Optical and Multimodality Molecular Imaging

Insights Into Atherosclerosis

Farouc A. Jaffer, Peter Libby, Ralph Weissleder

Abstract—Imaging approaches that visualize molecular targets rather than anatomic structures aim to illuminate vital molecular and cellular aspects of atherosclerosis biology in vivo. Several such molecular imaging strategies stand ready for rapid clinical application. This review describes the growing role of in vivo optical molecular imaging in atherosclerosis and highlights its ability to visualize atheroma inflammation, calcification, and angiogenesis. In addition, we discuss advances in multimodality probes, both in the context of multimodal imaging as well as multifunctional, or “theranostic,” nanoparticles. This review highlights particular molecular imaging strategies that possess strong potential for clinical translation. (Arterioscler Thromb Vasc Biol. 2009;29:1017-1024.)

Key Words: atherosclerosis ■ molecular imaging ■ optical ■ fluorescence ■ multimodality ■ nanoparticle

Imaging methods to identify high-risk atherosclerotic plaques have traditionally focused on stenosis detection, yet the majority of plaques responsible for acute coronary syndromes originate from non–flow-limiting lesions.1 Furthermore, numerous experimental and clinical studies reveal that biological processes such as inflammation modulate the risk of plaque disruption and consequent myocardial infarction and stroke.2 High-resolution molecular imaging approaches aim to bridge the gap between in vivo imaging and biological characterization of atheroma. Using novel targeted and activatable imaging agents, molecular imaging strategies now offer in vivo readouts for a diverse array of biological processes.3,4 This review discusses advances in atherosclerosis detection enabled by optical, specifically near-infrared fluorescence, molecular imaging, as well as by multimodality reporter agents.

Near-Infrared Fluorescence Molecular Imaging

The interaction of light with matter has yielded a number of powerful imaging modalities including fluorescence, absorption and Raman spectroscopy, optical coherence tomography, bioluminescence, and angioscopy (supplemental Table I, available online at http://atvb.ahajournals.org). Fluorescence imaging, in particular, near-infrared fluorescence (NIRF) imaging (excitation 650 to 900 nm), provides a highly versatile platform for in vivo molecular imaging.5,6 Advantages of fluorescence as a molecular imaging modality include (1) picomolar molecular sensitivity; (2) versatile targeting platforms such as peptides, proteins, antibody fragments, nanoparticles, phage, and aptamers; (3) a number of fluorescence detection imaging systems with microscopic resolution (<10 μm, eg, epifluorescence, laser scanning, confocal, and multiphoton microscopy), mesoscopic resolution (10 to 500 μm, eg, optoacoustic imaging, mesoscopic fluorescence tomography, optical projection microscopy), and macroscopic resolution (>500 μm, eg, reflectance imaging, fluorescence molecular tomography); and (4) a strong track record of clinical translation, with fluorescence imaging systems available for retinal angiography (via fluorescein or indocyanine green enhancement), endoscopy, bronchoscopy, coronary angiography, and coronary arterial bypass graft imaging.7

Compared to fluorescence imaging in the visible light or mid IR range, fluorescence imaging in the NIR bandwidth offers (1) markedly less photon absorption from blood hemoglobin, lipid, and water, enabling photon transmission centimeters deep into the body; and (2) substantially reduced tissue autofluorescence, enabling higher sensitivity detection of targeted NIRF molecular imaging agents against a low background (supplemental Figure I). For a greater discussion of the physics underlying efficient NIR photon delivery through tissues, fluorescence chemistry synthesis approaches, and fluorescence hardware systems, the interested reader can consult several reviews.6,7

In Vivo NIFR Molecular Imaging of Atherosclerosis

Imaging of Protease Activity

Inflammatory and destructive matrix metalloproteinases (eg, MMP-1, -2, -3, -7, -8, -9, -12, -13, and disintegrin metallo-
noninvasive FMT of cysteine protease activity in atheromata and therefore represents an important molecular imaging target for atherosclerosis. Recently, a novel class of protease-activatable agents has enabled in vivo NIRF imaging of protease activity. The chemical design and imaging amplification strategy of different types of protease-activatable reporters are discussed in detail in the online supplemental materials.

