase-based imaging in particular has enjoyed a large amount of success since it can be placed into vectors and delivered to cells, enabling a range of molecular biology techniques such as studying promoter function. Fluorescent proteins have the advantage that they can be seen in tissues and cells without a substrate, thus can be followed *in vitro*, *in vivo*, and often *ex vivo*.

TABLE 1.1 Imaging Modalities

Light-based imaging
Primarily CCD-based cameras
White light
With filters: fluorescence, near infrared, infrared
Raman spectroscopy
Penetration depth limited: external cameras, internal cameras such as endoscopes
Nuclear medicine
Gamma camera-based imaging
PET
Magnetization
MR
Sound
Ultrasound
Photoacoustic imaging (light input, sound output)
Thermoacoustic imaging (radiofrequency input, light output)
X-rays
Radiography
CT

A disadvantage is that fluorescence in the visible range has a higher incidence of scatter and background signal in tissues. Some of this may be obviated using near-infrared imaging, since at these wavelengths background signal from tissues is diminished. Another form of light-based imaging is Raman spectroscopy, which visualizes molecular vibrations based on inelastic scattering of monochromatic light. It may be used to interrogate intrinsic characteristics of superficial tissues without the need for contrast agents.

Among percutaneous imaging methods that may also be applicable in humans, nuclear medicine offers the highest sensitivity, in the nanomolar range. For such imaging, a radiopharmaceutical is delivered to the patient. It includes a radionuclide whose decay in the body is imaged. Radionuclides are unstable atoms. Atoms are made up of neutrons, proton, and electrons. In their ground state, nucleons (protons and neutrons) are stable, but if the ratio of neutrons to protons is not optimal or the nucleons are not in their ground state, they may release energy/particles, including gamma rays with characteristic energy. This released energy signature is used to distinguish radioactive decay arising directly from the radionuclide from background/scatter reactions that result in different energies from the source of interest. The de-excitation may be immediate or delayed. The latter is referred to as a metastable state. The decay of ^{99m}Tc (m for metastable) is commonly imaged. The released characteristic energy is imaged using a gamma camera, which may be used to perform 2D planar imaging or single photon emission computed tomography (SPECT) 3D imaging. Characteristic energy detection and collimation to avoid scatter adds specificity in imaging the radiopharmaceutical.

Nucleons may decay to more stable states by releasing particles. Positron emission reduces the number of protons in the nucleus by transforming a proton to a neutron and ejecting both a positron and a neutrino from the nucleus. A positron has the same mass but opposite charge as an electron. It loses kinetic energy after traveling a short distance (usually millimeters) and collides with an electron in an annihilation reaction that transforms their combined mass into energy, releasing not one but two gamma ray photons (each of 511 keV) traveling in opposite trajectories. This enables coincidence detection, which is capitalized upon in PET imaging to localize the annihilation event along a line called the line of response (LOR) that connects the two detectors that detected the two 511-keV gamma rays. In PET imaging, a ring of radiation detectors encircles the patient and detects the gamma rays on opposite sides within a specified period of time. Unlike SPECT, PET imaging does not require a collimator to help identify the source of activity.

Magnetic resonance imaging enables a wide range of contrast mechanisms. Most commonly hydrogen is imaged due to its abundance, although several other nuclei may be used. For MR, when a collection of nuclei with nonzero spin (odd number of protons, neutrons, or both) are placed in a strong magnetic field, a very small net greater amount align with the field creating a net magnetization which eventually decays. Additional time-varying magnetic fields are applied to convert the net magnetization to other forms such as radiofrequency (B_1 field). Magnetic field gradients are also applied in orthogonal directions to encode positional information. Different pulse sequences are used to obtain different types of MR contrast such as T1 weighting, T2 weighting, and T2* weighting. The variety of

