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In Vivo Molecular Imaging

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Abstract The relatively young field of molecular imaging is focused on the visualization of molecular phenotypes in whole organisms. This is achieved using imaging systems based on radionuclides, nuclear magnetic resonance, ultrasound, or the visible-IR region of the optical spectrum. Molecularly defined contrast in these modalities is generated by exogenous probes of the endogenous proteome, or through transgenes. Examples of exogenous probes include those that are transported and trapped (glucose, nucleoside analogs), those directed against extracellular receptors (somatostatin, opioid, melanotropin), and those activated by extracellular proteases. Transgenes that have been used in molecular imaging include the above receptors, non-mammalian enzymes that trap pro-drugs (HSV-tk, yeast CD), and optical reporter proteins (luciferase, fluorescent proteins). Cutting edge technologies in this field include in vivo assays for protein-protein interactions, and in vivo assays for mRNA expression patterns. The number of degrees of freedom in designing new agents is daunting, and advancements in this field will require a significant participation from molecular and cellular biochemists. J. Cell. Biochem. Suppl. 39: 231–238, 2002.

Key words: fluorescence; MRI; PET; SPECT; gene therapy

The past two decades have witnessed the beginnings of a revolution in the field of in vivo imaging from a discipline that was based on anatomy to one that is increasingly based on tissue function. Techniques are being developed on a regular basis to assess tissue behavior, tissue metabolism and biochemical patterns in living animals with higher and higher precision and information density. Because many of the imaging modalities are similar, one promise of this work is the straightforward translation from animal work to the clinic. The center of this revolution is the relatively new science of *Molecular Imaging*. Because this field represents a convergence of basic and clinical interests, it is being driven by productive alliances between medical physicists and radiologists on one hand and molecular and cellular biochemists on the other. These two cultures are working together in multidisciplinary teams and consortia to develop reporter constructs and approaches to dynamically visualize molecular phenotypes in living mammals. Although many scientists have played important roles in the development of this field, no one deserves credit more than Richard Klausner, whose visionary leadership of the NCI established cancer imaging as one of his priority growth areas in 1996 [Klausner, 1996]. As a consequence of this action, the Biomedical Imaging Program (BIP) was established at the NCI in Fall, 1997 under the leadership of Daniel Sullivan (http://www3.cancer.gov/bip). This program has been instrumental in nurturing the infrastructure and development of this nascent field. Even though this field is relatively young, it has been extensively reviewed and is prominently featured in a number of new journals, for example, [Jain, 2001; Lanza and Wickline, 2001; Nichol and Kim, 2001; Weissleder and Mahmood, 2001; Weissleder, 2002]. This communication will serve as a basic introduction and focus the discussion on applications of most interest to molecular and cellular biochemists.

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IMAGING AT THE MOLECULAR LEVEL

Molecular imaging grew out of advances which occurred about 25 years ago in the

then-disparate fields of medical physics and cellular biochemistry. In medical physics, positron emission and single photon emission tomographic imaging (PET and SPECT) were being developed to monitor the fate of radiolabeled compounds to be imaged in whole animals and human patients for the first time [Kuhl et al., 1978; Hoffman and Phelps, 1979]. Biochemists were actively involved in these developments, because many of these radiotracers were metabolic precursors. At the same time, ^{31}P and ^{1}H magnetic resonance spectroscopy (MRS) were being translated out of pure chemistry/biochemistry and applied to living systems [Salhany et al., 1975], which eventually led to spectroscopy and imaging of animals and humans [Mansfield and Maudsley, 1977; Chance et al., 1982; Budinger and Lauterbur, 1984]. At the same time that these developments were taking place in the translational medical arena, cellular biochemists were developing fluorescent chemicals with which to monitor specific macromolecular and metabolic behaviors in living cells [Tsien, 1980]. The powerful application of fluorescent and luminescent proteins has enabled the in vivo contrast to be genetically encoded [Prasher et al., 1992]. Such reagents have revolutionized the study of cellular behavior and have become an indispensable part of the cellular biochemists' armamentarium. These optical approaches have recently been applied to imaging gene expression in whole mice [Contag and Bachmann, 2002], and this has consummated the consortium between two previously distinct disciplines. Figure 1 shows examples of these different imaging modalities applied to mice.