Noninvasive Fluorescence Molecular Tomography of Plaque Protease Activity

Fluorescence molecular tomography (FMT) uses temporally and spatially resolved illuminators and detectors to detect NIR fluorochromes noninvasively and deep within the body. Advanced mathematical algorithms resolve photons deep within scattering tissue and render a quantitative 3-dimensional image of NIR fluorescence. Feasibility of noninvasive FMT of cysteine protease activity in atheromata first used apolipoprotein E–deficient (apoE<sup>−/−</sup>) mice. Twenty-four hours after injection of a first generation protease-activatable NIRF imaging agent (Prosense680, dose 5 nmol per mouse, 0.25 μmol/kg), noninvasive FMT demonstrated focal NIRF signal in the atherosclerotic aorta (Figure 1). Ex vivo macroscopic fluorescence reflectance imaging (FRI) demonstrated colocalization of NIRF signal with Sudan IV–stained plaques, and microscopic analyses demonstrated that the plaque NIRF signal colocalized with the lysosomal cysteine protease cathepsin B. This study demonstrated that FMT noninvasively visualized augmented plaque protease activity, and established cathepsin B as an inflammatory imaging biomarker for atherosclerosis. The first-generation of protease activatable agent is currently under development for clinical studies of atheroma and tumor cysteine protease activity.

To noninvasively visualize plaque gelatinolytic activity in vivo, Deguchi et al used a second-generation protease-activatable agent to investigate MMP-2 and MMP-9 activity. The MMP-2 and MMP-9 activity agent was synthesized by incorporating the gelatinase sensitive peptide substrate GG-PRQITAG into the first-generation protease activatable agent. Cholesterol-fed apoE<sup>−/−</sup> mice received the gelatinase NIRF imaging agent (dose 8 nmol per mouse; 0.4 μmol/kg). After 24 hours, FMT revealed augmented NIRF signals in areas corresponding to the aortic root, arch, and thoracic aorta, known sites of atherosclerosis in apoE<sup>−/−</sup> mice. Ex vivo FRI and NIRF microscopy validated the in vivo imaging findings. In addition, in situ zymography using quenched fluorescein-labeled gelatin demonstrated colocalization of plaque gelatinase NIRF signal with fluorescence signal from fluorescein.

As NIR photons may potentially travel >5 centimeters deep into the body, noninvasive FMT systems may eventually detect NIRF signals from human carotid atheroma, or from abdominal aortic aneurysms or plaques. Additional gains in FMT imaging may result from the use of nonfluid contact based imaging systems, integrated FMT-computed...
tomography (CT) systems, and multimodality imaging agents (discussed below).

Intravascular NIRF Catheter Sensing of Cysteine Protease Activity in Atherosclerosis

To develop a highly clinically translatable approach to image proteolytically active atherosclerosis in coronary-sized vessels, we developed an intravascular NIRF sensing approach based on a clinically utilized optical coherence tomography (OCT) guide wire. The guide wire was percutaneously tested in cholesterol-fed balloon-injured rabbits harboring inflammatory proteases in iliac atheromas. Twenty-four hours before imaging, the rabbits received an injection of the first-generation protease-activatable agent for cysteine proteases (Prosense750, dose 600 nmol/kg). In vivo, the NIRF guide wire detected strong signal emanating from inflamed plaques. Multiple catheter pullbacks in vivo yielded high plaque target-to-background ratios (TBR 6.8 versus saline control 1.3) through blood, without the need for flushing or occlusion, affirming the favorable photonic transmission properties of the NIR window. A good correlation was noted between in vivo and ex vivo TBR measurements and correlative fluorescence microscopy confirmed that the microscopic NIRF signal colocalized with immunoreactive cathepsin B. As the NIRF guide wire is a clinical platform and the Prosense agent should enter clinical trials in 2010 (personal communication), the current catheter based approach could enable identification of high-risk inflamed coronary atheromata.

