Intra-arterial catheter for simultaneous microstructural and molecular imaging in vivo

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Supplementary Figure 1 Dual-modality imaging probe. (a) Schematic diagram of the structure of the double-clad fiber (DCF). Due to a low-index polymer coating, the cladding of the DCF transmits light with high efficiency. (b) Picture of the imaging probe contained within a rotating cable. Scale bar, 1 mm. (c) Picture of the side-viewing ball-lens fabricated at the end of the DCF. Scale bar, 100 μm.
Supplementary Figure 2 Sensitivity analysis and distance calibration for NIRF. (a) Measured fluorescence emission signal obtained while repeatedly dipping the catheter into the solution. The concentration of the Cy7 solution was 100 nM. (b) SNR measurements using the dual-modality imaging catheter for Cy7 as a function of fluorochrome concentration. (c) A plot of the measured SNR and correlation with Cy7 concentration. (d) A plot of normalized NIRF intensity as a function of distance from the catheter. (e) A function, computed from the data in d, that was used to correct the NIRF signal intensity as a function of distance from the catheter to the target. (f) 2D NIRF image of a tube containing Cy7. The distance between the catheter and the tube varied along the pullback dimension, where the catheter was closer to the tube at the start of the pullback (bottom of f) and the catheter was further from the tube at the end of the pullback (top of f). As a result, the uncorrected NIRF intensity diminished during the pullback. (g) 2D NIRF image of the tube after the NIRF intensity was corrected using the data plotted in e and measurement of the distance between the catheter and the tube from the simultaneously acquired OFDI image.
Supplementary Figure 3 Image processing, fusion and display. 2,048 OFDI A-lines were acquired and processed to form a circular cross-sectional image while the probe rotated one revolution. (a) Back-reflected signal from the arterial wall microstructure is displayed in gray scale. (b) A NIRF emission value is acquired for every OFDI A-line acquisition, and then converted into a value that is proportional to dye concentration based on the correction function derived in Supplementary Figure 4. The NIRF signal (yellow scale), rendered on the artery’s surface, was mapped to the luminal contour determined from the OFDI data. (c) The fused cross-sectional image demonstrates the spatial localization of the fluorescence emission, superimposed on the structural OFDI data. (d) Three dimensional cutaway view of the rendered OFDI data with fluorescence overlay (yellow). Scale bar, 1 mm.
Supplementary Figure 4 Assessment of co-registration of OFDI and NIRF imaging signals. (a) Photograph of the phantom. The phantom consists of a thin glass tube filled with an NIR fluorochrome (ICG), and 2-µm OFDI-detectable microspheres. (b) Cross-sectional image of OFDI and NIRF at location denoted in a. OFDI shows strong scattering signal from the microspheres inside the glass tube (yellow asterisk). The simultaneously acquired NIRF signal from the ICG is displayed outside of the OFDI cross-sectional image. Yellow dotted lines indicate the mixed solution of microspheres and NIRF fluorochrome. (c) Intensity graph of the OFDI scattering and the NIRF emission signal along the rotational direction. The graph shows that OFDI and NIRF are precisely co-registered. (d) Reproducibility results of OFDI and NIRF co-registration for rotational and longitudinal directions, as assessed by analyzing five image data sets.

| OFDI/NIRF set | Rotational direction Offset (Angle, degree) | Offset (Length, µm)* | Longitudinal direction Offset (length, µm) |
|---------------|---------------------------------------------|----------------------|------------------------------------------|
| 1             | -0.06                                       | -1.57                | 20.59                                    |
| 2             | 0.07                                        | 1.90                 | -0.22                                    |
| 3             | -0.53                                       | -13.82               | 17.08                                    |
| 4             | -0.11                                       | -3.01                | -24.10                                   |
| 5             | 0.50                                        | 13.20                | 5.41                                     |