Table I lists techniques available for in vivo molecular imaging approaches and their sensitivities and resolutions. Dedicated small animal imaging systems have been commercially developed for all of these modalities and this is a rapidly growing area. Because this review will focus on applications, the reader is referred to one of the many excellent medical physics texts, e.g., [Bushberg et al., 2002] for further discussion of the design and principles for these different imaging platforms. Table II lists some of the common contrast media available for these different modalities. Generally speaking, nuclear and optical methods involve the release of photons and these are extremely sensitive, requiring sub nanomolar concentrations of contrast media in the imaging voxels. Optical photons (i.e., those between 200 nm and 10 μm in wavelength) are strongly absorbed by biological tissues, except in a red-near infrared window between approximately 800-1000 nm (Fig. 2). As scatter also decreases with wavelength, this range provides a "diagnostic window" within which there is hope of capturing and localizing optical photons emitted from deep tissues.

IMAGING THE PROTEOME AND GENE EXPRESSION IN VIVO

Molecular imaging can be divided into two general approaches for generation of image contrast. In one approach, contrast reagents are engineered to interrogate endogenously expressed proteins or nucleic acids and in the other, reporter genes are transfected for expression regulated by environmental or tissue factors [Luker, 2002]. Imaging of endogenous expression has a high potential for clinical translation and is already very useful in diagnosis and staging for many human pathologies. An additional promise of this work is the combination of imaging and therapeutic reagents such that they can be iteratively co-optimized. Transfection of reporter genes is a staple of cellular biochemistry and can be used to interrogate specific intracellular events. Additionally, transfection of reporter genes can be useful in quantifying and optimizing gene therapy protocols. In optical imaging, reporter genes are imaged directly, such as luciferase or fluorescent proteins. For nuclear and magnetic resonance approaches, reporter genes generally require the addition of exogenous contrast reagents that detect the presence of gene product.

Transporters

The simplest form of molecular imaging is through the use of small molecule substrates for specific transport processes that are upregulated in the target cells. The most widely used such system is ^{18}F-labeled fluorodeoxyglucose (FdG), which is avidly taken up and trapped by phosphorylation by many types of cancers [Anderson and Price, 2000a]. Uptake and retention of FdG is correlated with high rates of glycolysis and overexpression of the glucose transporter, GLUT-1. This is becoming a staple technique in the oncologists' armamentarium, as it allows single shot visualization of metastatic sites, for the purpose of staging and