NIRF Molecular Imaging of Plaque Cathepsin K Activity, Osteogenic Activity, and Angiogenesis

NIRF approaches to image cathepsin K protease activity, the spatiotemporal distribution of plaque osteogenic and inflammatory activity, and NIRF imaging of neoangiogenic vasculature in atheromata are discussed in the online supplemental materials and supplemental Figure II.

Emerging NIRF Imaging Agents

Promising NIRF agents for in vivo optical molecular imaging of atherosclerosis (Table) include sensors for detection of apoptosis by annexin sensors, oxidative stress by hypochlorous acid, integrin sensors based on RGD or small molecules (Intregrisense680), new plaque-targeted nanoparticulate NIRF agents, cysteine protease activity–based probes, NIR fluorescent deoxyglucose sensors for glycolytic flux/metabolism (similar to fluorine-18 deoxyglucose [18FDG] for PET metabolism studies), and matrix metalloproteinase presence via modified C-5–disubstituted barbiturates.

Multimodal Probes: Imaging of Atherosclerosis

Multimodal or multi-functional probes for molecular imaging fall into 2 categories: (1) probes that enable multiple in vivo imaging molecular readouts (multimodality imaging probes, ie, detectable by optical, MRI, and nuclear approaches simultaneously), and (2) probes that enable in vivo imaging and concomitant targeted therapy (therapeutic and diagnostic, or “theranostic,” probes). Favorable multimodality imaging probes harness capabilities from complementary imaging modalities to (1) achieve high sensitivity and high spatial resolution (eg, probes with reporter capabilities for both positron emission tomography and MRI) and to (2) enable both noninvasive and invasive molecular imaging (eg, via probes with MRI and NIR fluorescence reporter capabilities). A common platform for multimodality probes uses MRI to obtain concomitant anatomic, chemical, and physiological information. In addition to the above in vivo NIRF imaging considerations, fluorescent multimodal imaging offers the ability to perform precise target valida-
Multimodality Imaging of Plaque Macrophages

A powerful platform for multimodality molecular imaging of lesional macrophages uses dextran-coated crystalline iron oxide magnetic nanoparticles (MNP). Iron-oxide MNP are superparamagnetic imaging agents that exert field-dependent R2 and R1 effects. After phagocytosis by tissue macrophages, MNP induce strong relaxation effects detectable on T2-weighted MRI, and more recently by off-resonance positive contrast approaches.31–34 A class of MNPs show promise clinically in prostate cancer35 and carotid atherosclerosis MRI studies.36–40

In vivo testing of TNP was performed in apoE−/− mice using in vivo PET/CT and in vivo 7.0 T MRI systems. Several important results were obtained. First, TNP permitted concomitant in vivo MRI and PET imaging of plaque macrophages (Figure 3), with a 7- to 20-fold in vivo increase in sensitivity by PET- versus MRI-based detection. Second, the relatively longer half-life of copper-64 (12.7 hours versus fluorine-18, 1.8 hours) enabled autoradiographic studies that demonstrated TNP deposition into aortic atheromata.

<table>
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<th>Table. Promising Near-Infrared Fluorescence (NIRF) and Multimodal Molecular Imaging Agents for Atherosclerosis Detection</th>
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</table>

MMP indicates matrix metalloproteinase; CLIO, cross-linked iron oxide; MNP, magnetic nanoparticle; VCAM, vascular cell adhesion molecule.
injected dose/gram tissue). Third, the NIRF component permitted precise cellular localization and quantification fluorescence studies after radioisotope decay, and demonstrated that TNP deposited primarily in plaque macrophages (Figure 3). Fourth, compared to 18-fluorodexoyglucose (18FDG), a clinical PET tracer for imaging plaque metabolism/inflammation,3,4 TNP provided favorable plaque signal enhancement (standardized uptake value [SUV] 1.2 versus 0.8, \( P < 0.05 \)).