pulses allow for native tissue characterization, which is especially helpful for soft tissue imaging. Spectroscopy imaging may also be performed for evaluation of molecules such as choline and has enabled fields such as metabolomics. Contrast agents may be used to enhance tissue contrast. Targeted agents may also be employed. New developments include hyperpolarization of input molecules that increases sensitivity of MR for such molecules by tens of thousands times with low background, enabling imaging of not only their input molecules but also of their metabolic products. A disadvantage of hyperpolarization is only a few molecules hyperpolarize long enough (up to a few minutes) for practical imaging. Hyperpolarized gases have been used, including in patients, for lung imaging. The time resolution of MR and appropriate pulses also allows imaging of motion; moreover, with appropriate modeling, function can be assessed, for example, that of vessels using dynamic contrast-enhanced imaging.

Ultrasound images the reflectance of sound waves in tissues. It most commonly uses a piezoelectric transducer to create sound of different frequencies and shape. The reflected sound returns to the transducer that converts the vibrations into electrical pulses to create the image. Sound of different frequencies is used depending on the application and the species studied. Higher frequencies are used for smaller animals than those used in humans. Ultrasound is most commonly used to image intrinsic tissue reflectances. Its rapid temporal resolution enables evaluation of motion in real time, for example, for imaging cardiac motion. Doppler may be used to image blood flow. Newer applications include injectable ultrasound contrast agents that encapsulate gases such as perfluorocarbons for enhancing reflectance. They can be decorated with targeted moieties. Photoacoustic imaging delivers nonionizing laser pulses. Some of these are absorbed and converted to heat leading to thermoelastic expansion and ultrasonic emission that is detected by an ultrasound transducer. Photoacoustic imaging may be used on native tissue or with contrast agents. When radiofrequency, instead of light, is used to heat tissue, it is referred to as thermoacoustic imaging.

X-rays are commonly used in imaging. Electrons in a lower orbital shell have greater binding energy than those in a higher shell. When an electron in a lower orbital shell of an atom is lost, due to heating, for example, one from a higher orbital shell replaces it with resultant release of energy in the form of an X-ray. 2D radiography and 3D computed tomography (CT) use the absorption of X-rays by the subject to generate an image. Although excellent for anatomic imaging, these techniques have relatively poor sensitivity for molecular imaging. They are more commonly used to fuse images with other methods such as nuclear medicine to help localize signal. The temporal resolution of CT may be used for functional imaging such as that of the vasculature using dynamic contrast-enhanced CT.

1.3 IMAGING AGENTS

Molecular imaging may be performed using the native tissue contrast discerned by the instrument itself. For example, MR spectroscopy enables evaluation of molecular species in a defined volume. Clinically, metabolites such as choline and N-acetylaspartate (NAA) have been used in the brain to help distinguish malignant and live versus necrotic

regions. Choline and citrate levels have been used to distinguish prostate cancer from benign prostate tissue in the peripheral zone of the prostate gland.

1.4 CONTRAST

More commonly, molecular imaging uses a contrast agent. These generally have at least two domains—one for producing contrast and one for specificity to the target. The two domains may be part of the same molecule or attached to each other using a linker or a shell. Nuclear medicine provides examples of each. Most PET agents employing ^{18}F use the ^{18}F to replace another atom or group in the molecule. For example, ^{18}F-FDG may also be written as 2-deoxy-2-[^{18}F]fluoro-D-glucose, where ^{18}F replaces the hydroxyl (OH) group in the second position of glucose. Most gamma camera agents use a chelator to join two groups. For example, in ^{111}In-octreotide, which is used to image tumors such as carcinoid that overexpress somatostatin receptors, the imaging agent, ^{111}In, is connected to a peptide via a bifunctional linker called DTPA. DTPA is a chelator that entraps the ^{111}In and has a reactive group that is covalently linked to the peptide. This is needed because many radiometals are not readily reactive for direct binding to molecules or such binding would interfere with their biological function. Chelators are also used for MR imaging. For example, DOTA is used for entrapping gadolinium, which in its free form can be toxic. The same principles are used for light-based imaging agents. Example light-based imaging agents include small molecules such as rhodamine and quantum dots. Another method for linking an imaging agent to a targeting domain is to use polymers or liposomes. These have the advantage of potentially linking several targeting agents and/or imaging agents to potentially amplify signal. An example of a polymer with multiple imaging agents connected is PG-gadolinium [10]. An example of a liposome with multiple imaging agents is dual-Gd, which has ~10,000× the relaxivity per particle compared to classic clinically used Gd chelates [11]. Liposomes may encapsulate material. This has been exploited to capture gases such as perfluorocarbon in order to enable contrast-enhanced ultrasound imaging. These "bubbles" can be decorated with targeting agents so that the externally exposed targeting moiety is tethered to the lipid bilayer and the gas is entrapped within the liposome. The increased echogenicity enables imaging by ultrasound.