*Assuming the vessel diameter is 3mm.
Supplementary Figure 5 OFDI, NIRF and fusion images of a Cy7 labeled fibrin-binding peptide incorporated into a stent microthrombus implanted in a rabbit iliac artery. (a) Average z-projection of the OFDI data presented in an unfolded manner. 2,048 A-lines correspond to 360° angle of the rotation of the imaging probe. The longitudinal interval was 100 μm. (b) NIRF 2D map shown in the same coordinate system. (c) Fused image of OFDI and NIRF. Scale bar, 1 mm.
Supplementary Figure 6 Colocalization analysis. (a) Stack of segmented thrombus delineated from OFDI cross-sectional images. (b) Stack of segmented thrombus displayed in polar-coordinates. (c) 2D map of thrombus. Intensity in the figure represents the density of the thrombus. (d) NIRF 2D map of fluorescence intensity. Using two 2D images (c,d), the colocalization coefficients for each channel can be calculated.
Supplementary Figure 7 3D cutaway view of the native unstented aorta of a rabbit acquired by the dual-modality intra-arterial imaging catheter in vivo. The artery wall was color-coded in red and the fluorescence signal was also rendered on the luminal surface in yellow. No fluorescence signal was observed in this data set.
Supplementary Figure 8 OFDI-NIRF imaging of atherosclerotic rabbits ($n = 4$) injected with Prosense in vivo and corresponding histopathology. (a) Dual-modality OFDI-NIRF image of an injured aorta. In the OFDI image, a large portion of the artery wall shows evidence of atherosclerotic plaque (2 to 10 o’clock). A strong NIRF signal is observed in the atherosclerotic region. (b) Corresponding H&E histology demonstrates a large plaque with the same spatial distribution. (c) Corresponding immunohistochemistry demonstrates cathepsin B staining within the lesion. (d) Dual-modality OFDI-NIRF image of an uninjured aorta. Small plaques are evident in this OFDI cross-sectional image. The NIRF signal is strong in the area of the plaque. (e) Corresponding H&E staining demonstrates multiple focal lesions. (f) Corresponding immunohistochemistry shows that the lesions were immunoreactive to cathepsin B. (g) Scatter plot of H&E histology and OFDI measurements of plaque percent circumference shows a very strong correlation ($r = 0.97$, $P < 0.0001$, $y = 1.04x-2.43$). (h) The average NIRF intensity was highly correlated with cathepsin B positive percent area ($r = 0.82$, $P = 0.0004$, $y = 0.57x+3.30$). (i) Quantitative analysis for reproducibility of OFDI showed an excellent correlation between the percent plaque circumferences measured by OFDI for test and retest pullbacks ($r = 0.99$, $P < 0.0001$, $y = 0.99x+2.35$). (j) Average NIRF intensities of test and retest images demonstrated a very high correlation ($r = 0.93$, $P < 0.0001$, $y = 0.88x - 1.42$). Scale Bars, 1mm.
**Supplementary Video 1** Simultaneously acquired OFDI and NIRF datasets. Simultaneous dual-modality imaging of a cadaveric coronary artery with an implanted NIR fluorescent-fibrin labeled stent, obtained ex vivo. The movie shows the OFDI cross-sectional images and the corresponding NIRF signals that are acquired during helical scan of the dual-modality catheter.
Supplementary Video 2 Flythrough movie of color-coded 3D volume rendering of simultaneously acquired OFDI and NIRF datasets. Three-dimensional rendering of an OFDI-NIRF dataset obtained from a rabbit iliac artery with an implanted NIR fluorescent-fibrin labeled stent in vivo. The following components of each of the OFDI images were segmented and rendered in color: artery wall (red); stent (white); thrombus (purple). The NIRF signal (flashing yellow) was overlaid on the luminal surface of the artery wall prior to volume rendering.
**Supplementary Methods**