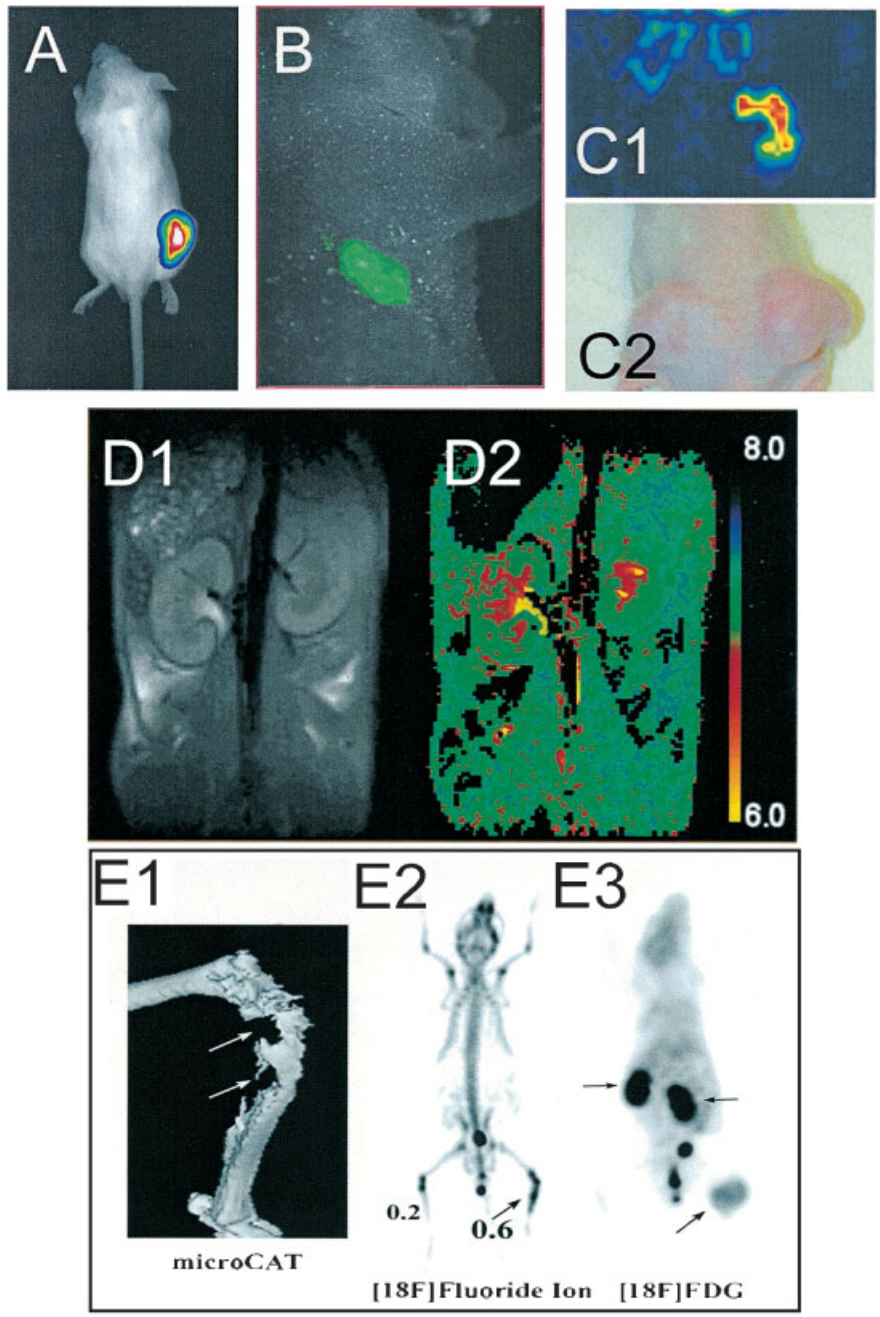

Fig. 1. Examples of molecular imaging. **A**: In vivo bioluminescence of mouse bearing *P. pyralis* luciferase-expressing C6 glioma tumor. From author's lab. **B**: Spectrally enhanced registered in vivo fluorescence image of mouse bearing *Aequora* GFP-expressing tumor. Image courtesy Richard Levenson, M.D. of CRI, Inc. rlevenson@cri-inc.com. **C1**: SPECT image of mouse expressing adenovirus encoded somatostatin-2 receptor, probed with ^{99m}Tc labeled somatostatin analog, and (**C2**) corresponding bright field image. Images courtesy Kurt Zinn, DVM/PhD, Univ. Alabama, kurtzinn@uab.edu, and Harrison Barrett, PhD, Univ. Arizona barrett@radiology.arizona.edu. **D1**: Fat-supressed proton-density MR image of mouse torso, and (**D2**) corresponding image of interstitial pHe calculated from relaxivity of pH-sensitive Gd-containing contrast reagent [Raghunand et al., 2003; 5460/id]. From author's lab. **E1–E3**: MicroCT, $Na^{18}F$ and [^{18}F]deoxyglucose MicroPET scans in a mouse with CL-1 prostate cancer xenograft (transversal arrows). CT demonstrates the osteolytic character of the lesion, and microPET of $Na^{18}F$ shows high bone turnover. [^{18}F]dG shows high metabolism and kidney uptake (horizontal arrows). Figure courtesy S. Gambhir, MD/PhD, Crump Institute, UCLA (SGambhir@mednet.ucla.edu; Berger et al., 2002].

TABLE I. In Vivo Molecular Imaging Modalities and Their Approximate Sensitivities

Modality	Spatial resolution	Time resolution	Sensitivity
Magnetic resonance			
MR imaging	Sub-mm	1–2 s	mM Gd; nM Fe (~10 cells/voxel)
^{1}H spectroscopy	>mm	>5 min	>10 μM
Nuclear medicine			
PET	mm	s-min	pM tracer
SPECT	Sub-mm	min	Sub-nM tracer
Optical			
Bioluminescence	mm	min	~100 cells/voxel
Fluorescence	μm	ms	pM
X-ray computed tomography			
Contrast-enhanced	Sub-mm	1–2 s	mM I
Ultrasound			
Micro bubbles	μm	ms	1 bubble

therapeutic decision making. Transporters have not been used extensively as reporters for other imaging modalities, nor have they been used extensively for monitoring of gene therapy, despite the availability of FDA-approved substrates. It is expected that this will be a growth area with the development of specific transporter-substrate pairs.