As starch-based iron oxide MNP have been tested clinically,35–40 the TNP agent may ultimately enable clinical noninvasive PET/CT and MRI detection of carotid plaque macrophages, particularly as promising integrated PET/MRI scanners translate into the clinic to provide one-stop molecular, anatomic, and physiological imaging capabilities. In addition, the fluorescence capability of TNP could enable invasive catheter-based NIRF detection of coronary plaque macrophages.

**Vascular Cell Adhesion Molecule–1**
The development of multimodal NIRF and MRI nanoparticles for in vivo imaging of VCAM-1 expression46,47 is discussed in detail in the online supplemental materials.

**Emerging Multimodality Imaging Probes**
Available multimodality probes with potential utility in atherosclerosis (Table) include apoptosis sensors for NIRF/MRI48 and angiogenesis-targeted perfluorocarbon paramagnetic emulsions for MRI/ultrasound.49 Additional promising nanoparticle platforms that could be useful for in vivo multimodality imaging include perfluorocarbon based microemulsions, microbubbles, micelles, quantum dots, liposomes, and lipoproteins, as comprehensively discussed in several recent reviews.50,51

**Multimodal Theranostic Probes: Targeted Imaging and Therapy of Atherosclerosis**
Integrated diagnostic imaging and therapeutic molecules, or “theranostic” particles, are increasingly appreciated as a valuable advance for targeted therapy. Addition of a diagnostic imaging moiety to a targeted therapeutic enables temporal and spatial monitoring of the therapeutic agent. Imaging information in theranostics can serve to confirm delivery of therapy at the desired target, identify the need for modified dosing/redosing strategies, quantify and track the “molecular efficacy” of the therapeutic in vivo, and identify likely responders and nonresponders at the onset of therapy. Although theranostics are in the early stages for targeting atherosclerosis, several recent advances merit discussion.
Integrated Imaging and Therapy of Plaque Angiogenesis

Reduction of neovascularization in atheromata is an intriguing strategy to limit plaque growth and intraplaque hemorrhage. Building on an integrin $\alpha_v\beta_3$-targeted perfluorocarbon platform for molecular MRI of plaque angiogenesis, Winter et al developed an angiogenic atherosclerosis theranostic strategy by incorporating fumagillin, a naturally secreted antibiotic from *Aspergillus fumigatus*, into the surfactant layer of an integrin $\alpha_v\beta_3$-targeted nanoparticle. Hyperlipidemic rabbits with aortic atheromata were then injected with a single dose of control MNP or fumagillin-targeted MNP ($\approx 26 \mu g/kg$ body weight). Immediate postinjection MRI of aortic plaques demonstrated similar evidence of angiogenesis for both groups. After 1 week, rabbits in both groups were reinjected with the control MNP (angiogenic targeted MNP without fumagillin). Rabbits that originally received the fumagillin MNP showed a marked reduction of plaque angiogenesis in vivo ($>60\%$ reduction in averaged aortic MRI signal enhancement), whereas control rabbits showed unchanged levels of plaque angiogenesis. Furthermore, the initial signal enhancement on the original MRI scan predicted the net fumagillin MNP-mediated reduction in plaque angiogenesis ($R^2=0.62$). In vivo MRI findings were corroborated by PECAM immunohistochemical analyses demonstrating a $>60\%$ reduction in the number of neovessels per plaque section in the fumagillin MNP group compared to the control MNP group. In a very recent study, Winter et al further used fumagillin MNP to demonstrate an antiangiogenic synergism of fumagillin and statin therapy by in vivo MRI. Even more recently, a dual angiogenesis-targeted fumagillin theranostic nanoparticle (integrin $\alpha_v\beta_3$ and integrin $\alpha_\text{v}\beta_3$) appears more effective than a single angiogenesis-targeted fumagillin NP (integrin $\alpha_v\beta_3$).}

Emerging Theranostic Agents for Atherosclerosis

Additional agents (Table) such as photosensitizers with intrinsic NIR fluorescence capabilities or those with discrete NIR fluorochromes could offer the ability to characterize inflamed atheromata before photodynamic therapy. Another platform with substantial promise for atherosclerosis include NIR fluorescent magnetic nanoparticles carrying therapeutic siRNA molecules.