**OFDI-NIRF system**

The OFDI system is described in detail in prior manuscripts. The light source of the OFDI system was a high-speed wavelength swept laser that used a semiconductor optical amplifier (SOA, Covega) and an optical tunable filter based on a polygon-mirror and a grating. The laser had a center wavelength of 1320 nm, provided a sweep range of 111 nm (1264–1376 nm), a repetition rate of 52 kHz and an average power of 60 mW. A fiber optic coupler (1:9) divided the laser output to the reference arm and the sample arm. An acousto-optic frequency shifter was used to remove depth degeneracy and double the ranging depth. Back-reflected light from the tissue microstructure generated an interference signal when combined with the reference beam. A series of interference OFDI signals, acquired by the dual-balanced detector, was processed by DFT in k-space to form depth-profiles (A-lines). The axial resolution of the OFDI was 7 µm in tissue (refractive index n = 1.4) and the sensitivity was 110 dB. Ranging depth was 4.6 mm in saline.

NIRF excitation light was generated by a 750-nm laser (RPMC lasers) coupled into a multi-mode fiber with a core diameter of 100 µm. A laser-cleanup filter (Chroma, 750/10 nm) was used after the fiber output to reject any unwanted wavelengths. The excitation fiber’s maximum output power was 116 mW. Fluorescence excitation and emission were separated by a dichroic mirror (Chroma, LP at 770 nm). Fluorescence emission from the sample was transmitted through a band-pass emission filter (Chroma, 810/90 nm) and detected by a photo multiplier tube (PMT) (Hamamatsu). The A-line acquisition of the OFDI and the analog signal acquisition of the NIRF were synchronized to timing pulses generated by the swept source at a rate of 52 kHz, so that OFDI and NIRF signals could be acquired and saved simultaneously.

**Optical rotary junction and dual-modality catheter**

A custom-built dual-modality optical rotary junction, based on a dichroic mirror and collimators, served as the interface between the stationary OFDI-NIRF fibers and the catheter’s rotating and translating imaging probe. The catheter’s DCF and inner cable were contained within a through-hole inside the shaft of a velocity-controlled DC motor. The OFDI sample arm beam was transmitted through a long-pass dichroic mirror (Chroma, LP at 1,000 nm) and then coupled into the DCF’s core. NIRF excitation light was reflected by the dichroic mirror then coupled into the multi-mode inner cladding of the catheter’s DCF. OFDI and NIRF light returned from the sample was collected by the ball-lens and traveled through the DCF back to the system. The entire optical rotary junction was mounted on top of a translation stage to allow pullback of the catheter’s inner components. The rotational velocity of the optical imaging probe was 25.4 revolutions per second (rps) and the translational speed for the pull-back motion was 2.5 mm s\(^{-1}\).

The intra-arterial imaging catheter comprised an imaging probe, contained within a rotating cable and an 800 µm-diameter (2.4 F) transparent outer sheath that incorporated a guide-wire provision.
optical core was made of a double-clad fiber (DCF), which had two concentric light-guiding channels (Supplementary Fig. 1a). DCF has recently been employed in optical imaging fields for increasing the light efficiency or simultaneous acquisition of multiple signals\textsuperscript{4,5}. Due to the unique design of the DCF, it enables dual-modality optical imaging efficiently. The DCF used in this study had a core diameter of 7.7 μm and an inner cladding diameter of 124.9 μm. The core’s mode field diameter of 9.2 μm and numerical aperture (NA) of 0.12 were designed to closely match those of SMF-28 so that it could be optimally coupled to the rest of the OFDI system that was fabricated from SMF-28 fiber. The inner cladding was coated with a low index polymer, so that it transmitted multimode fluorescence excitation and emission light efficiently with a high NA of 0.48. A side-viewing ball-lens was fabricated at the distal end of the DCF. First, a short length of a coreless fiber was spliced at the distal end. Then a ball-lens was formed by melting the tip of the coreless fiber using a fusion splicer. After polishing one-side with 41 degree angle to induce the total internal reflection, the optical core was assembled with the rotating cable and the fiber connector. Supplementary Fig. 1b,c show the pictures of the imaging probe contained within a rotating cable and the fabricated side-viewing ball-lens. Diverging beams out of the distal end of the DCF were reflected by the polished surface of the ball-lens then focused onto the tissue by the curvature of the ball-lens. The transverse resolution, determined by the measured full-width at half-maximum diameter of the focused spot, was 27 μm and 109 μm for OFDI and NIRF, respectively. The catheter obtains co-registered NIRF and OFDI data of an arterial lumen as the inner cable is rotated and translated in a helical pattern within the external transparent sheath (Supplementary video 1).