Receptors

Receptor ligands are distinguished from transporter substrates as they are larger and must be endocytosed in order to be trapped and amplified through concentration. The larger size of these ligands allows more degrees of freedom in their design and hence, higher specificity. In some cases, the probes are directed against endogenously expressed targets, while others rely on genetically engineered overexpression. This latter approach is successfully being used to monitor the efficiency of gene therapies.

In nuclear medicine, success has been had with a number of receptors, including the δ2-dopamine and somatostatin-2 receptors. ^{18}F labeled ligands specific for the dopamine receptor have been developed to image, using PET, specific activation of nuclei within the limbic system, especially during risk/reward behavior [Barrio et al., 1997; Drevets et al., 2001]. The δ2-dopamine receptor has also been used as a transfected reporter protein, using micro-PET [MacLaren et al., 1999]. However, the relatively slow pharmacokinetics for ligands generally requires longer-lived isotopes than those available for PET. Using SPECT, ^{99m}Tc and ^{111}In labeled analogs of α-MSH have been used to identify and image malignant melanoma with high specificity and sensitivity [Chen et al., 2002]. Similarly, ^{99m}Tc and ^{111}In labeled somatostatin analogs are used to identify and image neuroendocrine cancers that over express somatostatin receptors. Because these are FDA approved, it was cleverly reasoned that the receptors themselves could be used as reporters for gene transfection, i.e., in gene therapy [Rogers et al., 2000; Chaudhuri et al., 2001]. In MR, endogenous receptor expression levels

TABLE II. Common Contrast Media for Molecular Imaging

Modality	Media[a]	Comments
Positron emission tomography (PET)	^{11}C (20.4 m); ^{18}F (110 m)	^{18}F is used in >80% of all PET scans
Single photon emission computed tomography (SPECT)	^{111}In (2.8 d); ^{123}I (13.2 h); ^{99m}Tc (6 h)	^{99m}Tc is most widely used clinically; ^{111}In half-live useful for targeted agents
Magnetic resonance imaging (MRI)	Gadolinium; Iron	Gd is T1 agent (10–100 μM); Fe is T2 agent (1–10 μM)
Ultrasound (US)	microbubbles	Stabilized liposomes. Can be targeted with surface ligands
X-ray computed tomography (CT)	Barium; Iodine	Dose required is generally too high for effective molecular targeting
Bioluminescence	Genetically encoded luciferases from *P. pyralis*, *Renilla*, *bacteria*	Protein-substrate engineering has not been fully explored for in vivo imaging
In vivo fluorescence	Genetically encoded fluorescent proteins: green, cyan, yellow, red (dyes: Cy5.5)	Proteins have tendency to aggregate in vivo. Dyes work best in near-IR wavelengths

[a]For radionuclides, half-lives given in minutes-hours-days.