The types of contrast induced depend upon the imaging system employed. For light-based agents, most commonly a fluorophore is employed, for example, fluorescein or rhodamine. To decrease tissue background, an emitter of non-visible light such as near infrared may be used, for example, Cy5.5. For nuclear medicine, both gamma- and positron-emitting substances are exploited; examples include ^{99m}Tc, ^{111}In, and ^{131}I for the former and ^{18}F, ^{68}Ga, and ^{64}Cu for the latter. For MR, both T1- and T2-shortening agents are most commonly used. T1-shortening agents are the workhorse, and among these, gadolinium is exploited most frequently in conjunction with a T1-based sequence. T2-shortening agents have found more popularity in small animal imaging than clinically, and among these, those based on iron are exploited most frequently. For ultrasound, encapsulated echogenic gases are employed. For CT, agents that alter X-ray penetration, such as chelated iodine, are employed.

1.5 TARGETING

The targeting moiety may be one of a variety of molecules. Antibody-based agents are popular because they may provide a very high degree of selectivity. The specificity of the antibody resides in the variable domain. The species of antibody used is important to avoid an immune response to the antibody. Whole antibodies tend to stay in circulation for a long time, on the order of days. Imaging with such has been performed successfully, commonly one day or more [12] after delivering the agent. For imaging, washout from normal structures is essential for visualizing the targeted material. In order to speed washout, smaller versions of antibodies/fragments have been created, including diabodies and single-chain fragments of the variable domain (scFv's). These preserve the variable region for specificity. The smaller size permits earlier imaging, including within the same day. Peptides have also found utility. These may be designed, but often are discovered, like antibodies, using a library containing many variants (10^9–10^{12}) that is screened for the one most ideal binder. Peptides may be stabilized to prevent digestion within the body. An example of a clinically important peptide-based agent is ^{111}In-octreotide. The octreotide portion mimics the hormone somatostatin. Small molecules are also commonly used and again libraries of such may be screened. These may also be designed based on the characteristics of the target to which they will bind. An example of a clinically important small molecule is ^{99m}Tc-labeled methyl diphosphonate (MDP). It mimics calcium phosphate and is incorporated into the mineralized matrix of newly formed bone. More exotic targeting moieties include aptamers, which consist of stabilized oligonucleotides or peptides of different shapes that bind specific molecules. The targeting moiety may be designed but more commonly is selected from a random library of a large number of variants and is then "panned" against the target to select those few that bind the target and wash away those that do not. This process is repeated until the best binders are selected.

Once the targeting moiety is selected, it may be labeled for imaging as mentioned earlier. This process is most applicable for evaluating delivery since these agents, like drugs, are given to the patient. Delivery may be via various routes such as intravenous, intraarterial, intratumoral, intralymphatic, intraperitoneal, intrapleural, intravesical, inhalation, or oral. The route of delivery influences bioavailability and whether the drug itself or its metabolite causes functional change. Most commonly, imaging agents are not delivered via an oral route for several reasons including to avoid portal flow and metabolism in the liver. If a metabolite is the active compound, an alternative imaging strategy may be to label it, instead of the parent drug.