The single pass insertion loss for OFDI through the rotary junction was less than 1.2 dB and the rotational uniformity, defined by the ratio of the minimum to the maximum intensity while rotating 360 degrees, was 0.98. The excitation insertion efficiency of NIRF through the rotary junction and the optical core was 21%, and the rotational uniformity was 0.95. The uni-directional light loss due to the dichroic mirror inside the rotary junction was less than 5% for both imaging modalities. The resulting power on the sample was 39.4 mW and 4.9 mW for the OFDI and NIRF, respectively.

**Sensitivity analysis of the NIRF signal**

We measured the sensitivity of the dual-modality catheter for detecting the NIRF signal by measuring the fluorescence emission of fluorochrome phantoms. The lowest concentration of the fluorophore determined the sensitivity limit of the catheter. Cy7 solutions (ex/em 747/775nm) at concentrations ranging from 1–100 nM were prepared for the sensitivity analysis. The signals were measured by immersing the catheter in the solution. The signal to noise ratio (SNR) was calculated as $SNR (dB) = 20 \log_{10} \left( \frac{S}{\sigma} \right)$, where $S$ was the maximum intensity of the detected signal after background subtraction and $\sigma$ was the standard deviation of the background signal. For the sensitivity analysis, 32-bit analog voltage from the PMT was recorded with a sampling rate of 48 kHz. An analog electronic filter (Stanford Research Systems) was used with a cut-off frequency of 10 kHz followed by the software filtering with a cut-off frequency of 1 kHz using LabView (National Instruments) to reduce the noise. Supplementary Fig. 2a shows the measured fluorescence signal. Supplementary Fig. 2b,c depicts the SNR as a function of concentration of the fluorochrome, Cy7. The measurement results indicate that the sensitivity of the developed catheter system was less than 1nM of Cy7.
**Distance calibration of NIRF signal**

We measured the NIRF intensity as a function of sample distance from the catheter in order to characterize the relationship between the NIRF intensity and the distance. This information was then used to correct for the variation of the NIRF signal due to distance and provide a corrected NIRF value that is proportional to dye concentration. A capillary glass tube (ID 0.8mm/OD 1.2mm) was filled with 100 nM Cy7, positioned next to the OFDI-NIRF catheter, and then imaged using the OFDI-NIRF catheter. **Supplementary Fig. 2d** shows the normalized NIRF intensity as a function of distance. The measurements were fitted using an exponential function, $f(x) = \exp(ax^2 + bx + c)$, where $x$ is the distance from the catheter to the specimen. Then we generated a calibration function $weight(x) = 1/f(x)$, for distance correction as shown in **Supplementary Fig. 2e**. Briefly, after measuring the distance from the catheter to the luminal surface of the artery wall from the OFDI images, the NIRF signal was multiplied by the calibration function accordingly. The 2D NIRF image of the capillary tube filled with NIRF agent was corrected, enabling the quantification of the dye concentration (**Supplementary Fig. 2f, g**). This procedure is valid for fluorescence signals that arise from superficial tissues, which is the case for our *in vivo* experiments.

