A clearer vision for *in vivo* imaging

Progress continues in the development of smaller, more penetrable probes for biological imaging.

Ralph Weissleder

Thinking small can be very effective when designing targeted probes for imaging biological processes. In particular, the substitution of small peptides for bulky monoclonal antibodies is proving a particularly promising strategy for targeting probes to receptors in tissues that would otherwise be difficult to penetrate or access. Such agents are also often less immunogenic and show better pharmacokinetic properties. A paper in this issue by Becker *et al.*[1] describes the latest demonstration of this approach for *in vivo* optical imaging—an analog of somatostatin coupled to a near-infrared (NIR) cyanine fluorochrome that allows *in vivo* visualization of tumors displaying somatostatin receptors. This is one of many probe types and detection technologies under development that are allowing new ways of imaging biological systems at the cellular and even molecular level.

Several diagnostic imaging technologies developed over the past three decades have had a profound impact on clinical medicine, including magnetic resonance imaging, x-ray computed tomography, and nuclear tomographic imaging. Whereas the resultant multibillion-dollar industries have primarily focused on developing next-generation clinical imaging systems and applications, interest has also been growing in using these technologies as laboratory tools for imaging small animals at high resolutions. Although clinical imaging systems are primarily used for displaying anatomical, physiological, and metabolic parameters, experimental animal systems are additionally being developed to image at the cellular and molecular level *in vivo*. The various existing imaging technologies differ in five main aspects: resolution, depth penetration, energy expended for image generation (ionizing versus non-ionizing), availability of injectable, biocompatible molecular probes, and the respective detection threshold of probes for a given technology.

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Noninvasive *in vivo* imaging with light photons represents an intriguing avenue for extracting relevant biological information. Whereas light in the visible range is routinely used for intravital microscopy[2], imaging of deeper tissues (>500 μm to cm) requires the use of NIR light. Hemoglobin and water, the major absorbers of visible and infrared light, respectively, have their lowest absorption coefficient in the NIR region around 650–900 nm (Fig. 1A). Light photons can be used to measure different native parameters of tissue through which they travel—for example, absorption, scattering, polarization, spectral characteristics, and fluorescence. The major impact for imaging molecular information *in vivo*, however, has come from the recent development of targeted NIR fluorochromes[3], activatable NIR fluorochromes[4], red-shifted fluorescent proteins[5], and bioluminescent probes[6].

The paper in this issue[1] and several other previous publications by the same authors and others[7] demonstrate that NIR fluorochromes coupled to peptides can be used to image receptors using reflectance imaging. The strategy of coupling biocompatible, NIR fluorochromes to peptide ligands, rather than monoclonal antibodies[3], may well represent a step toward smaller, more penetrable reporter probes and may have particular applications in unique clinical situations where nuclear imaging is not an option (e.g., for reasons of resolution, during endoscopy, or in surgery). The peptide-coupled NIR fluorochromes are also expected to have significant advantages over nonspecific fluorochromes, such as indocyanine green, as the latter primarily reflects initial vascular distribution (through binding to plasma proteins) and subsequent hepatobiliary excretion.

The concept of tagging NIR fluorochromes can potentially be extended to a myriad of other peptide/small-molecule receptor systems. The technology may also be useful for *in vivo* screening of limited peptide libraries and/or for identifying structure/activity relationships. Several toxicology and safety issues of the above probes remain to be addressed before their clinical use. Some of the NIR fluorochromes, such as indocyanine green, however, have been used in tens of thousands of patients with reported side effects of <0.15%, an extremely favorable index compared with other reporter agents[8].

One potential caveat of using targeted conjugates is the fact that target-to-background ratios can be limited by receptor density and/or availability, limited clearance kinetics from the interstitial space, and/or

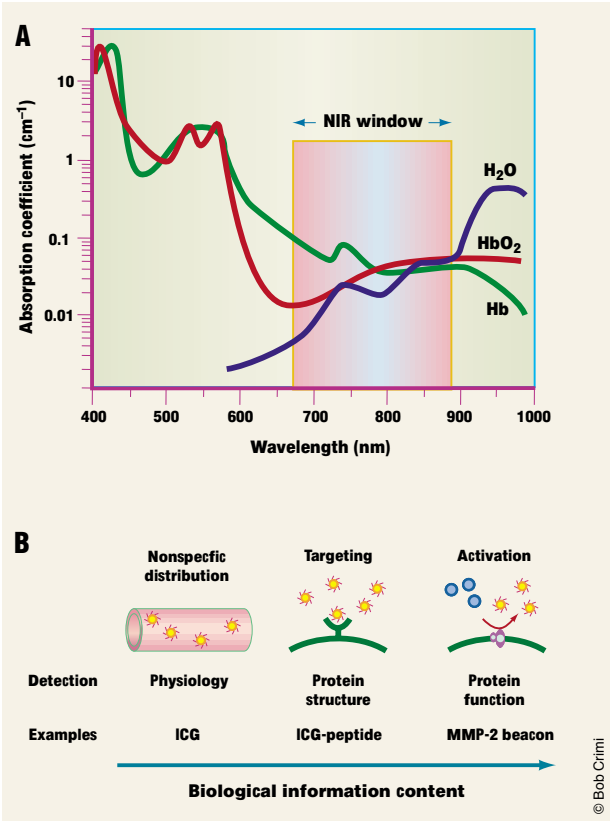

Figure 1. Getting the right image. (A) The NIR window is ideally suited for *in vivo* imaging because of minimal light absorption by hemoglobin (<650 nm) and water (>900 nm). (B) Approaches of NIR fluorescent imaging probes. Isotope and fluorochrome reporters can be used interchangeably for nonspecific and targeted agents; however, fluorochromes can also be used to make activation-sensitive agents for read-out of protein function.

nonspecific cellular uptake or adhesion of certain fluorescent probes. In particular, it may be difficult to differentiate specifically bound from unbound ligands (Fig. 1B), and this is the reason why imaging is usually performed after nonspecifically distributed surplus has cleared.