Conclusions

Molecular imaging of atherosclerosis offers new opportunities to study the evolution of biology in vivo, as well as new clinically translatable strategies to identify high-risk coronary and carotid plaques. Rapid growth of optical, specifically near-infrared fluorescence, molecular imaging strategies show promise for imaging plaque inflammation, osteogenic activity, and angiogenesis with increasingly clinical-type imaging systems such as intravascular catheters or noninvasive tomography. Additional growth in multimodality probe technology, with imaging agents detectable by 2 or more imaging systems, as well as theranostic agents enabling spatiotemporal monitoring of targeted therapies, are also poised to strengthen emerging in vivo biological approaches to understanding and treating atherosclerotic vascular disease.

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Disclosures

Dr. Jaffer and Dr. Weissleder are shareholders in VisEn Medical.

References


51. Jaffer et al Optical and Multimodality Molecular Imaging


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Supplement Material

Protease-activatable NIRF imaging agents. To image protease activity and not merely protease presence, Weissleder et al. developed a protease-activatable substrate for NIRF imaging (Figure 1). After circulation and delivery of the imaging agent to a protease-rich environment, lysosomal cysteine proteases such as cathepsin B, L, K, and S cleave the lysine-lysine cleavage site, liberating fluorochromes from the backbone and consequently generating strong fluorescence. Low background signal from the uncleaved circulating imaging agent and low tissue autofluorescence in the NIR range enables sensitive detection of the NIRF signal in vivo. In addition to this first generation multi-cathepsin protease-activatable agent, second-generation agents have been engineered for various enzyme selectivities including matrix metalloproteinase 2 and 9, thrombin, cathepsin K, cathepsin D, urokinase-type plasminogen activator, and cathepsin S.

NIRF Intravital fluorescence microscopy (IVFM) of plaque cathepsin K activity. Cathepsin K is a potent elastase that colocalizes in human atheromata and may contribute to plaque destabilization. To investigate plaque cathepsin K (catK) activity, Jaffer et al. developed a catK-sensitive imaging agent by attaching multiple copies of a catK peptide substrate (GHPG•GPQ) to a first generation protease-activatable NIRF agent. ApoE-/- mice received an intravenous injection of the catK NIRF imaging agent (dose 5 nmol per mouse, 0.25 μmol/kg). After 24 hours, carotid plaques were surgically exposed and underwent intravital fluorescence microscopy (IVFM) using a prototypical laser scanning microscope (IV110, Olympus, Japan). IVFM of carotid plaques yielded exquisitely high-resolution images of catK protease activity (5x)
magnification objective, 13x13x10 µm resolution). Histological analyses of plaque sections shed new pathophysiological insight into in vivo catK activity by demonstrating that macrophages predominantly furnished the enzymatically active form of cathepsin K. While surgically-based molecular imaging of atherosclerosis is likely to remain restricted, hand-held confocal fluorescence microendoscopes are clinically available\textsuperscript{15} and could be used to interrogate surgically-exposed carotid, coronary, or aortic plaques. In addition to laser scanning confocal microscopy, additional intravital fluorescence microscopy approaches on the horizon include multiphoton microscopy\textsuperscript{16} and spinning disk confocal microscopy.\textsuperscript{17}

**Intravital fluorescence microscopy of plaque osteogenesis.** The spatiotemporal distribution of plaque osteogenic activity was visualized in atheromata using an NIR fluorescent bisphosphonate compound targeted to osteoblasts.\textsuperscript{18,19} Using a serial, multichannel NIRF IVFM approach in apoE\textsuperscript{-/-} mice, Aikawa et al. demonstrated that plaque macrophages (assessed with the multimodality nanoparticle CLIO-VT680) preceded the development of osteogenic activity (assessed with a spectrally distinct NIRF bisphosphonate agent Osteosense750), thereby establishing an in vivo link between plaque inflammation and plaque calcification (Supplemental Figure 2). Temporal studies revealed that plaque inflammation (macrophage content) peaked in mice between 30 and 72 weeks of age, while plaque osteogenesis continued to increase with age. Intriguingly, IVFM technology visualized early evidence of arterial calcification (osteogenic activity) that was not detected by computed tomography or by von Kossa histological staining. In addition, statin therapy limited the development of plaque calcification and plaque macrophage content, with a 2-fold reduction in both components in age- and sex- matched mice. In vitro, macrophage-conditioned media
promoted an osteogenic phenotype (alkaline phosphatase mRNA expression) in vascular smooth muscle cells. The overall results suggest that plaque macrophages precede and promote lesion calcification, thus strengthening the postulate that atheroma calcification involves inflammation.\textsuperscript{20}