**Data processing and display**

After imaging, the OFDI and NIRF data were processed offline using Matlab (Matlab 7.5, The MathWorks, Inc.), ImageJ (ImageJ 1.43j, Wayne Rasband, US National Institutes of Health), and OsiriX (OsiriX 2.75, The OsiriX Foundation). Reconstructed circular cross-sectional OFDI images (**Supplementary Fig. 3a**) were comprised of 2,048 A-lines. A background image was measured by averaging A-lines without any sample and then subtracted from all OFDI images to suppress noise. Stationary images of the ball-lens and the transparent sheath were also removed to enable clear visualization of luminal structures. The following components of each of the images were segmented and rendered in color for further visualization: artery wall (red); stent (white); thrombus (purple). Also, the distance from the catheter to the artery surface was measured for each A-line of the OFDI images. As described above, this distance was used to calibrate the intensity of the NIRF signal. In synchrony with the 2,048 OFDI A-lines acquired for each cross-sectional image, 2,048 NIRF intensity levels were detected and digitized at 16-bits. The NIRF signals were multiplied by a factor that corrected for the signal variation as a function of distance between the artery wall and the catheter. Using the titration experiment result and the distance calibration data, the NIRF signal was converted into a parameter that is an estimate of the concentration of the dye. Then, we rendered the NIRF data onto the luminal surface of artery wall defined in the OFDI cross-sectional image, since the NIRF signal is surface-weighted due to the penetration limit. First we segmented the OFDI image of the artery wall and generated a binary mask of the luminal surface with a thickness of approximately 100 μm for clear visualization of the overlay. The NIRF signal was then mapped into a two-dimensional image in Cartesian coordinates. By applying the luminal surface mask on the 2D NIRF map using an “AND” operation, we generated NIRF 2D image (yellow, red, or red-hot), located at the luminal surface of the artery wall (**Supplementary Fig. 3b**). OFDI and NIRF images were then fused (**Supplementary Fig. 3c**). This process was repeated for the entire 3D dataset, which was subsequently volume rendered (**Supplementary Fig. 3d**). The processing routine for combining OFDI and
NIRF data sets was semi-automatic, taking approximately 30 minutes to complete. 3D visualization with segmentations took several hours to create, as many of the segmentation steps were semi-automatic or manual.

Reliability of OFDI-NIRF co-registration

To evaluate the reliability of co-registration of simultaneously acquired OFDI and NIRF data, we prepared a phantom and performed multiple pullbacks for calculating the mean variation of co-registration. Supplementary Figure 4a shows a phantom image. The phantom comprised a capillary glass tube (ID 0.8mm/OD 1.2mm) filled with a mixed solution of NIR fluorochrome (1 μM ICG; ex/em 805/835 nm, MP Biomedicals) and scattering microspheres (1% w/w Latex microspheres, diameter 2 μm, Duke Scientific Corp.). Both ends of the glass capillary were enclosed by the epoxy and it was glued to a polycarbonate tube (ID 1.4mm/OD 1.8mm), which accommodated OFDI-NIRF catheter. The entire phantom was submerged into the saline solution. To assess the reliability of the co-registration of OFDI and NIRF, we measured the phantom 5 times and analyzed the data. The rotational velocity of the optical imaging probe was 25.4 rps and the translational speed for the pullback motion was 2.5 mm s\(^{-1}\) with a frame interval of 100 μm. The NIRF signal was displayed outside of the corresponding OFDI image with excellent co-registration (Supplementary Fig. 4b). OFDI scattering and NIRF emission intensity graph shows that the two signals are highly co-registered (Supplementary Fig. 4c). Post-processing for co-registration was not required since the OFDI and NIRF imaging beams were intrinsically aligned and imaged simultaneously. The measured variation of spatial offset between the OFDI scattering and the NIRF emission was 0.37 degree in the fast rotating direction, which corresponds to 10 μm assuming the vessel diameter was 3 mm. The variation in the longitudinal direction (pull-back direction) was measured to be 18 μm (Supplementary Fig. 4d). The overall registration variance of combined OFDI-NIRF catheter was smaller than the spot diameter of OFDI.