1.6 GENE EXPRESSION IMAGING

Molecular imaging has made inroads into the realms of molecular biology. The central dogma teaches that DNA encodes genes that are transcribed into messenger RNA (mRNA), and this is translocated out of the nucleus into the cytoplasm where it is translated into protein by the sequential addition of appropriate amino acids based on the mRNA code. One may design agents for imaging the building blocks of these

FIGURE 1.2 SPECT imaging of SSTR2-based gene expression. Coronal view of the lungs demonstrates imaging of gene expression after *in vivo* transfer of a somatostatin receptor-based reporter. The reporter was made to express in a human lung tumor on the left side and bound its ligand, ^{111}In-octreotide, as demonstrated by the increased uptake (pink). For color details, please see color plate section.

processes, such as labeled nucleotides that are components of DNA or RNA or such as labeled amino acids, like methionine, that are components of proteins. One may also want to image the end product, i.e., the protein that is built, since this will inform regarding the robustness of the entire process. Imaging of gene expression can be performed using appropriate reporter technology. A reporter is a gene product that can be imaged due to its intrinsic nature, or more commonly, because it binds or enzymatically acts on an imaging agent. Light-based reporters include green fluorescent protein and luciferase. The former fluoresces. The latter acts on a systemically delivered substrate, luciferin, to produce light. For imaging larger animals and humans percutaneously, reporters that have the greatest potential currently include those that can be imaged using nuclear medicine-based techniques such as those based upon the somatostatin receptor type 2 (SSTR2). These have the advantage of a human origin to limit immunogenicity, can be tagged to distinguish endogenous versus exogenously delivered material [13], and can be made signaling deficient to prevent disrupting the cellular milieu [14]. They can also be imaged using a labeled somatostatin analogue such as ^{111}In-octreotide and can be quantified *in vivo* using imaging [15–17], thus, have significant potential for clinical translation (Fig. 1.2).

The reporter is commonly delivered in a vector that contains elements for expressing a gene, such as a promoter for initiating and maintaining transcription, sometimes an enhancer to improve promoter activity, the gene with a stop codon for appropriate termination, and a poly A site to stabilize the transcript. The reporter concept allows evaluation of this entire process, or parts of it, for example, variation of the promoter for optimizing promoter function. The reporter gene may also be linked to other genes such as a therapeutic gene to follow its linked expression. Linkage may be performed using tools such as an IRES [18, 19] or bifunctional promoter [20]. For delivery, multiple different kinds of vectors may be used. The vector may be labeled to image delivery [20]. However, there are a large variety of vectors that would involve substantial work for labeling each; more simply and widely, delivery and expression may be evaluated using reporter technology that uses the same reporter-imaging agent pair. Thus, one would be able to decrease workload

and evaluate the system from delivery to the ultimate goal of expression of the gene product in one imaging session.

Example applications of reporter imaging include evaluating delivery vectors for localization, as well as degree and duration of expression; promoter function for selectivity of expression in a particular normal tissue or pathology, degree of expression, and control of expression; monitoring expression of a linked therapeutic gene; studying mechanisms of action of therapeutics; understanding therapeutic efficacy such as onset of response and why response may have waned, for example, loss of expression; understanding toxicity such as due to inappropriately located expression. These are just some of the applications of imaging of gene expression. This technology has significant potential to positively impact research and clinical needs.

1.7 SUMMARY

Molecular imaging has already impacted research and clinical medicine. Established and new technologies are enabling understanding of molecular events and physiological and pathological process in living systems. These technologies are based on physical characteristics such as detecting light, sound, magnetization, and X-ray absorption but are often made even more powerful by adding contrast agents. These techniques enable the study of basic biological processes and their alterations in disease. Not only do they enable approaching research questions such as the mechanism of action but also clinical questions such as disease localization and prognosis. This book will explore molecular imaging techniques and their applications.

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