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Contents

Preface ........................................................................................................... ix
Contributing Authors ................................................................................ xi

1 Digital Radiography and Fluoroscopy ......................................................... 1
Wei Zhao, Katherine P. Andriole, and Ehsan Samei*

2 Mammography and Other Breast Imaging Techniques ............................. 25
Libby Brateman* and Andrew Karellas

3 Computed Tomography ............................................................................... 59
Thomas G. Flohr, Dianna D. Cody, and Cynthia H. McCollough*

4 Nuclear Medicine ..................................................................................... 101
Robert E. Zimmerman* and Michael King

5 Magnetic Resonance Imaging ................................................................. 123
John B. Weaver*, Douglas J. Ballon, and Anthony B. Wolbarst

6 Medical Ultrasonic Imaging .................................................................... 151
Jeffrey Brian Fowlkes* and Anthony B. Wolbarst

* Corresponding author.
7 Molecular Imaging .................................................. 181
Xavier Michalet*, Laurent A. Bentolila, and Shimon Weiss

8 Overview of Medical Imaging Informatics ......................... 201
Katherine P. Andriole

9 Evolving and Experimental Technologies in Medical Imaging1 ............................................. 229
Anthony B. Wolbarst and William R. Hendee

10 Biological Effects of Low Doses of Ionizing Radiation .................. 255
Antone L. Brooks*, Matthew A. Coleman, Evan B.Douple, Eric Hall,
Ronald E. J. Mitchell, Robert L. Ullrich, and Andrew J. Wyrobek

11 Radiation Therapy ..................................................... 287
Laurence E. Court and Lee M. Chin

12 Magnetic Nerve Stimulation ............................................ 317
Anthony T. Barker

*Corresponding author.
CHAPTER 7

Molecular Imaging

Xavier Michalet,* Laurent A. Bentolila, and Shimon Weiss

7.1 Introduction 182
7.2 Imaging Modalities 182
  7.2.1 Imaging with Radionuclides 183
  7.2.2 Imaging with Nuclear Spins: MRI 186
  7.2.3 Ultrasonography 186
  7.2.4 X-Ray CT 186
  7.2.5 Bioluminescence and Fluorescence 187
  7.2.6 Fluorescence Lifetime Tomography 188
  7.2.7 Two-Photon Light Microscopy 188
  7.2.8 Optical Coherent Tomography and Diffuse Optical Tomography 189
  7.2.9 Quantification of Molecular Imaging Data 190
7.3 Probes for Molecular Imaging 190
  7.3.1 Probe Delivery to the Target Site 190
  7.3.2 Nanoparticles 192
  7.3.3 Toxicity Issues 193
7.4 Clinical and Fundamental Applications 194
  7.4.1 Oncological Imaging 194
  7.4.2 Non-Oncological Imaging 195
  7.4.3 Reporter Gene Imaging 195
  7.4.4 Drug Discovery and Development 196
  7.4.5 Combined Imaging/Therapy 197
7.5 Conclusion 198
7.6 References 198

*Corresponding author.
7.1 Introduction

Molecular imaging covers a wide variety of noninvasive techniques that enable the quantitative detection and visualization of biological entities or processes in living organisms down to the cellular level (Weissleder 2006; Massoud and Gambhir 2003). Given the lack of intrinsic signal or contrast from the organelles, cells, or tissues themselves, these techniques depend on the use of either exogenous probes or contrast agents, which leads to their separation into two distinct categories.

The first consists of methods aimed at detecting and visualizing inherent molecular targets in a biological sample, such as the product of a biochemical reaction (e.g., a protein derived from a particular gene), a tumor marker, or a molecule expressed or displayed as an internal pathology. To detect these molecules, an exogenous probe (such as a gamma-emitting radiopharmaceutical) that can be detected by some suitable detector or imager (a gamma camera) is usually employed. The probes can be as small as ions or larger macromolecules, molecular pairs or aggregates, etc., but nanoparticles specifically tailored for improved signal strength or specificity have recently been generating considerable research interest.

The second category of molecular imaging approaches uses molecules or nanoparticles as contrast agents, which are labeled analogs of biological entities (metabolites, proteins, etc.). Once delivered into the body, they localize with more or less specificity into subregions of an organism, tissue(s), or cell(s). This localization depends on interactions that are not necessarily well characterized (if at all), and the agent molecules/nanoparticles function merely as passive markers of their often serendipitously discovered "targets."

Both categories include methods that are enhancements of some standard imaging techniques, along with others that are uniquely "molecular imaging" modalities. Single photon emission computed tomography (SPECT) and positron emission tomography (PET) were fields of molecular imaging long before the term was invented. Magnetic resonance imaging (MRI) and x-ray computed tomography (CT) are established techniques that have recently become "molecular" due to the development of specific contrast agents. Planar fluorescence imaging, on the other hand, is new, and there are few clinical applications for it yet.

The various molecular imaging techniques differ in the physical nature of the detected signal [high-energy or radiofrequency (RF) photons, acoustic waves, etc.] and the origin of this signal (gamma-ray or annihilation photon emission, spin relaxation, fluorescence emission, etc.). In principle, any detectable physical observable providing enough contrast (signal-to-background and signal-to-noise ratios) could be adopted for molecular imaging. In practice, however, only a few modalities have turned out to be of clinical utility, largely because of sensitivity issues.

The "classical" imaging techniques have mostly been anatomic ones: The whole organism is visualized, or a good fraction of it, and most organs or anatomical regions are distinguished from their neighbors by a different signal level. A pathological or physiological stage is identified by a morphological difference from a reference state, but this may require the expertise of a well-trained physician for proper diagnosis. Molecular imaging techniques, on the other hand, may not provide a complete picture of the organism, and in some cases will only allow the viewing of only a few thousand cells; they tend to do so with extreme specificity, however, relieving the physician or scientist from the complex process of image interpretation. In practice, the combination of molecular imaging with an anatomic imaging modality can provide the best of both worlds, as the recent development of clinical PET/CT and SPECT/CT scanners is demonstrating (von Schulthess 2003).

Some in vivo molecular imaging work is a direct extension of fundamental in vitro or ex vivo (cell cultures) observations to living organisms. It allows a validation, with similar techniques, of well established observations performed in model systems, for instance by studying the effect of the physiological environment on cellular and molecular events (such as cell-cell and protein-protein interactions, molecular pathways, gene expression, etc.). It also opens the possibility to observe molecular and cellular events that are occurring exclusively in living organisms, such as cell migration and homing; to monitor multiple molecular events simultaneously, by means of different labeling or contrast agents; to track drug delivery at the cellular level (using labeled drugs), and the effects on the presence of biomarkers and other molecular species (using labeled probes against these targets); to quantitatively measure the progression of pathologies and monitor therapy at the molecular level; and potentially to be combined with molecular therapy, integrating diagnosis and therapy in a single technological platform.

Other technologies have been developed specifically for small animal molecular imaging research, which has proven to be a mainstay of this young and vibrant field. These include microPET, microCT, microSPECT, planar fluorescence imaging, microMRI, micro-ultrasound, and boliminescence imaging (Figure 7–1).

In any case, the extreme sensitivity and versatility of molecular imaging comes largely from recent advances in molecular biology, physico- and biochemistry, and pharmacology, which have been driven largely by the human genome program. These permit a careful crafting of the solubility of molecular probes in physiological media, for example, and their affinity for specific targets.

7.2 Imaging Modalities

The field of molecular imaging covers clinical and research techniques to obtain diagnostic information by noninvasive
Means using x-ray and CT, SPECT and PET, MRI, ultrasonography (US), bioluminescence and fluorescence, and other methods. Table 7-1 summarizes the performances and limitations of the various imaging modalities that the following sections will briefly review.

7.2.1 Imaging with Radionuclides

The probes for PET and SPECT contain radioactive materials that emit positrons (the positively charged antiparticles to electrons) and gamma rays (high-energy photons), respectively. Either way, a radionuclide is generally conjugated to an active compound, carrier, or drug to create a radiopharmaceutical that has a natural tendency to concentrate in a specific organ system or structure. Several (or more) minutes after the radiopharmaceutical is administered into the body, usually by intravenous injection, it will have had sufficient time to reach and accumulate in its target, and one can begin to take image data.
Single Photon Emission Computed Tomography (SPECT)

As with standard nuclear medicine, SPECT is used to image the spatial distribution of a gamma-emitting radiopharmaceutical within the body. But SPECT represents a significant advance and refinement over conventional gamma camera technology: Just as CT pushes x-ray imaging into the third dimension, so too does SPECT.