One approach to facilitate the differentiation of target and background fluorochromes is to use quenched precursors that are activated by specific targets *in vivo* ("molecular switches" or "beacons"; Fig. 1B)[4]. In addition, this approach has the advantage that one target (e.g., an enzyme) can convert many individual beacons. As such, this strategy thus presents several levels of amplification (10 to 1,000 fold) over simple tagging.

Several activation-sensitive peptide–fluorochrome conjugates have also recently been introduced with specificity for cathepsins[4], matrix metalloproteinases, and other enzymes. These probes are currently being used to detect diseases at their earliest stage (e.g., small cancers, vulnerable plaque, or rheumatoid arthritis), image transgenes, or test the *in vivo* efficacy of enzyme inhibitors within hours after administration. Related molecular beacons capable of recognizing specific nucleic acid sequences have also been described[9].

Inherently linked to the development of the described probes is the need to develop tomographic three-dimensional imaging systems that can accurately quantify NIR fluorochrome concentrations and fluorescence activation in deep heterogeneous media *in vivo*. Whereas simple reflectance-type imaging systems have been used in previous development work, these systems are not quantitative and the image information is surface-weighted (i.e., anything closer to the surface will appear brighter compared with deeper structures).

Recently, optical tomography with NIR light has been in the frontier of research for resolving and quantifying light in deep tissues. In part, this has been the result of rigorous mathematical modeling of light propagation in tissue and technological advancements in photon sources and detection techniques. NIR light has been shown to travel at least 10 cm through breast tissue, and 4 cm of skull/brain tissue or deep muscle using microwatt laser sources (Food and Drug Administration (FDA) class 1[10]. With higher power levels (FDA class 3), light has been shown to penetrate through 7 cm of muscle and neonatal skull/brain. Advanced technology for single-photon counting or very low noise detection systems can now be used to reconstruct the activation of beacons in sub-millimeter-size cancers in deep tissues and may potentially yield a highly sensitive early cancer detection modality.

It is clear that the newly described probe armamentarium and NIR photon detection technology stand a good chance of making a significant impact on our capability to image molecular targets *in vivo*. With further advances in technology and chemistry, *in vivo* optical imaging and sensing is destined to truly come into focus.

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Toward the phosphoproteome

Two chemical protocols for the rapid analysis of protein phosphorylation by mass spectrometry promise to expand the scope of proteomics research.

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With the availability of a burgeoning sequence database, genomic applications demand faster and more efficient methods for the global screening of protein expression in cells. However, the complexity of the cellular proteome expands substantially if protein post-translational modifications are also taken into account. Two reports in this issue[1,2] describe new chemical protocols for selectively modifying phosphopeptides or phosphoproteins within complex mixtures. Modified peptides are enriched by covalent or high-affinity avidin–biotin coupling to immobilized supports, allowing stringent washing to remove nonphosphorylated peptides. Both methods proved successful in enriching phosphopeptides from defined peptide contaminants as well as from whole yeast cell extracts. They also presage the development of new strategies for rapidly screening phosphorylation events in cells.

Protein phosphorylation represents one of the most prevalent mechanisms for covalent modification, reflected in as many as one-third of eukaryotic gene products. Detection and sequencing of phosphopeptides is a well-studied problem; nevertheless, standard techniques of site identification by mass spectrometry or ^{32}P-labeling coupled with Edman sequencing are inefficient when dealing with highly complex mixtures of proteins or peptides and still require relatively pure starting samples. One solution is to selectively enrich phosphopeptides from unpurified mixtures. A first-generation technique that is easy and cheap is immobilized metal affinity chromatography (IMAC), in which phosphopeptides are bound noncovalently to resins that chelate Fe(III) or other trivalent metals, followed by base elution[3]. Unfortunately, this method proves ineffective in many cases as a result of inefficient adsorption of phosphopeptides or nonspecific adsorption of nonphosphorylated peptides. Clearly, alternative chemistries are needed.

The method of Zhou *et al*[1]. begins with a proteolytic digest that has been reduced and alkylated to eliminate reactivity from cysteine residues. Following N-terminal and C-terminal protection, phosphoramidate adducts at phosphorylated residues are formed by carbodiimide condensation with cystamine. The free sulfhydryl groups produced from this step are covalently captured onto glass beads coupled to iodoacetic acid. Elution with trifluoroacetic acid then regenerates phosphopeptides for analysis by mass spectrometry. Trial experiments showed that 80% of peptides recovered from yeast extracts were phosphorylated.

In contrast, Oda *et al*[2]. start with a protein mixture in which cysteine reactivity is removed by oxidation with performic acid. Base hydrolysis is used to induce β-elimination of phosphate from phosphoserine and phosphothreonine, followed by addition of ethanedithiol to the alkene. The resulting free sulfhydryls are coupled to biotin, allowing purification of phosphoproteins by avidin affinity chromatogra-

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