**NIRF Imaging of plaque angiogenesis.** Neoangiogenic plaque vasculature may (1) facilitate plaque expansion via delivery of cellular and soluble moieties,\textsuperscript{21} (2) promote acute plaque expansion via intraplaque hemorrhage,\textsuperscript{22,23} and (3) identify high-risk plaques at risk of progression and rupture.\textsuperscript{24} Matter et al. developed a NIRF sensor targeted to the extra-domain B (ED-B) of fibronectin, a 91 amino acid molecule inserted into fibronectin under tissue conditions promoting neoangiogenesis.\textsuperscript{25} A miniantibody (L19) derivatized with the NIR fluorochrome Cy7 visualized plaque angiogenesis ex vivo in 4 month old apoE\textsuperscript{-/-} mice. Excellent plaque target-to-background ratios (18:1) were achieved in contrast to control apoE\textsuperscript{-/-} mice receiving saline. In a drug efficacy study, ex vivo fluorescence imaging of a NIRF derivative of L19 demonstrated that the cholesterol-absorption inhibitor ezetimibe abolished the plaque NIRF signal apoE\textsuperscript{-/-} mice.\textsuperscript{26} Interestingly, recent work suggests that ED-B-targeting via a single chain antibody complex might indirectly assess plaque inflammation.\textsuperscript{27}

**Multimodal NIRF and MRI of vascular cell adhesion molecule (VCAM)-1 expression.** VCAM-1 (CD106) is a sialoglycoprotein member of the immunoglobulin superfamily whose expression increases during hypercholesterolemia in experimental atherosclerosis.\textsuperscript{28} VCAM-1 modulates leukocyte adhesion to inflamed endothelial cells and modulates atherogenesis.\textsuperscript{29} VCAM-1 expression both indicates and promotes plaque inflammation and thus could serve as a target for high-risk plaque imaging as well as a therapeutic target.
Using in vitro phage display technology, Kelly et al. identified the cyclic peptide VHSPNKK homologous to integrin very late antigen (VLA) that avidly bound VCAM-1, and developed a multimodal nanoparticle for VCAM-1 sensing.\textsuperscript{30} To develop a more sensitive VCAM-1 imaging sensor, Nahrendorf et al. identified the linear peptide VHPKQHR via in vivo phase display with three rounds of iterative positive selection from aortic vasculature of apoE\textsuperscript{-/-} mice.\textsuperscript{31} This peptide shares sequence similarity with integrin VLA-4, a known ligand for VCAM-1 capable of entering cells. Multiple copies of the peptide were next attached to fluorescent iron oxide MNPs to form VINP-28 (VINP=VCAM-1 internalizing NP), a multimodal MRI and NIRF imaging particle for VCAM-1 visualization in vivo. Competitive inhibition experiments with soluble VCAM-1 demonstrated specificity of VINP-28 for VCAM-1 expressing human vein endothelial cells. Ex vivo dual agent multiwavelength fluorescence microscopy revealed a distinct subintimal atheroma targeting profile by VINP-28 (cyanine 5.5, ex 675nm/694nm) compared to a co-injected, underivatized conventional NIRF MNP (CLIO-AF750, ex 752mm/779nm) targeted to macrophages.