A SPECT camera consists typically of two or three gamma camera heads that rotate together around the patient, so we should begin there. An optical camera can create an image on film of an object emitting or reflecting light and, similarly, a gamma camera can create a planar image out of a pattern of gamma rays. It is not possible to focus gamma rays with a lens, however, so another method is needed to localize the source of their emission. This involves placing a thin, flat lead collimator, riddled with a large number of narrow, parallel holes, on the front of each gamma camera head; a collimator allows only gamma photons that happen to be traveling directly from the gamma-emitting source through the holes, to pass on to the surface of the large sodium iodide (NaI) scintillation crystal beyond it and contribute to image formation.

Reconstruction of the source location is performed from successive “images” obtained by positioning the detectors at a set of different angles around the subject. Due partly to the collimation, the sensitivity of SPECT detectors is inferior to that of PET detectors, but higher spatial resolutions can be achieved in specific animal SPECT imaging devices using micropinhole apertures (Beekman et al. 2002).

Positron Emission Tomography (PET)

PET exploits the beta-plus ($\beta^+$) decay that occurs in certain radionuclides. This involves the transformation of one proton within the nucleus into a neutron, in effect, accompanied by the emission of a positron and a neutrino, which plays no role in any imaging procedure.

A positron, ejected into tissue typically with a hundred or so keV of kinetic energy, slows nearly to a stop within a few hundred micrometers through ionization of the surrounding material. It then collides with an unsuspecting atomic electron, and the two annihilate one another, with the creation of a pair of 511 keV annihilation photons (each the energy equivalent of the mass of an electron or positron). The two photons leave the scene of the interaction in almost opposite directions, and reach two detectors on opposite sides of the PET device (and of the subject) at virtually the exact same time. The original positron-emitting nucleus must have been situated somewhere on the line of response (LOR) linking the two triggered detectors; so the region of intersection of all the LORs indicates the location of the radioactivity source.

Current PET systems can attain a spatial resolution of a few cubic millimeters ($\text{mm}^3$) at the center of the imager. Their sensitivity depends on the type of imaging mode [two or three dimensions (2-D or 3-D)], and is limited by the detectors’ solid-angle coverage, resulting in detection efficiencies of only a few percent of the actual positron annihilation events. In practice, very low levels of radioactive materials (concentrations down to $10^{-12}$ mol/liter) accumulated by a few million cells can be detected and localized with millimeter precision (Phelps 2000).

Radionuclides for SPECT and PET

From the perspective of the SPECT or PET user, a radionuclide is either delivered from a commercial radiopharmacy shortly before it is to be administered or produced on-site in a generator or in an accelerator (i.e., a cyclotron). The use of

<table>
<thead>
<tr>
<th>Table 7-1. Performance characteristics of modalities employed in molecular imaging.</th>
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</thead>
<tbody>
<tr>
<td><strong>Modality</strong></td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>PET</td>
</tr>
<tr>
<td>SPECT</td>
</tr>
<tr>
<td>MRI</td>
</tr>
<tr>
<td>Bioluminescence</td>
</tr>
<tr>
<td>Fluorescence</td>
</tr>
<tr>
<td>Ultrasound</td>
</tr>
<tr>
<td>CT</td>
</tr>
</tbody>
</table>
cycloclotrons is confined almost exclusively to the production of positron-emitting radionuclides for PET studies.

In a radionuclide generator, a “parent” radionuclide with a long half-life \( (t_\text{p}) \) decays into a “daughter” species of value for imaging (Table 7–2). The generator is kept in the imaging department, and replaced periodically as it runs down.

The most familiar example is the molybdenum “cows” that produce metastable technetium, \( ^{99}\text{Mo} \rightarrow ^{99m}\text{Tc} \). Parent and daughter nuclides are not isotopes of the same element, so they are easily separated through ion exchange column chromatography. The molybdenum, which itself is created in a nuclear reactor facility, has a half-life of 66.7 hr, while that of its daughter is only 6.0 hr; the cow is milked once or twice a day, and has to be replaced with a fresh one weekly.

\(^{99m}\text{Tc}\) is by far the most commonly used radionuclide today, incidentally, because it exhibits nearly ideal nuclear imaging characteristics. Its 140 keV principal photon is of high enough energy to escape the tissues of the body, but sufficiently low to interact strongly with the higher density, higher atomic number \((Z)\) detector materials. Its radio-pharmacology is fairly straightforward and flexible, and much of it can now be carried out with the aid of commercially prepared kits. Its 6-hr half-life provides plenty of time to carry out the chemistry, but it will not irradiate the patient and others long after completion of the examination.

SPECT. SPECT relies either on beta-minus \((\beta^-)\) decay or on electron capture by certain radionuclides. In \(\beta^-\) decay, a neutron transforms into a proton, an electron (beta particle), and an antineutrino; in effect, the electron and antineutrino escape, and the proton stays behind, incrementing the \(Z\) of the nucleus by one. In electron capture, an electron from the inner electronic shell is drawn into the nucleus, in effect, and combined with a proton to give a neutron and a neutrino, here decreasing \(Z\) by 1. In either case, the parent nucleus is usually left in an excited state, and it returns to its ground state with the emission of one or more characteristic gamma-ray photons, which are used in imaging.

The change in \(Z\) of a nucleus will, of course, also cause a disruption of its cloud of atomic electrons as well. Beta decay of \(^{11}\text{I}\), for instance, leads to the emission not only of 173 keV and 247 keV gamma-ray photons, but also of 23 keV and 26 keV x-rays. (The distinction between gamma- and x-rays relates to their origin, not their energy). Lower-energy gamma- and x-rays are readily absorbed and scattered in tissue, and they rarely play any constructive role in imaging.

For the gamma-emitting radionuclides used typically in SPECT, the excited state is relatively long-lived (metastable), while allowing for a temporal separation between the beta and gamma emissions. With \(^{99m}\text{Tc}\), the work-horse of any conventional nuclear medicine department, for example, decay of the \(^{99}\text{Mo}\) parent leaves the \(^{99m}\text{Tc}\) daughter in a metastable state (hence the \(m\)); its \(t_\gamma = 6\) hr makes it possible to carry out some fairly complex radiochemistry, binding the technetium to a number of medically interesting agents.

**PET.** The positron-emitting isotopes used most commonly in PET \((^4\text{C}, ^\text{13N}, ^\text{15O}, \text{and } ^\text{18F})\) are readily incorporated in biological molecules by means of automated chemical synthesizers similar to DNA or peptide synthesizers.

The most important positron-emitting isotopes have quite short half-lives \((t_\text{p} = 20.3, 10.0, 2.1, 110\) min for \(^{11}\text{C}, ^\text{13N}, ^\text{15O}, \text{and } ^\text{18F}\), respectively), which can severely constrain the radio-pharmacology possible, and necessitate the proximity of a cyclotron facility. On the other hand, the signals for some last sufficiently briefly to allow repeated observations without interference from remnant signal from previous injections. Others, such as \(^{64}\text{Cu}\) have longer half-life \((t_\gamma = 12.7\) hr\), which makes it possible to radiolabel a greater range of agents.

Heavier PET radionuclides \((^{80}\text{Cu}, ^{86}\text{Br}, ^{89}\text{Rb}, ^{86}\text{Ga})\) can be complexed with chemical moieties covalently bound to the molecule of interest, using well-known chelating groups. Yet others \((^{124}\text{I})\) can be used directly as metabolites (Smith 2004).

Table 7–2. Generator systems used in nuclear medicine.

<table>
<thead>
<tr>
<th>Generator</th>
<th>Parent ( t_\text{p} )</th>
<th>Daughter ( t_\gamma )</th>
<th>Daughter ( \gamma ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{99}\text{Mo})–(^{99m}\text{Tc})</td>
<td>2.78 day</td>
<td>6 hr</td>
<td>140 keV (90)</td>
</tr>
<tr>
<td>(^{81}\text{Rb})–(^{81m}\text{Kr})</td>
<td>4.7 hr</td>
<td>13 sec</td>
<td>190 keV (65)</td>
</tr>
<tr>
<td>(^{113}\text{Sn})–(^{113m}\text{In})</td>
<td>115 d</td>
<td>1.7 hr</td>
<td>393 keV (64)</td>
</tr>
<tr>
<td>(^{68}\text{Ge})–(^{68}\text{Ga})</td>
<td>280 d</td>
<td>68 min</td>
<td>511 keV</td>
</tr>
<tr>
<td>(^{68}\text{Zn})–(^{68}\text{Cu})</td>
<td>9.3 hr</td>
<td>9.8 min</td>
<td>511 keV</td>
</tr>
<tr>
<td>(^{82}\text{Sr})–(^{82m}\text{Rb})</td>
<td>25 day</td>
<td>1.3 min</td>
<td>511 keV</td>
</tr>
</tbody>
</table>

Routes of administration into the body. While radionuclides can be used directly in both SPECT and PET, they are generally conjugated to active compounds, carriers, or drugs to create “radiopharmaceuticals” that will localize to a specific organ system or structure after being administrated into the body.