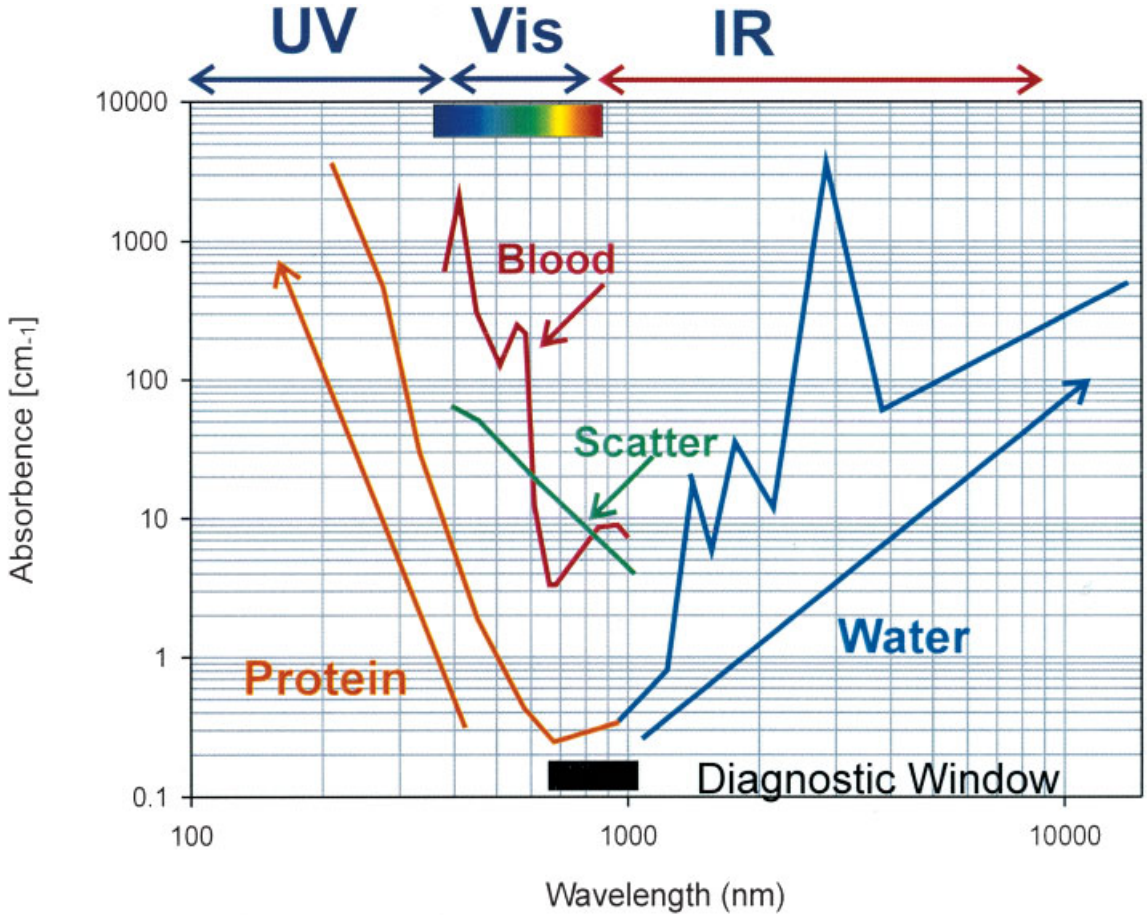

Fig. 2. Photon absorbence and scatter by tissues. Optical sensitivity is degraded by absorbence. In the UV region, photons are absorbed strongly by macromolecules such as nucleic acids and proteins. This is reduced by 4 orders of magnitude between 200 and 800 nm (orange line). Above 1,000 nm, water absorbs photons through rotational and vibrational energy transitions (blue). Hence, the red to near-IR region is known as the diagnostic window. Absorbance by blood (shown in red) is high in the red and reaches a local nadir near 900 nm. On a whole animal level, this is not problematic as blood volume is small. Optical resolution is degraded by elastic scattering which decreases with wavelength, as shown in green.

are generally too low to generate sufficient signal without amplification. The most well-developed ligand–reporter combination is a down-regulation incompetent transferrin receptor (TfR) which is interrogated using iron-oxide containing transferrin (Tf) oligomers [Moore et al., 1998]. Iron provides contrast in MR images because it perturbs the magnetic field, causing distortions that can be visualized. Integrins have also provided important targets for molecular imaging because they are accessible through the vasculature and their isotype expression is sub-tissue specific [Trepel et al., 2002]. Consequently anti-integrin targeted contrast agents are being developed for PET [Haubner et al., 2001a,b], SPECT [Sivolapenko et al., 1998; Posey et al., 2001], MR [Sipkins et al., 1998; Anderson et al., 2000b], ultrasound [Lanza and Wickline, 2001] and fluorescence.