In vivo VCAM-1 expression in atheromata of apoE\textsuperscript{-/-} mice was visualized using 9.4T, ECG-gated MRI following intravenous injection of VINP-28 (30 mg Fe/kg, 48 hour circulation time). T2*-weighted MRI plaque signal enhancement (signal loss from iron oxide-induced higher R2 relaxation rates) occurred at predicted sites in the aortic root and lesser curvature of the aortic arch (contrast-to-noise (CNR) ratio 77% for in vivo MRI, and target-to-background (TBR) ratio 9.2 on ex vivo NIRF imaging). On plaque sections, NIRF signal was correlated with immunopositive VCAM-1 in the intima. VINP-28 also reported in vivo effects of statin pharmacotherapy on VCAM-1 expression. ApoE\textsuperscript{-/-} mice randomized to high cholesterol diet
(HCD) or HCD+ atorvastatin 0.01% wt/wt treatment for 8 weeks underwent VINP-28 enhanced molecular MRI. Statin therapy reduced VCAM-1 expression on in vivo MRI and ex vivo NIRF imaging (40 and 47% decreases in CNR and TBR, respectively). The reductions in VCAM-1 expression detected by MRI exceeded the cholesterol lowering effects of statin therapy (16% reduction), consistent with anti-inflammatory effects of statins beyond lipid lowering. VINP-28 also localized in plaques of juvenile apoE⁻/⁻ mice and in resected human carotid endarterectomy specimens. Given these promising findings, a clinical PET imaging trial utilizing the above VCAM-1 peptide is planned to identify inflamed atherosclerotic plaques (P. Libby and R. Weissleder, personal communication).

REFERENCES


### SUPPLEMENTAL TABLE I

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*Note that the combination of reporter probes (Table 2) and imaging system often imparts molecular specificity. A, Absorption; E, emission; S, scattering; Fl, fluorescence. In cleared specimen.

**Table 1.** In vivo optical imaging systems. Reproduced by permission from reference 33.
**SUPPLEMENTAL FIGURE LEGENDS**

**Figure 1.** Advantages of fluorescence imaging in the near-infrared (NIR) window. In the NIR window (defined typically between 650nm-900nm), photon absorption from oxy- and deoxy-hemoglobin, lipids, and water have local minima, enabling orders-of-magnitude better penetration of NIR light compared to visible light (bottom panel, comparative photon counts in nude mice with a posteriorly placed excitation source either at 532nm or 670 nm). The insert (upper right) shows the autofluorescence spectrum of tissue with excitation range from 337-610nm (vertical axis) and emission range 360 to 750 nm (horizontal axis). At longer wavelengths in the near-infrared (>650nm), tissue autofluorescence is minimized, allowing more efficient detection of NIR fluorochromes in vivo. Reproduced by permission from reference 33.

**Figure 2.** Serial, multichannel intravital fluorescence microscopy of plaque osteogenic activity and plaque inflammation. ApoE<sup>−/−</sup> mice were injected prior to imaging with two spectrally distinct NIRF imaging agents: CLIO-VT680 (for macrophages, 15 mg Fe/kg) and Osoteosense750 (for osteogenic activity, 2 nmol/mouse). After 24 hours, IVFM of carotid plaques was performed at age 20 weeks (A) and then at follow-up at 30 weeks (B), and revealed that the presence of macrophages predicted future osteogenic activity in atheromata. (C) Significant reductions in both plaque inflammation and plaque calcification were noted following statin treatment, as detected by multichannel IVFM. (D) Quantification of IVFM data demonstrates the temporal relationship of osteogenesis and macrophages in apoE<sup>−/−</sup> atheromata, and the effects induced by statin therapy. Arrows show the internal elastic lamina, L=lumen, scale bar=500 µm. Modified with permission from reference 19.
SUPPLEMENTAL FIGURE 2

A. Macrophages  Osteogenesis  Merged  H&E

B. 20 week old apoE-/- mouse

C. 30 week old apoE-/- mouse

30 week old apoE-/- mouse treated with statin

D. Plaque area (pixels)

<table>
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- Macrophages
- Osteogenesis

P<0.001  P<0.001  P<0.01  P<0.01  P<0.01  P<0.05

NS