The route of administration of the radiopharmaceutical (or of any probe or contrast agent, for that matter) into the body critically influences its pharmacokinetic properties (i.e., uptake, distribution, and elimination) as much as the specificity of the carrier alone does. Typically, the probe is administrated into the body by injection in liquid or aggregate form, inhalation in gaseous form, local applications or, rarely, injection after micro-encapsulation (Table 7–3). Some specialized studies require the labeling of a patient’s own cells ex vivo with a radionuclide (lymphocyte scintigraphy and red cell scintigraphy), and reinjection into the body for imaging. Since they are administrated to patients or animals, probes need to be sterile and pyrogen-free (i.e., free of any agents that may cause a rise in temperature). Their production also needs to
comply with the strict quality control measures that apply to conventional drugs.

### 7.2.2 Imaging with Nuclear Spins: MRI

Hydrogen nuclei (protons) act like spinning, charged bodies, and each generates its own minute magnetic field. Application of a strong, external magnetic field to a sample of water, lipid, etc., will cause the proton spins and fields to align along it, and the composite field that they themselves create is referred to as their net magnetization.

At thermal equilibrium, the net magnetization lies parallel to the external field. When the system is somehow disturbed, such as with a pulse of RF energy of the right frequency, the interval characteristic of its return to equilibrium is referred to as the relaxation time $T_1$. The values of $T_1$ and of the related $T_2$ are influenced by the biophysical and biochemical environment of the protons of interest, and can differ significantly among various tissue types or pathologic states.

**Nuclear magnetic resonance (NMR)** is a technology that allows, among other important things, the determination of $T_1$ and $T_2$ in a sample by monitoring the behavior of the net magnetization. MRI extends NMR, providing a way to assess the local proton magnetization throughout a portion of the body so as to generate 3-D maps that indicate, in effect, local variations in $T_1$ and $T_2$, proton density, chemical shifts, water diffusion, and other parameters and ongoing processes there. NMR and MRI are discussed in detail in chapter 6.

Despite this rather involved and indirect way of obtaining the information, MRI turns out to have very good spatial resolution. MRI produces anatomical information, for the most part, but it can be transformed in a molecular imaging technique through the adjunction of contrast agents. Contrast agents such as superparamagnetic iron oxide nanoparticles or gadolinium (Gd) chelates, for instance, have a concentration-dependent influence on $T_1$ and $T_2$, and therefore can enhance the contrast of labeled domains (Persiaghel, Heindel, and Bremer 2005). These effects are weak, however, and require micromolar ($\mu$M) or higher concentrations (Massoud and Gambhir 2003), which means that MRI is much less sensitive than PET or SPECT; the spatial resolution of MR molecular imaging, however, is at least an order of magnitude better.

### 7.2.3 Ultrasonography

The heart of a medical ultrasonography (US) device is the transducer, which interfaces with the subject. A transducer is typically a planar array of minute piezoelectric or capacitive elements that are driven to oscillate by an RF generator. Together they produce pulses or a continuous stream of high-frequency (typically 2 to 10 MHz) sound waves that enter the body and can be focused into a narrow beam and swept back and forth within it. After the waves reflect off internal tissue boundaries, the echoes return to the transducer and are converted back into electrical signals. US devices have the remarkable ability to transform the jumble of returning data into clinically useful images.

The reconstructed image is commonly obtained from measured echo times and intensities, or frequency changes related to Doppler signals, with no need for contrast agent. Contrast agents such as ligand-targeted acoustic nanoparticles or emulsions (microbubbles), however, have allowed the imaging of specific cell-surface receptors, bringing US into the realm of molecular imaging (Goldberg, Liu, and Forsberg 1994; Massoud and Gambhir 2003). The resolution of US can be better than 1 mm, but its sensitivity is poor. It remains a highly affordable imaging technique, but it is used mostly for morphological characterization.

### 7.2.4 X-Ray CT

X-ray computed tomography (CT) is a mandatory player in the discussion of molecular imaging, first as a technique providing the high-resolution anatomic background needed for SPECT and PET (von Schultheiss 2003), and also because of recent developments of x-ray CT contrast agents have been recently reported (Rabin et al. 2006). X-ray CT has several advantages that would justify researching ways of transforming it into a molecular imaging tool per se: it is omnipresent in the clinical setting; it is an established small animal imaging tool; it has high resolution; and it has been successfully combined with PET and SPECT.

Standard contrast agents are composed of elements having high atomic numbers, such as iodine and barium, and are injected or ingested, respectively, to enhance the contrast of the vasculature or the gastrointestinal tract. Obviously, such
agents are not specific. The same advantage that makes x-ray CT such a powerful anatomic imaging tool renders the development of contrast agents that can be targeted extremely difficult. Since the technique relies on photon absorption, any agent needs to have a much larger absorption cross section than the surrounding biological tissues. Currently, the only reported attempt to develop molecular imaging agents for x-ray CT has employed large crystalline nanoparticle (~30 nm in diameter) consisting of bismuth sulfide coated with a polymer preventing their nonspecific absorption (Rabin et al. 2006). Following the injection of μM quantities of these nanocrystals in the blood circulation of mice, highly contrasted signals are observed in the liver, spleen, and lymph nodes, regions known to harbor phagocytic cells; elimination of these particles takes place via the kidney and urinary tract in a matter of a few days. Although very preliminary, this study illustrates the interesting potential of functionalized nanoparticles with high x-ray CT contrast for targeted molecular imaging.

7.2.5 Bioluminescence and Fluorescence

**Bioluminescence** commonly refers to spontaneous light emission during the oxidation of the protein luciferin by way of a luciferase enzyme. This natural phenomenon can be used to detect the expression of target genes by inserting a luciferase gene so as to be under regulation by the same promoter (Contag and Bachmann 2002). By providing luciferin (an albumin with a heterocyclic prosthetic group that does not cause an immune response) to the genetically engineered animal, luminescence is generated at the time and location of expression of the target gene of interest, permitting a noninvasive monitoring of its expression level, virtually free of background.

Fluorescence is a more general property of certain light-absorbing and -emitting materials (here molecules or nanoparticles). Incident light of wavelength belonging to the absorption spectrum of the probe is absorbed by the fluorophore, elevating an electron into an excited state. After losing some of its additional energy to molecular vibrational and rotational modes, an electron rapidly drops back to the ground state of the molecule, in the process emitting a photon of lower energy than that of the one originally absorbed (Lakowicz 1999). The emission spectrum of a fluorescent material is thus shifted towards the red with respect to the absorption spectrum, and the peak-to-peak distance between the two is referred to as the **Stokes shift**.

This spectral difference implies that fluorescence can be excited with shorter wavelength photons than those actually detected, permitting a simple rejection of unabsorbed/scattered excitation photons, which would otherwise swamp the detector with a uniform background, masking the signal coming from fluorescent molecules. **Fluorescence imaging** can be employed for gene-expression studies similar to those of bioluminescence, using natural or engineered gene-encoded fluorescent proteins (GFP, DsRed, etc.). Unlike bioluminescence, however, no substrate (luciferin) is necessary for fluorescence, which is triggered at will in space and time by the researcher through directed light excitation.

**Fluorescent proteins (FP)** have found applications beyond reporting gene expression, as **fusion proteins** that are composed of the protein of interest with a FP moiety co-translated at one of its termini. The expressed chimeric fluorescent (but functional) proteins can, by way of high-resolution microscopy, be tracked or seen to interact with other species fused with different color FPs; this has led to tremendous progress in our understanding of cellular processes (Tien 1998; van Roessel and Brand 2001).

Fluorescence imaging is far from limited to FP, however, since many biocompatible organic and inorganic materials exhibit fluorescence. The most often used fluorophores, by far, are organic dye molecules, which are attached easily and site-specifically to proteins, DNA, RNA, and other biological macromolecules, or simply used as ion sensors. Recent developments have increased the panoply of usable fluorescent probes, from rare-earth doped nanocrystals to quantum dot nanocrystals, described later. The range of biological applications of fluorophores is broad, including in vitro spectroscopic analysis of protein-protein interaction and biochemical reactions, high-resolution microscopy of single molecules, cultured cells, tissues or embryos, and finally the imaging of small animals (Michalet et al. 2003).