Coronary stent coated with NIR fluorochrome-labeled fibrin

To create the stent-thrombus implant model, we prepared a balloon-expandable coronary stent (Abbott Laboratories, MULTI-LINK VISION Coronary stent) coated with fluorescently-labeled fibrin. Fibrin labeling of human clots was performed using specialized molecular imaging agents, specifically fibrin-targeted nanoparticles bearing the NIR fluorochrome (CLIO-Cy7-GPRPP (oligopeptide), ex/em 747/775 nm). Human clots were created from fresh frozen plasma (FFP)\(^3\). First, a centrifuge tube was filled with 180 μL of FFP. An undeployed metal stent with a length of 12 mm and an expanded diameter of 2.75 mm was soaked into the FFP. Then 10 μL of 0.4 M CaCl\(_2\), 10 μL of thrombin (0.1 U μL\(^{-1}\) PBS), and 12 μL of CLIO-Cy7-GPRPP were added all at once to maximize the binding. Then the mixture was incubated at 37 °C for approximately 24 hours. Human FFP was obtained via the protocol approved by the Partners Internal Review Board for the MGH blood Bank.

Imaging of a fibrin-rich thrombi on a coronary stent in vitro

To demonstrate and verify the ability of dual-modality intra-arterial imaging, we performed dual-modality imaging of a NIRF fibrin-coated stent implanted into an excised cadaveric coronary artery. Prior to implantation, the coronary stent was rendered fluorescent in the NIR by incubating the stent with
human fresh frozen plasma and a fibrin-targeted peptide derivatized with the NIRF agent, as described above. A 30 mm-long segment of a cadaver coronary artery was excised for the in vitro imaging study. Thereafter, a guide wire (0.014") was advanced to the distal end of the artery. Then, we delivered the coronary stent to the middle of the artery and deployed it at 10 atm. After implanting the stent, we inserted the dual-modality imaging catheter and advanced it over the guide wire to the distal coronary artery. The artery was filled with saline before the image acquisition. The imaging system simultaneously acquired OFDI and NIRF data at an A-line rate of 62 kHz, while the rotary junction rotated the imaging probe of the catheter at 30 rps and pulled it back with a velocity of 3 mm s⁻¹. The entire dataset of 30 mm-long segment was successfully acquired with a longitudinal spacing of 100 µm in 10 seconds.

Analysis of OFDI-NIRF colocalization

The results of intravascular imaging of stent-microthrombosis in vivo showed a good qualitative correspondence between the OFDI and NIRF. To quantify the degree of colocalization, NIRF fluorescence signal and thrombus segmentation of OFDI were analyzed in 2D coordinates. For the colocalization analysis, we prepared the thrombus 2D map in the same coordinate system (Supplementary fig. 6). First, a coordinate transformation was performed on stack of thrombus segmentations defined in OFDI cross-sectional images. Then, applying re-sectioning following z-projection with summation, a 2D thrombus map was acquired. Finally, colocalization coefficients were calculated by using MATLAB (Mathworks)⁹. The colocalization coefficients are defined by

\[ M_1 = \frac{\sum R_{i,\text{coloc}}}{\sum R_i} \]  

where \( R_{i,\text{coloc}} = R_i \) if \( G_i > 0 \) and \( R_{i,\text{coloc}} = 0 \) if \( G_i = 0 \) and

\[ M_2 = \frac{\sum G_{i,\text{coloc}}}{\sum G_i} \]  

where \( G_{i,\text{coloc}} = G_i \) if \( R_i > 0 \) and \( G_{i,\text{coloc}} = 0 \) if \( R_i = 0 \). The colocalization coefficients are proportional to the amount of pixel values of the co-localizing objects in each component of the image, relative to the total pixel values in that region. The range of the value of the colocalization coefficient is from 0 to 1 and larger number means stronger colocalization. Here, \( R_i \) is the OFDI-delineated thrombus (Supplementary fig. 6c) and \( G_i \) is the fluorescently-labeled fibrin (Supplementary fig. 6d). For OFDI and NIRF, colocalization coefficients of 0.98 and 0.67 were measured, respectively. The lower colocalization coefficient value for the NIRF channel suggests that, during the segmentation of the thrombus in OFDI, small and thin clots were missed. Also, uneven staining of fluorochrome to fibrin or uneven distribution of fibrin in clots could affect the colocalization.