Proteases

Secreted proteases are critically important in tissue remodeling during development, wound healing and cancer metastasis. Molecular imaging probes have been developed to exploit the self-quenching of the near-IR dye, Cy 5.5 [Weissleder et al., 1999]. Fluorescence quenching is strongly dependent upon proximity, dropping off with distance to the sixth power. In these constructs Cy 5.5 dyes are covalently linked together through peptide linkers that contain protease-specific primary sequences. In the presence of active protease, the linkers are cleaved and the dyes are free to diffuse,

whereupon they lose quenching and exhibit high intensity fluorescence. This approach has been coupled to a specially designed fluorescence optical tomography system to generate 3-D images of protease activity in living mice [Ntziachristos et al., 2002].

Intracellular Metabolite Interconversion

The next big challenge for molecular imaging is to develop probes for signal transduction events that occur intracellularly. In whole animals, such approaches can be used to monitor the efficacy of targeted therapies. ^{18}F-labeled thymidine (FT) has been developed as an in vivo reporter of cell proliferation. This agent is trapped in proliferating cells via phosphorylation by thymidine kinase, TK-1, whose activity is cell cycle dependent [Rasey et al., 2002]. The most well-studied exogenous reporter gene is herpes simplex virus thymidine kinase (HSV-TK) which has been used in gene therapy to convert pro-drugs, such as ganciclovir, into active chemotherapeutics. Radiolabeled ganciclovir analogs are trapped in cells in the same way as FT [Iyer et al., 2001; Tjuvajev et al., 2002]. In this case, the therapy and imaging agents can have the same biodistribution, giving a direct and quantifiable readout on the efficacy and distribution of gene therapy. Similarly, yeast cytosine deaminase will convert 5-fluoro cytosine into the biologically active 5-fluoro uracil, and this has been monitored using ^{19}F-magnetic resonance spectroscopy [Stegman et al., 1999].

Challenge of Imaging Intracellular Macromolecular Interactions

A major challenge for molecular imaging is to visualize protein–protein, nucleic acid–nucleic acid, and protein–nucleic acid interactions in vivo. This will requires significant input from researchers who have developed such approaches for in vitro use. For example, optical reporters for protein-protein interactions have been available for years in vitro, methods are only now being applied to probe these events in vivo [Luker et al., 2002; Ray et al., 2002].

A more significant challenge will be to image intracellular events without genetic encoding in order to visualize endogenous expression of macromolecule behavior. A few approaches have been proposed, but this is an area that will require significantly more effort involving collaboration with cellular biochemists. One approach to visualize endogenous gene expression is through the use of radio- or optically labeled antisense RNA, which holds promise for exquisite specificity [Zhang et al., 2001; Younes et al., 2002]. However, there are serious obstacles due to complex in vivo pharmacokinetics of these agents. An exciting aspect of this research is that imaging and therapy are combined in a single agent and thus, quantitative molecular imaging and therapy response can be iteratively optimized together. Contrast can also being delivered into cells using polycationic complexes, such as the HIV Tat-peptide [Josephson et al., 1999; Bhorade et al., 2000]. This is especially exciting because the contrast media can be of variable size and have the potential to be targeted. As above, the imaging and therapeutic agents can be combined, yielding potential for iterative co-optimization.

CONCLUSIONS AND CHALLENGES

A major driving force for the development of molecular imaging comes from the potential for in vivo human clinical use. Although it is assumed that chemically engineered exogenous reporters will be more useful than genetically encoded reporters, this need not be the case. Genetically encoded reporters can be used to monitor gene therapy and have great potential to report on in vivo pharmacodynamics for targeted therapies. Thus, even at a relatively young age, this field has a daunting number of degrees of freedom in the design of specific imaging strategies. Choices must be made regarding the molecular target, the contrast-generating mechanism, the imaging platform and the methods for data analysis. At this time, the number of potential targets are too numerous to count. Receptors, transporters extracellular enzymes and intracellular macromolecules are all potential targets for molecular imaging. This minireview has highlighted some approaches that are in development, as well as some areas where more progress is needed. At the cutting edge are new approaches for visualizing intracellular macromolecule interactions in living systems. The need for novel approaches can be satisfied either through de novo engineering, or through the translation of existing in vitro diagnostics to in vivo use. Although the move from in vitro to in vivo used to be rate-limiting, this is being opened up through the development of a number of imaging "cassettes"

from which to choose. Hence, there is a great opportunity at the present time for molecular and cellular biochemists to impact on this rapidly developing field.

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