In the study of small animals, bioluminescence and fluorescence molecular imaging both involve visible to near-infrared photons (3 to 1 eV), many thousands of times less energetic than gamma- or x-ray photons used in PET or SPECT (tens of keV to 500 keV). They much more readily undergo numerous scattering events or are absorbed by the surrounding biological medium, and the signal is reduced by an order of magnitude or so for every centimeter of tissue penetrated. The few photons emerging from the subject and reaching the collection optics and the detector have, in fact, undergone a complex diffusion process within the various layers of biological media, which renders the reconstruction of their original locations much more difficult a task than for CT, PET, or SPECT. For this reason, bioluminescence and fluorescence imaging is for the time limited to the observation of subcutaneous lesions or small (therefore thin) animals.

The spatial resolution of both techniques decreases linearly with the depth of imaging, being at best <1 mm. The sensitivity of bioluminescence imaging is unmatched at the level of 10⁻¹⁵ mol/l or better, due to the absence of background signal. That of fluorescence imaging depends on a number of factors (wavelength, depth of imaging, etc.), but it is generally comparable to that of PET and SPECT (~10⁻⁹ to 10⁻¹² mol/l).

Luminescence imaging could initially be performed only in two dimensions (2-D), but can now be carried out in three
dimensions (3-D), using tomographic principles related to those in x-ray CT. Fluorescence tomography (FT) employs either of two geometries in the reconstruction of the distribution of fluorescence sources in small animals (Figure 7–2). In the planar reflectance mode, excitation light is sent toward the subject and collected on the same side. This designation is actually appropriate only for the excitation light spectrum scattered (and spectrally unshifted) by the tissues, and used to reconstruct a 2-D or 3-D contour of the subject. The fluorescence light, which is actually emitted within the subject and not reflected off the surface, is collected separately using a different filter in front of the detector. A single image allows only the 2-D visualization of the source locations projected on the animal surface; rotating the detector around the animal and obtaining data from multiple angles makes possible a 3-D reconstruction of the luminescence sources location, and a quantification of their intensity. This approach is applicable also for bioluminescence (Figure 7–3), and it is often used to acquire a (series of) reflectance image(s) for reference.

In the transmittance mode of FT, the excitation light source is situated on the opposite of the animal from the detector (Figure 7–2). It is then possible to measure the transmittance of the sample by recording the signal collected at the excitation wavelength. The emission signal recorded at the same location can thus be corrected for absorption at both excitation and emission wavelengths.

Since the probes used in optical imaging are nonradioactive, the corresponding techniques pose even less hazard for the subject or researcher than SPECT and PET.

7.2.6 Fluorescence Lifetime Tomography

Fluorescence tomography (FT) has been discussed, so far, in the context of continuous excitation and detection, in which only the intensity of the signal is recorded. The combination of FT with time-resolved detection offers the specificity of molecular imaging, the penetration and resolution of optical tomography (see below), and the possibility to measure the fluorescence lifetime of the probe. The fluorescence lifetime of a probe is of particular interest since it is independent of its concentration but directly related to the type of tissue and the molecular environment (pH, presence of quencher, etc.) in which it resides.

Two general approaches derived from well-established fluorescence microscopy and microscopy techniques (Lakowicz 1999; Michalet et al. 2003) have been demonstrated for in vivo time-resolved fluorescence microscopy, employing frequency-domain (Godavarthy 2005) and time-domain measurements (Veras et al. 2005), respectively. Although at a very early stage of development, this technology could possibly render in vivo fluorescence molecular imaging as sensitive and as versatile as nuclear imaging, but at a much lower cost to the patient and with no ionizing radiation involved.

7.2.7 Two-Photon Light Microscopy

A fluorescent material is normally excited with single photons that have a wavelength within its absorption peak. It can also be stimulated through the simultaneous absorption of two photons with twice this wavelength, since together they carry the same total energy. Most fluorescent dyes have a one-photon excitation spectrum in the visible range, so two-photon excitation (2-PE) of these dyes requires near-infrared (NIR) wavelengths.

The probability for 2-PE of fluorescence to take place is constrained by quantum mechanics and is usually very low, but it can occur with sufficiently intense pulsed laser sources. Typically, average incident powers of several milliwatts are needed (instead of a few microwatts for one-photon excitation), with pulses only hundreds of femtoseconds to tens of picoseconds in duration to reach the very high instantaneous peak intensities required.

Two-photon excitation (2-PE) of fluorescence is a significantly more complex procedure than its single-photon relative (1-PE), but there are several good reasons for the extra effort.

NIR radiation is less absorbed by tissues, and therefore 2-PE can image deeper within an organism. Still, the technique is limited to shallow depths (<1 mm) because of both
scattering and absorption in tissues and optical aberrations, which lead to poor excitation and collection efficiencies away from the surface (Denk, Strickler, and Webb 1990).

Also, 2-PE can do away with a pinhole system for rejecting light coming from out-of-focus regions, as is needed for 1-PE confocal microscopy. But the laser beam does need to be focused by a high numerical aperture (NA) microscope objective lens to reach the large peak intensity required for 2-PE. (The numerical aperture measures the maximum collection angle of the lens, and is inversely proportional to the focusing power.) Focusing also limits the excitation to a volume of a few femtoliters ($10^{-15}$ l) centered around the objective lens focal point. With raster-scanning of the laser beam, this allows high-resolution 3-D imaging with no need to de-scramble the emission light and funnel it through a pinhole, greatly simplifying the optics and increasing the collection efficiency.

As a corollary to this, 2-PE limits photobleaching of the sample away from the focus, unlike the case with 1-PE which does cause premature bleaching.

Finally, 2-PE with well chosen frequencies can excite different dyes emitting at different wavelengths, allowing multicolor imaging to be performed without having to worry about aligning different lasers lines with one another.

The high-resolution (submicrometer) capability of the technique allows the detailed observation of single fluorescently labeled cells deep in tissues or live animals at video rates. Noninvasive observations of lymphatic organs, kidney, heart, skin, eye, and brain have been reported. The extraordinary resolution of 2-PE fluorescence microscopy has permitted the reconstruction of networks of neuronal connection via intracellular staining or expression of fluorescent proteins: for example, the functional imaging of individual neurons using calcium-sensitive dyes, and the evolution of Alzheimer disease plaques and their relationship with neighboring neuronal structures (Helmchen and Denk 2005). Although 2-PE excitation of cellular and tissue autofluorescence has been used, labeling with fluorescent agents possessing a large 2-PE absorption cross section is generally preferred. Fortunately, most standard fluorescent molecules [dyes, FP, quantum dots (qdots)] have reasonable to excellent 2-PE absorption properties, and they are therefore efficient molecular probes.

### 7.2.8 Optical Coherent Tomography and Diffuse Optical Tomography

Optical coherence tomography (OCT) and diffuse optical tomography (DOT) rely on different principles, and can both benefit from the addition of contrast agents. OCT is an interferometric approach relying on the backscattering of a broadband source of light from anatomical details of the tissue (Huang et al. 1991), whereas DOT, mainly using NIR light, can measure both scattering and absorption, the latter giving access to information on tissue metabolism (NIR absorption is primarily due to hemoglobin and therefore depends on its degree of oxygenation) (Hielsher et al. 2002). They are both characterized by a moderate penetration depth (approximately 2 to 3 mm in tissue).

In OCT, the light source is split in two, with one half sent to the sample, the other reflected in an interferometer arm; the two are being recombined to form an interference pattern. The imaging depth is thus easily changed by modifying the length of the interferometer. The lateral resolution is determined by the diameter of the source, which is raster-scanned over the sample to form images. Different technical variants of OCT exist, using time-domain, frequency-domain, or Fourier-domain detection schemes. OCT was first demonstrated in the early 1990s with ~30 mm axial resolution, but has now reached submicrometer axial resolution with the advent of large bandwidth light sources (emitting wavelength over a spectral window >100 nm). OCT has been used in ophthalmology, dermatology, dentistry, gynecology, gastrointestinal medicine, cardiology, and laryngology (Bouma 2001). Some interesting attempts to combine OCT with the adjunction of molecular contrast agents have recently been proposed, but the approach still lacks sensitivity (Yang 2005).

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**Figure 7-3.** Series of 2-D projections of bioluminescence images acquired with the IVIS® 3D Imaging System. The series shows PC3Huc metastatic lesions 28 days after injection of $2 \times 10^6$ PC3M cells in 100 µl of PBS into the left ventricle of the heart of a nude mouse. The bioluminescence intensity is represented in false color, superimposed on the reflectance images. Subsequent tomographic reconstruction allows a precise localization and quantification of the bioluminescence sources within the mouse. (Reproduced with permission. © 2005, Xenogen Corporation.)
7.3 Probes for Molecular Imaging

The development of molecular imaging has been critically dependent on indicator probes that show when and where, in living cells and organisms, genetically or biochemically defined molecules, signals or processes appear, interact and disappear, with high spatial and temporal resolution. The classic example is that of radioactive tracers that, in combination with 3-D imaging systems such as PET and SPECT, help clinicians characterize the molecular status of tumors. Other types of imaging probes rely on bioluminescence and fluorescence of genetically encoded proteins (originally found in fireflies and jellyfish), of entirely synthetic fluorochromes, or of hybrid combination of both.