Histopathology of the stented iliac artery

After imaging, the animal was euthanized and the stented segment of right iliac artery was excised and fixed in 10% neutral buffered formalin. Vascular rings were then embedded in polymer for
histopathological analysis. 5-µm sections were cut from the distal portions of the stent every 250 µm to find matches with OFDI-NIRF cross-sections. All sections were stained with hematoxylin-eosin (CVpath Institute). Tissue sections were viewed and digitized using a whole slide scanner (Nanозoomer, Hamamatsu Corporation).

**Histopathology of the atherosclerotic arteries**

After imaging, the animals were euthanized and the aortoiliac arteries were excised. In order to facilitate the registration of the OFDI-NIRF images obtained *in vivo* to histopathology sections, we additionally performed OFDI-NIRF imaging *in vitro* after putting pins, as fiducial markers, at multiple injured and uninjured portions of the arteries. Vascular rings were cut with a length of 3 mm at the pin positions. The vertically oriented tissues were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance) and frozen in −80 °C freezer for histopathological analysis. Serial 5-µm frozen sections and one thick section (20 µm) of the segments were cut. Plaque morphology was demonstrated with hematoxylin-eosin staining. Rabbit macrophages were identified with a mouse monoclonal antibody (RAM-11, Santa Cruz Biotechnology). Immunoreactive cathepsin B was detected with an anti-cathepsin B antibody (R&D system). Tissue sections were viewed and digitized using a whole slide scanner (Nanозoomer, Hamamatsu Corporation). Corresponding OFDI-NIRF sections were found using the distance from fiducial markers and anatomic landmarks such as side branches. For a representative case, microscopic fluorescence signals were captured by an epifluorescence microscope (DM6000 B, Leica) equipped with a 20× objective lens and NIR filter-set for Prosense fluorescence. Autofluorescence was observed in the green fluorescence protein (GFP) channel. Since the field of view of the microscope was smaller than the arteries, multiple images were acquired with overlapping fields of view. Then, the images were stitched together (http://rsb.info.nih.gov/ij/plugins/stitch-bliss.html) to visualize the entire artery cross-section.

**Quantitative comparison between OFDI-NIRF, obtained *in vivo*, and histology**

A total of 13 corresponding OFDI-NIRF/histology image pairs from four rabbits injected with Prosense were randomly selected for analysis. OFDI-NIRF/histology registration was accomplished by matching distances from fiducial markers and side branches. Plaque by OFDI was considered to be present when the intima was thickened. An experienced OFDI reader (H.Y.), blinded to the histology results, measured the percent plaque circumferences from the OFDI cross-sectional images. The percent plaque circumference was defined as a ratio of the length of the circumferential extent of the plaque, measured at the luminal surface, divided by the total luminal circumference of the vessel. Plaque by histology was defined as intimal thickening containing macrophages. Digitized H&E slides were reviewed and analyzed by a pathologist (G.J.T.) who was blinded to the OFDI cross-sectional images and the percent plaque circumferences for the histologic sections were measured and calculated as described above. We then compared morphological measurements of percent plaque circumferences obtained from OFDI cross-sectional images and corresponding H&E histology sections using linear regression. We also evaluated the relationship between NIRF measurements *in vivo* and protease content. Cathepsin B percent positive area was measured from digitized immunostained sections following bimodal thresholding of the hue channel (Matlab 7.5, The MathWorks, Inc.)⁹. The average NIRF signal intensity for each image
was calculated and correlated with cathepsin B immunostaining percent positive area from the corresponding histologic section using linear regression.

**Reproducibility of OFDI-NIRF in vivo**

We compared images from serially-acquired test and retest pullbacks, obtained from the arteries of four rabbits injected with Prosense in vivo. Test-retest images were selected from the same artery locations used in the histopathologic correlation study described above. Test-retest image pairs were registered using distances from side branch landmarks. In order to evaluate the reproducibility of OFDI structural measurements, test and retest percent plaque circumferences were measured from the OFDI data and compared using linear regression. The reproducibility of NIRF was analyzed by correlating the average NIRF signal intensities of the test and retest images. Statistical analysis was performed using PRISM (PRISM 5, GraphPad Software, Inc.).
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