The development of these probes has become easier during the past decade thanks to the availability of combinatorial and rational chemical design techniques, high-throughput screening assays, robotics, bioinformatics, large databases of molecular properties and structure, etc., and because of dedicated and painstaking research from multidisciplinary collaborations. This research path has been somewhat dispersed and inefficient; however, because the market for imaging reagents is dwarfed by that for therapeutic drugs. Specific, targeted molecular imaging agents generally cost less to develop than therapeutic drugs, but they can at best generate a tenth of the revenue. Some of the radionuclide reagents discussed earlier are costly to prepare and of limited lifetime, which explains the attractiveness of new, nonradioactive approaches. But it is possible that new developments in biotechnology may eventually lead to more cost- and time-effective synthesis of radiopharmaceuticals, as recently demonstrated with the synthesis of radiolabeled probes using microfluidic techniques (Lee et al. 2005). The molecular imaging approaches used in the clinic of tomorrow will depend as much on the bottlenecks of probe production, availability, or ease of use as on their scientific merits.

7.3.1 Probe Delivery to the Target Site

This section considers the fabrication, functionalization, and delivery routes of some of the diverse labeling agents used in molecular imaging. Their rapid growth in sophistication may add levels of complexity to the enterprise, but it also provides new degrees of freedom in tailoring each probe for a particular use.

**Non-targeted probes**

Some imaging agents accumulate naturally in specific biological compartments. The non-targeted biodistribution of the pertechnetate ion ($^{99m}$TcO$_4^-$) resembles that of the iodide ion (I$^-$), both concentrating primarily in the thyroid, salivary glands, gastric mucosa, and choroid plexus.

For optical imaging, best transmission of light through biological tissue occurs in the NIR (650 to 850 nm), between
the absorption peaks of hemoglobin and water (Weissleder 2001; Gambhir 2002). Fluorescent indocyanine green (ICG), for example, is a nontargeted NIR fluorophore used routinely in the clinic for testing hepatic and heart functions, as well as to perform fluorescence angiography in ophthalmology (Bremer, Ntziachristos, and Weissleder 2003). ICG and other cyanine dyes derivatives have also shown great potentials as contrast agent for tumor detection in both animals and humans (Bornhop et al. 2001). Although these probes have no target-seeking moieties, the value of contrast-enhanced tumor detection relies upon a more distinct perfusion of tumor tissues compared to normal areas given their increased vasculature.

Also target-nonspecific, aromatic NIR lanthanide chelates have shown promise in detecting cellular transformation from dysplasia to colon cancer in rats (Bornhop et al. 1999). The sensitivity for this class of abnormal tissue marker is such that femtomole per pixel (picomolar) quantities can be used in topical examinations.

**Targeted probes**

Target-specific probes can provide increased molecular specificity in imaging technologies utilizing both ionizing (radionuclides) and non-ionizing (luminescence) radiations.

Radioactivity-based molecular imaging techniques mainly use radionuclide tracers attached to biologically active compounds, carriers, or drugs. These have been prepared in the form of metabolic imaging probes (e.g., $^{18}$F-FDG or $^{18}$F-FLT) (Been 2000; Gambhir 2002; Buck 2003; Francis et al. 2004), or as radioimmunoconjugates (as in CEA-scan) (Gambhir 2002; Been 2004) or small ligands that bind to intracellular or cell surface receptors (Been 2000; Gambhir 2002; Buck et al. 2003). Technetium’s biodistribution, for example, can be altered to specifically image the blood, the skeleton, the lung, the kidneys, the heart, or the brain, simply by complexing it to difference pharmaceuticals. More than 30 radiopharmaceuticals are commercially available to investigate different parts of the body.

The size of the targeting moieties ranges from bulky macromolecular carriers such as human albumin or transferrin (Becker et al. 2000), to monoclonal antibodies, single-chain antibodies, diabodies, and small peptides (Becker et al. 2001). Smaller carriers tend to be less immunogenic, show better pharmacokinetic properties, and provide increased probe penetration (Weissleder 2001). The strategy of coupling NIR fluorophores to peptide ligands offers an alternative to nuclear imaging when higher resolution is required.

The targeted-conjugates approach can achieve high target specificity using a low amount of probes, but the signal-to-background ratio can be affected by several parameters. The receptor target density and/or availability establish primary limitations, as do a significant background level from unbound probes, nonspecific cellular uptake, or nonspecific adhesion of the probe. These problems can be partially avoided by imaging after the excess of contrasting agent has been cleared by the hepatobiliary excretion system, when the studied pharmacokinetics allow it.

**Activatable probes**

The crucial issue of improving the signal-to-background ratio has driven the design of more complex activatable probes, principally for fluorescence imaging, but also for MRI.

For fluorescence, these probes, generally referred to as molecular switches or molecular beacons, consist of a fluorescent moiety and a quenching moiety; they are in close contact before the probe has reached its target, and are therefore initially nonfluorescent (the actual mechanism of fluorescence quenching can vary, but is generally based on fluorescence resonance energy transfer to a nonfluorescent moiety, or electron transfer). After activation by a specific target in vivo, the probe undergoes a conformational change, which results in separation of the fluorescent moiety from the quencher, restoring fluorescence. Their first use was the specific recognition of nucleic acid sequences in vitro (Tyagi and Kramer 1996). More recently, optically quenched near-infrared fluorescence (NIRF) probes targeted to the folate receptor were developed and used to improve the in vivo detection of tumors in mice (Moon et al. 2003).

Other probe designs have combined the use of a quencher linked to the fluorescent moiety peptide sequences that are targets of tumor-associated proteases in vivo (Weissleder et al. 1999; Kircher, Weissleder, and Josephson 2004). In the presence of the enzyme, the fluorescence signal is strongly amplified (up to several hundredfold) by release of the quencher since one target enzyme can activate multiple beacon molecules. Importantly, the selectivity of these protease-sensing optical probes can be tailored to various proteases that are found at higher levels in highly aggressive and invasive cancers (Bremer, Ntziachristos, and Weissleder 2003 and references therein). To date, different protease-sensitive fluorescent probes for in vivo NIRF imaging have been linked to enzymes such as cathepsin-B, matrix-metallo-proteinase-2, cathepsin-D and thrombin (Bremer, Ntziachristos, and Weissleder 2003). This molecular contrasting strategy is efficient because of the low background fluorescence of the free probe, the high level of amplification due to serial dequenching of the probe, and a probe selectivity that can be customized to different target proteases.

A similar design can be used to synthesize probes for MRI. As in activatable fluorescent probes, where emission is quenched in the absence of the molecule activating the probe, a magnetic resonance contrast agent such as gadolinium can be rendered largely ineffective by protection groups reducing access of water to it. In these enzyme-activated probes, the presence of the enzyme frees the gadolinium chelate from these protecting groups, restoring its activity as a MRI contrast.
agent (Alauddin et al. 2003). Deprotection can also be triggered by the presence of molecules other than enzymes, such as intracellular messengers. A recent example has illustrated this concept with DOPTA-Gd, whose relaxivity is modulated by Ca²⁺ concentration (its relaxivity increases almost doubles when Ca²⁺ is added to a Ca²⁺-frees solution) (Li et al. 2002).

7.3.2 Nanoparticles

The use of artificial nanostructures and nanoparticles in imaging has recently gained considerable momentum. There is now a vast array of particulate systems, both organic and inorganic, capable of targeting different cells and extracellular entities in the body as diagnostic agents.

Superparamagnetic iron oxide crystals (SPIO)

Superparamagnetic iron oxide (SPIO) nanocrystals can serve as negative contrast agents in MRI (i.e., appearing predominantly dark on MRI images). They consist of an inorganic core of iron oxide 4 to 5 nm in diameter (magnetite Fe₃O₄, maghemite, or other insoluble ferrites), perhaps coated with a polymer such as dextran or polyethylene glycol (PEG) (Bonnemain 1998). Such nanoparticles are commercially available in sizes from 30 to 300 nm, which imparts them with different half-lives and biodistributions for different imaging applications.

SPIO surfaces can be functionalized with small molecules and/or functional groups amenable to the bioconjugation of proteins, antibodies, or oligonucleotides for targeted imaging (Schellenberger et al. 2004). Likewise, SPIO particle colloids have been used to monitor gene expression or to detect pathologies such as brain inflammation, arthritis, atherosclerosis plaques, and lymph-node mapping (Moghim, Hunter, and Murray 2005).

Semiconductor quantum dots

Over the last decade, fluorescent semiconductor nanocrystals (also known as quantum dots or qdots) have emerged as new labels that are thought to have unmatched potential as novel intravascular probes (Michalet et al. 2005). Qdots are nanocrystals (1 to 10 nm) of inorganic semiconductor material in which absorption of a photon of sufficient energy (a few electronvolts) can result in the creation of an exciton (bound electron-hole pair). Upon recombination of the pair, a photon is emitted at a longer wavelength, in a classical fluorescence process. Qdot electronic and optical properties are size dependent and governed by quantum confinement effects (Alivisatos 1996). Perhaps their most useful size-dependence property is the shift of absorption and emission spectra with particle diameter; as the nanoparticle gets bigger, its emission shifts from the blue to the red end of the visible spectrum. By playing on the material composition and sizes of qdots, the whole visible, NIR, and even far infrared spectra can be covered.

Other unique optical properties set qdots apart from conventional dyes. They are brighter, emitting many more photons per nanocrystal, which means that even a very small number of qdots are sufficient to produce an adequate signal. Also, they are more photostable, allowing for the acquisition of crisp and well-contrasted images over long periods of time (minutes to hours). And they have a broader excitation spectrum, meaning that a mixture of nanocrystals of different sizes can be excited by a single wavelength (laser line) and simultaneously detected in separate color channels; that is, qdots enable users to multiplex many different biological signals in complex environments, such as the living cell or the whole organism, with an unprecedented kaleidoscope of colors.

Qdots can be made to emit in the NIR “diagnostic window” of the spectrum, where autofluorescence is considerably reduced and where few good dyes exist. The potential of qdots as fluorescent contrast reagent for tumor imaging in the NIR spectrum has been demonstrated in live animal studies in which NIR qdots were injected and imaged through animal skin (Gao et al. 2004) or used as a background-free reporter for lymph-node surgery (Kim et al. 2004). The use of qdots for tracking metastatic tumor cell extravasation has also been demonstrated (Voura et al. 2004).

The large surface area of a qdot (tens to hundreds of nm²) means that a number of surface attachment groups with different functionalities can be grafted on a single qdot, producing multimodality probes. PEGylated qdots having DOTA (a chelator for heavy metals such as the radioactive ⁶⁷Cu PET marker) on their surface, for example, have been dynamically imaged in a small animal microPET scanner, and later localized with high accuracy by fluorescence microscopy in the liver (Figure 7–4) (Michalet et al. 2005). By extension, qdots (and thus their target’s) biodistribution in a living organism can be studied from the level of the whole body down to nanometer resolution, using different imaging platforms such as microPET, two-photon light microscopy, single-molecule localization, and electron microscopy (Dahan et al. 2003).

Dendrimers

Dendrimers are highly branched polymeric macromolecules, with branches emanating from a central core. Polymer growth can be precisely tuned to achieve the desired final size, typically of a few nanometers. They are an attractive platform for bioimaging because of the presence of cavities in the core structure and multiple terminal groups on its exterior, all of which are amenable to bioconjugation and functionalization. A variety of PET, fluorescent, and MRI contrast agents have been conjugated to dendrimers and site-specifically delivered into the body when combined with a targeting moiety (Venditto, Regino, and Brechbiel 2005).
Polymeric micelles

A *micelle* is a microscopic sack consisting of a monolayer of amphipathic molecules (i.e., containing both polar and non-polar domains) separating two different media (for instance, water on the outside, and nothing or some hydrophobic material inside). As for other types of fluid membranes, the formation of micelles is largely a result of the amphipathic nature of their constituting phospholipids or block-copolymers; the hydrophobic tails segregate away from water, while their polar heads are immersed in the aqueous environment. These dynamic systems, which are usually below 10 nm in diameter, can also form in the presence of insoluble molecules, which segregate within their hydrophobic cores; insoluble contrast agents can be emulsified in this manner. The encapsulation of qdots for fluorescence imaging in a living organism has been demonstrated (Dubertret et al. 2002); the size of qdots sets the dimension of the micelle core, and the phospholipids of the original micelles merely coat the hydrophobic qdot surface.

Liposomes

*Liposomes* are closed bilayer vesicles that form upon hydration and sonication of dry phospholipids. Different shearing rates create either large multilamellar liposomes (up to 1 μm onion-like structures) or unilamellar vesicles (ranging from a few dozens to a hundred nanometers in size). Contrast agents can be entrapped in the process, the liposomes acting as carriers to introduce them into target cells upon fusion to the cell surface. Magnetite-loaded liposomés conjugated with targeting antibodies, for example, have enabled tumor-specific contrast enhancement in MRI (Ito et al. 2005).

7.3.3 Toxicity Issues

Labeled molecular probes and contrast agents, or genetically engineered fluorescent or bioluminescent proteins, are used to generate a measurable exogenous signal, and should fulfill this role while interfering as little as possible with the physiology. Whereas increasing the signal level is usually obtained by increasing the probe concentration, adverse effects on cell function or even survival usually limit the useful concentration range that can be used in practice. Cytotoxic effects of molecular probes and contrast agents are various in cause and nature, due to the diversity of physical principles used in molecular imaging approaches. For instance, radioactive labels may cause radiation damages to DNA, which will not be a concern with fluorescent quantum dots. On the other hand, heavy metals such as cadmium present in quantum dots may result in completely different effects, such as cell apoptosis, by interference with the activity of some critical enzymes or by the generation of radical oxygen species (Tsay and Michalet 2005). Due to these various possible cellular perturbation mechanisms, cytotoxic effects are generally difficult to characterize and quantify precisely unless they result in massive cell death or anatomical pathology, allowing the definition

![Figure 7-4. MicroPET and fluorescence imaging of qdots in normal adult live nude mice. Qdots having DOTA (a chelator used for radiolabeling) and 600-dalton PEG on their surfaces were radiolabeled with copper-64 (a positron-emitting isotope with half-life of 12.7 hours). The qdots were then injected via the tail vein (~80 μCi per animal) and imaged in a small animal scanner. (a) Rapid and marked accumulation of qdots in the liver quickly follows the injection; this could be avoided by functionalizing qdots with higher molecular weight PEG chains, as other studies have shown. (b) Overlay of DIC and fluorescence images of hepatocytes from a mouse shows the accumulation of qdots within liver cells. Scale bar, 20 μm. A further step could involve TEM imaging of the precise localization of qdots in cells. This illustrates the potential of qdots as probes at the macro-, micro-, and nanoscales. (Reprinted with permission from Science, vol 307, "Quantum dots for live cells, in vivo imaging, and diagnostics," X. Michaeli, F. F. Pinaud, L. A. Bentolila, J. M. Tsay, S. Doose, J. J. Li, G. Sundaressan, A. M. Wu, S. S. Gambhir, and S. Weiss, pp. 538–544. © 2005, AAAS.)](image-url)
a median lethal dose (LD50). This concentration has to be compared to the range of concentration that results in a measurable signal and sufficient signal-to-noise and signal-to-background ratios. A difference of several orders of magnitude between these two concentration regimes is necessary to define a safe probe. Unfortunately, final concentrations in an organism, and in particular in the targeted organs or cells, are difficult to assess. Large particles in particular can be accumulated in the liver, resulting in locally higher concentrations (Figure 7-4b).

In addition to the final concentration of the probes, the duration of exposure to the probe has to be taken into account. Again, this might be a difficult parameter to measure. Exogenous probes might be rapidly recycled and eliminated by excretion, resulting in limited exposure time, but they could as well be accumulated and retained for several days, as has been observed for large particles, resulting in long-term toxic effects, long after the probes have ceased emitting any measurable signal.

Cytotoxicity studies will be critical for the future development of molecular imaging approaches in human medicine, and have logically received the attention of health agencies, such as the National Cancer Institute, which has established a Nanotechnology Characterization Laboratory\(^1\) to perform pre-clinical efficacy and toxicity testing of nanoparticles and other nanoconstructs and to facilitate regulatory review of nanotechnology intended for cancer therapies and diagnosis.

### 7.4 Clinical and Fundamental Applications

Today's achievements in molecular imaging are clearly the early indications of the arrival of a period of explosive growth both in fundamental research and in the development of clinical applications.

In small animals, medical imaging techniques can be used to study gene expression, cell or protein trafficking, or drug metabolism. They can also be used in patients to detect, characterize, monitor, and follow the treatment of a disease. Clinical applications, at present, mostly involve PET, SPECT, MR, and US, and there is considerable research on new reporters and devices; there is also a growing amount of work dealing with optical imaging. Thus there is a natural division between applications that are used in the clinic and those that are the topic of current research in animals.

This section will briefly review some examples of both clinical- and research-level molecular imaging applications. It will describe cancer-related clinical molecular imaging approaches, methods for targeting other diseases (brain function, cardiovascular diseases, inflammation, etc.), and three different areas of animal research using reporter gene techniques: imaging of cell progeny, monitoring gene therapy, and examining in vivo protein interactions.

#### 7.4.1 Oncological Imaging

Cancerous tissues at different stages can be detected by way of different approaches. Late stages can be detected morphologically in the clinic by conventional immunocytochemistry techniques, but only molecular imaging can provide the sensitivity needed to pinpoint small tumors at their earliest stages, or their metastases.

Three main molecular imaging strategies are used to detect cancerous lesions, relying on: the increased metabolism of tumors; the increased vascularization of neoplastic tumors; and expression of specific markers of tumors.

**PET and SPECT**

\(^{18}\)F-FDG (fluoro-2-deoxy-D-glucose), a fluorinated analog of glucose in which the OH groups have been replaced by the positron-emitting radioisotope fluorine-18, is a PET reporter of high cellular metabolism. In general, cancer cells exhibit increased rates of glycolysis and pentose phosphate cycle activity, and slightly reduced rates of respiration. Cancer cells (and some others) tend to accumulate the non-cell-permeable FDG-phosphate, resulting in an enhanced contrast with respect to surrounding tissues. Similarly, \(^{18}\)F-FLT (fluoro-deoxy-thymidine) can act as a tracer of DNA synthesis and detect actively replicating neoplastic cells, as can other fluorinated derivatives (von Schultethes 2003). Assessments of different detection methods of lung and colorectal cancers have shown that \(^{18}\)F-FDG PET can bring about an 8% to 43% increase in detection accuracy for primary and metastatic cancers; as importantly, the approach can lead to a change of therapeutic choice in 20% to 40% of cases, mainly because PET imaging showed the disease to be more extensive than other conventional methods indicated (Phelps 2000). These characteristics are, in fact, common to most cancers studied with \(^{18}\)F-FDG PET, including breast cancer. Non-\(^{18}\)F-FDG PET studies are rarer, but promising because of the increased specificity of their targeting (von Schultethes 2003).

SPECT molecular imaging of cancer benefits from multiple tumor-specific markers. Iodine-131 \((^{131}\text{I})\) readily accumulates in the thyroid, for example, and serves to identify thyroid carcinoma. Indium-111 \((^{111}\text{In})\) chelated by DTPA (diethylenetriaminepentaaetic acid-D-phenylalanine) conjugated to octreotide, an artificial analog of the peptide neurotransmitter somatostatin, is used for staging of somatostatin receptor-expressing tumors such as carcinoid tumors.

**MRI**

Targeted MRI has not reached the clinical stage yet, but has been used to visualize cells undergoing apoptosis using annexin V cross-linked to SPIOs, and in a wealth of studies...
using specific antibodies cross-linked to SPIOs or Gd chelates. These include visualization of E-selectin overexpression, usually a characteristic of angiogenesis, inflammation, or atherosclerosis; fibrins in vascular plaques; integrins in tumor vessels; Alzheimer amyloid plaques; human transferring receptor; Her-2 receptors, etc. (Persigehl, Heindel, and Bremer 2005).

MRI can also be used, with nontargeted (perfusion) contrast agents such as ultra-small SPIO or gadolinium chelates, to measure blood flow parameters and vascular permeability (Padhani 2002).

US
Similarly, nontargeted US contrast agents can help assess the vascularization, and therefore detect the higher density of intratumoral vessels, as has been demonstrated in breast, prostate, and liver tumors (Dayton and Ferrara 2002). Animal studies have also shown a correlation between some tumor sites or sentinel lymph nodes and excess accumulation of different types of nontargeted microbubbles, although the exact mechanism of this phenomenon remains unclear (Dayton and Ferrara 2002).

Planar fluorescence imaging
Planar fluorescence imaging of tumor-associated proteases (cathepsin B or D, metalloproteinase 2) with NIR activatable probes has been demonstrated in mice, as has fluorescence imaging of cell-surface receptors and tumor-related ligands (annexin V, somatostatin receptor-binding peptides, hydroxyapatite-binding ligand) coupled to NIR fluorophores (Graves, Weissleder, and Ntziachristos 2004). Although sensitive, these applications suffer from the poor spatial resolution, and particularly the lack of depth information, inherent in planar fluorescent imaging. Promising developments in FT approaches have recently been reported in subcutaneous tumor and lung carcinoma murine models (Graves, Weissleder, and Ntziachristos 2004).

7.4.2 Non-Oncological Imaging

PET and SPECT
The unique ability of [18F]FDG PET to detect regions of high metabolism is also of great value in non-oncological brain imaging studies of infection, epilepsy, dementia, and cerebrovascular diseases, and of the early onset of Alzheimer disease in patients well before MRI could detect it (Pfleps 2000). It also plays significant roles in cardiac studies of coronary artery disease and of heart muscle viability following myocardial infarction, in the examination of inflammation of various tissues and infections, and elsewhere.

SPECT imaging applications in nonmalignant diseases cover a similar spectrum. For instance, coupled SPECT/CT of antigranulocyte [99mTc]-antibodies can provide the precise anatomic localization of post-surgical infections (e.g., osteomyelitis), and [99mTc]-labeled antibodies against red blood cells will help delineate the source of gastrointestinal bleeding before surgery (von Schultheiss 2003).

MRI
MRI's limited sensitivity relative to nuclear imaging renders its molecular imaging applications rather infrequent, and mostly confined to unique ex vivo studies. As an indication of this difference of sensitivity, visualization of hepatocytes with apoferritin-gadolinium complexes (8 to 10 of them entrapped per apoferritin molecule) necessitates up to 40 million complexes per cell (Aime et al. 2002).

US
US imaging of targeted contrast agents (antibody- or peptide-functionalized microbubbles, liposomes, or perfluorocarbon emulsion nanoparticles) has been applied in animal studies of inflammation, thrombus, and atherosclerosis. For instance, administration of perfluorocarbon emulsion nanoparticles (~250 nm diameter) targeted against fibrin with a monoclonal antibody in dogs resulted in a contrast enhancement at the thrombus site that resulted in an increase in detection rate from 2% to 96% after two injections of the contrast agent (Dayton and Ferrara 2002).

7.4.3 Reporter Gene Imaging

Gene expression levels have become a defining signature of the stage of the cellular cycle in which a population of cells resides. Such studies have been made possible by the combined advent of genome sequences and DNA chip technologies; the next step, beyond monitoring the level of messenger RNA (the transcriptome), is likely to be to monitor the level of expressed proteins (the proteome). The ultimate goal of these (still in vitro) efforts is to characterize the complex and dynamic network of interactions between expressed proteins, proteins and DNA or RNA, and to achieve a complete understanding of the spatiotemporal pattern of biological processes taking place in normal (and abnormal) cells.

How this can possibly be integrated into a humanly manageable picture is a question beyond the scope of this section, but it serves to justify the more modest approach used in current studies. Indeed, for simplicity's sake, it is generally assumed that normal cellular behavior corresponds to a typical expression level for one or few characteristic proteins, and any significant deviation from this average level signals the onset of some pathology. Even if things might turn out to be much more complex in future detailed studies, the success of this simplifying view has some strong experimental support from a variety of organisms, from both the bacterial and the eukaryotic kingdoms, and from both unicellular and multicellular organisms, as seen above. Some of those approaches, however, monitor the expression level of proteins via a targeted
probe only indirectly, and are sensitive to issues such as probe circulation and pharmaco-kinetics, physiological barrier penetration, affinity and temporal stability, etc.; the other approaches monitor the activity of endogenous enzymes, which can be provided with a label substrate that does not interfere with metabolism.

Reporter imaging technologies have been developed to circumvent these issues by generating the signal simultaneously and at the location of gene transcription. This is achieved in several ways, the first being by fusion of the gene of interest with an enzyme—such as a luciferase gene for bioluminescence imaging, or an herpes simplex virus type 1 thymidine kinase gene, which will accumulate radionuclide-labeled small molecules in the target cell, such as 18F-fluoropenciclovir for PET or 131I-uracil nucleoside derivatives for SPECT imaging. Alternatively, the gene of interest may be fused with a receptor protein, such as a transferrin receptor that will transport iron oxide particles into targeted cells for contrast in MRI imaging. Yet another involves a fluorescent protein gene, for fluorescence imaging.

Apart from the special case of transgenic animals, three types of studies can be carried with these approaches: Imaging the fate of labeled cells and their progeny; monitoring the level of expression of a gene injected for gene therapy; and study of in vivo protein interactions. Animal studies utilizing these approaches abound, and have been surveyed in several recent publications (Ray et al. 2001; Contag and Bachmann 2002; Massoud and Gambhir 2003).

**Monitoring gene therapy**

Even though gene therapy has suffered serious setbacks in recent years, the prospect of treating otherwise incurable, debilitating genetic diseases motivates continuing efforts. Means to better assess the localization, efficiency, duration of gene delivery, and expression in vivo are direly needed, and molecular imaging might provide a solution.

Several different strategies involving reporter genes have been adopted to monitor the expression of replacement genes injected in the targeted cells or organs. These include: fusion of the gene of interest with that of a gene that codes for a reporter protein, to express a functional chimeric reporter protein; insertion of an internal ribosomal entry sequence (IRES) between the gene of interest and the reporter gene, to produce a single polycistronic mRNA translated in two separate proteins; cloning in two different vectors, but under control of the same promoter; cloning of the two genes in a single vector, but under control of two different promoters. These approaches have been used with success in animal models of prostate cancer “suicide gene” therapy, in localized expression of the heme oxygenase-1 gene in lung alveoli, and in adenoviral treatment of ovarian cancer (Massoud and Gambhir 2003).

**In vivo molecular interactions.** Study of protein-protein interactions in vivo is necessary to validate the growing amount of data emerging from the field of proteomics, which, starting from experiments providing information on pairwise interactions, builds networks of protein interactions that may in part be artificial or tissue-dependent. Inspired by high-throughput cell culture studies, two general approaches have been explored, one for proteins interacting in the nucleus, the other for interactions anywhere in the cell (Massoud and Gambhir 2003).

The first uses the yeast two-hybrid approach, which is based on splitting a transcription factor that regulates the expression of a reporter gene (e.g., the luciferase gene), and fusing each split moiety to one protein of an interacting pair. When the two proteins bind, the transcription factor is reconstituted, and this can trigger reporter gene expression by binding to its promoter site, eventually resulting in a detectable signal (Ray et al. 2002).

The other involves splitting the protein itself (e.g., the luciferase protein) and, again, fusing each moiety to one protein of an interacting pair. Reconstitution of the active protein can occur by complementation or via an intein-mediated reaction (Paulmurugan, Umezawa, and Gambhir 2002).

**7.4.4 Drug Discovery and Development**

Molecular imaging allows the monitoring of a labeled molecular target in a live organism, and for some imaging modalities, allows quantifying this target over time: this is all that is
needed to study the effect of a drug on a molecular target. In this respect, the potentialities of molecular imaging to transform drug development (and discovery) are enormous (Rudin and Weissleder 2003).

Molecular imaging methods have several advantages over the current methods used to assess drug action using biopsies. First of all, compared to painstaking dissection and histological methods, using molecular imaging methods would shorten the time needed to evaluate a new drug candidate, as well as reduce its cost by avoiding sacrificing countless animals. This, in turn, would permit following the same animal over time, enabling longitudinal studies in a single animal. Such studies would provide critical information on the optimal timing and dosing of drugs. Finally, the increasing sensitivity of novel molecular imaging approaches would provide earlier indications of therapy success (or failure) than current methods do. It is therefore likely that molecular imaging will permit a reduction in development time of new drugs and also facilitate clinical studies. However, a number of obstacles will need to be overcome before these methods are used by the pharmaceutical industry.

(i) In terms of regulation, drug validation has relied thus far on long trials characterized by very crude criteria, such as survival. A paradigm in which a biomarker would be followed continuously over a shorter period of time, but with exquisite sensitivity and accuracy is at odds with this practice and it might take time for it to be accepted as a valid alternative. Extensive validation of the molecular imaging techniques will be needed before they can be accepted by regulatory agencies.

(ii) Molecular imaging biomarkers still need to be developed for a large number of targets and pathways of interest. As mentioned before, this is far from being an easy task.

(iii) Molecular imaging agents have consistently undergone longer approval processes than therapeutic agents before their clinical use. In addition, their market has yet to demonstrate a significant return on investment, both effects reinforcing each other and resulting in a slow development process.

(iv) The drug development and molecular imaging research communities have largely evolved without much interaction, whereas the development of biomarkers useful for drug discovery would require a close collaboration.

(v) Molecular imaging methods will have to prove their advantages over competing technologies such as genomics, metabolomics, and proteomics, which have made tremendous progress in assessing gene-, metabolite-, and protein-expression levels associated with disease and useful in monitoring response to a therapy.

Despite these issues, it is in the interest of the pharmaceutical industry to start taking a closer look and investing in developments in molecular imaging, to tailor them for their specific needs and, in particular, to respond to the requirements of future highly specific therapies requiring patient selection and close monitoring of the therapy response. Not only will this speed up drug discovery, it will also ultimately reduce costs and result in better medicines.

7.4.5 Combined Imaging/Therapy

One of the early visions of molecular imaging emphasized its potential to get as detailed (i.e., on the molecular level) an understanding of a patient’s disease as possible, both to customize the type, dose and location of the treatment and subsequently to follow the evolution of the disease. Current technologies have made great progress in fulfilling this goal, and another vision is now driving some research: Molecular probes are reaching targets that are crucial to the characterization of some diseases, so why not equip them with the tools needed also to cure them?

This idea can, in principle, be implemented easily with nanocrystal probes. The relatively large surface area (tens to hundreds of nm²) of qdots and other nanomaterials can be utilized to graft several different functionalities to the same particle. This concept was demonstrated with a so-called peptide toolkit to impart PET and fluorescence contrast agents simultaneously to the same particle (Michalet et al. 2005). In a similar fashion, qdots can be coated simultaneously with disease-specific combinations of large molecular weight PEG molecules (to increase their retention time in the blood circulation), antibodies or other affinity molecules (to target molecular markers), chelators (for adding MRI and PET contrast agents), quenchers (for molecular switching), and virtually any other available functionality.

Such multifunctional probes could be used in the future for cancer diagnosis and treatment monitoring, and perhaps for the treatment itself (Figure 7–5). After injecting such a probe into the patient, for example, it would target and home to cellular markers of diseased tissues and organs, for imaging them at the desired scale (from whole body down to the organ using PET and then at the tumor level using fluorescence imaging). The curing function would then be activated, and its success could be monitored over time.

As an example, one can envisage intravenously injecting patients at high risk for colorectal cancer, prior to colonoscopy, with color-coded qdots coated with engineered antibodies specific for colon cancer-associated cell surface markers. The colonoscopy could then be performed using next generation endoscopes that will be able to excite and image the qdot
fluorescence, providing an in situ color-coded fingerprint of expressed markers. Likewise, an optical fiber confocal-based catheter could be guided to the site of a known tumor (using fluoroscopy to reach the region of interest previously highlighted by PET) for real-time, in vivo “virtual optical biopsy.” If confirmation is needed, tissue removed in real biopsy could then be analyzed for the presence of cancer cells using fluorescence microscopy (Michalet et al. 2005). Nanometer scale, subcellular imaging could be further pursued using electron microscopy. Such an approach could be repeated during treatment to follow response and tailor the therapy.

Once diagnosis is made, therapy could be ensued selectively, locally, and temporally by depositing energy (monochromatic x-rays for k-shell absorption or laser IR radiation) into the targeted qdots, in a manner similar to the recently reported photo-thermal tumor ablation with gold nanoshells (O’Neal et al. 2004). It might also be possible to graft therapeutic enzymes to the qdot surface and activate them by light, or produce free radicals (such as singlet oxygen) by optically cycling the qdots.

Although still a vision, these scenarios are based on existing technologies and demonstrated capabilities, and are suggestive of things to expect from molecular imaging in the not too distant future.

7.5. Conclusion

Molecular imaging has currently two faces. One embraces a set of well-established imaging approaches that have reached the clinical domain, such as [18F]FDG PET, and various SPECT approaches that are mostly built around 99mTc. The other focuses on a large number of experimental studies exploring virtually all traditional imaging modalities, and on a number of others, as well.

This duality reflects the limited business opportunities for expensive probes with a small market, a leitmotiv applicable also to the drug development arena. But the future holds great promise, as methods of rapid and automated synthesis of highly specific contrast agents and targetable probes are developed, providing early, unambiguous, and spatially defined diagnostics. It is to be expected that the preparation costs of these tools will surely drop; that, along with the advent of the complete human genome sequence and the ever-increasing knowledge of protein function, networks, and dynamics, will enable molecular imaging to play a vital role in the creation of a new medical paradigm that may, indeed, replace the current “one symptom, one disease, one drug for all” approach (Rudin and Weissleden 2003).

7.6 References


bocyanine-labeled human serum albumin and transferring.”


Molecular Imaging Central: http://www.mi-central.org. Hosted by the Molecular Imaging Program at Stanford, this website offers a glossary of terms, links to review articles and books, institutes and research groups, vendors, and in general all information relevant for molecular imaging.


