

In vivo imaging of atherosclerosis disease progression response in ApoE deficient mice.

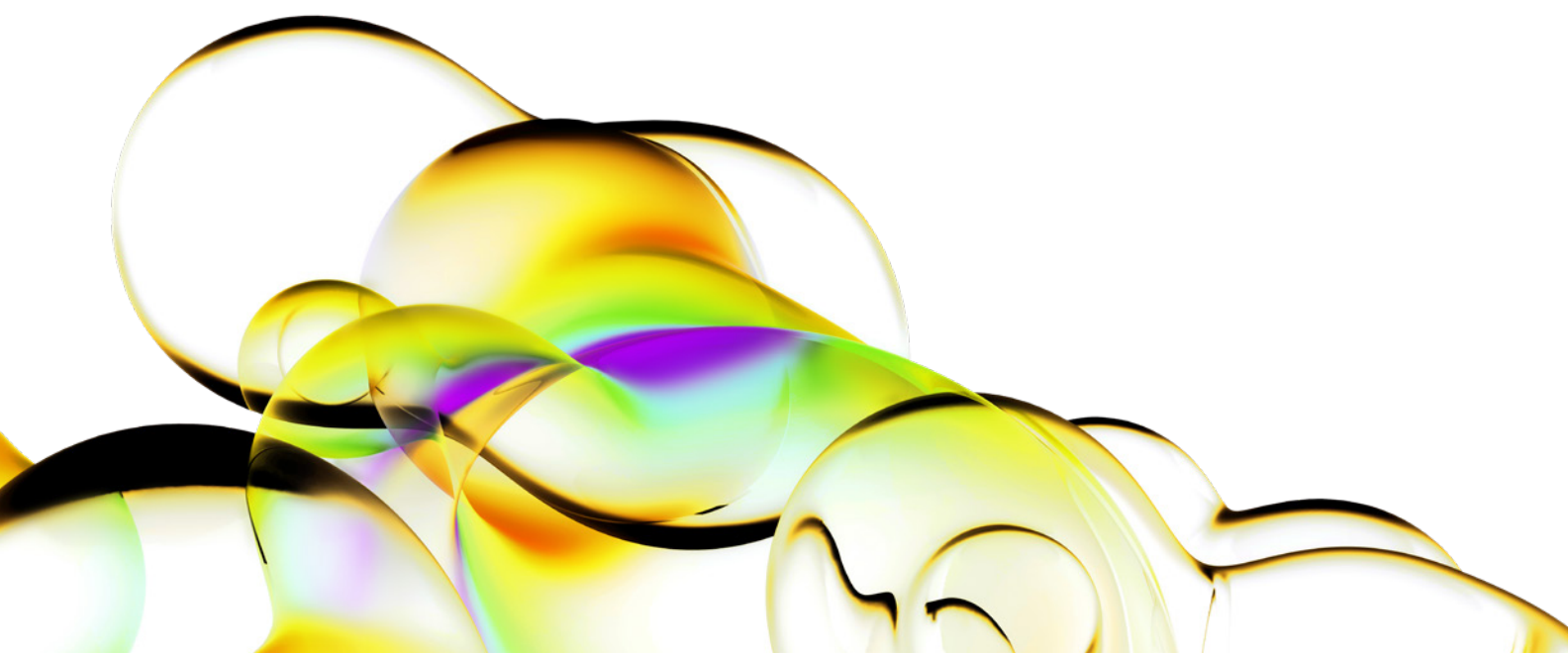
Author

Jeffrey D. Peterson, Ph.D.

Revvity, Inc.

Abstract

Current means of measuring disease in preclinical models of atherosclerosis include *ex vivo* assessment of disease tissues post-mortem and non-invasive imaging primarily of structural and anatomic features of lesions, *in vivo*. A non-invasive, quantitative means of imaging known biologic profiles associated with atherosclerotic disease, *in vivo*, would enable a robust additional understanding and analysis of disease progression and therapeutic response in research and drug development. We report the utility of the near infrared (NIR) protease-sensing, IVISense™ Pan Cathepsin 750 Fluorescent Pre-clinical Imaging Agent, in combination with fluorescence molecular tomography (FMT) for the non-invasive quantitative measurement of atherosclerotic disease biology and related response to therapy in apolipoprotein (apo) E-deficient mice *in vivo*. FMT imaging measured significant increases in aortic region protease activity with a range of values that were comparable to the range seen in the *ex vivo* aortic arches assessed by fluorescence reflectance imaging (FRI). Significant disease was detected by FRI in apoE^{-/-} aortic tissue as early as 20 weeks with animals on high cholesterol diet, and with a steady increase in disease progression at 25 and 30 weeks.



Non-invasive FMT imaging showed a similar progression of disease over time with an excellent correlation to tissue FRI. Atorvastatin treatment showed an anticipated modest decrease in plasma cholesterol and a more dramatic decrease in IVISense Pan Cathepsin agent levels assessed both *in vivo* and *ex vivo*. FMT imaging other biomarker-specific NIR agents was shown to detect and quantitate additional biologic profiles important in atherosclerosis pathogenesis, including agents targeted at cathepsin K (IVISense Cat K 680 FAST agent), cathepsin B (IVISense Cat B 750 FAST agent), and integrin upregulation (IVISense Integrin Receptor 680 agent). These *in vivo* studies demonstrate the ability of fluorescence molecular tomography with Revvity's IVISense NIR agents to non-invasively image and quantitate early-stage inflammation in atherosclerotic lesions, *in vivo*.

Introduction

Cardiovascular disease is the leading source of morbidity, disability, and mortality in Western industrialized countries, and atherosclerosis is unquestionably the main underlying pathology. In the United States alone, approximately 1.5 million heart attacks occur annually, and more than 11 million Americans have chronic coronary artery disease. In addition, thirty percent of persons older than 50 years have some evidence of carotid artery disease.

The first step of atherogenesis in the artery wall is the development of so called "fatty streaks", which are small sub-endothelial deposits of monocyte-derived macrophages. Promoted by low density lipoproteins (LDL) and inadequate removal of fats and cholesterol by functional high density lipoproteins (HDL), this tissue insult then drives an inflammatory cascade that recruits additional macrophages to the artery wall. The macrophages, which ingest oxidized LDL, slowly turn into large "foam cells", containing numerous internal cytoplasmic vesicles with high lipid content. Foam cells eventually die and further propagate the inflammatory process.

ApoE-deficient mice capture many aspects of the pathogenesis of the human disease. When placed on high cholesterol diet, apoE^{-/-} mice develop severe hypercholesterolemia and lesions that progress from fatty streaks to fibrous plaques in lesion-prone areas throughout the aorta. Early evidence of monocyte attachment to

endothelial cells can be seen at 5 weeks of age, and at 10 weeks atherosclerotic lesions containing foam cells develop in the aorta and coronary and pulmonary arteries. Lesion severity continues to increase significantly from 10 to 40 weeks, and despite inbreeding, the mouse-to-mouse variability in lesion area is tremendous. Drugs known to reduce serum lipids, such as the new and potent cholesterol-absorption inhibitors, dramatically reduce atherosclerotic lesion area in apoE^{-/-} mice (Davis, 2001).

We used a well characterized near infrared (NIR) protease-sensing agent, IVISense Pan Cathepsin 750, in combination with fluorescence molecular tomography to establish an alternative, non-invasive quantitative measurement of atherosclerotic disease biology and response to therapy. Through careful assessment of tomographic imaging datasets, we were able to establish appropriate analysis guidelines that allowed the robust quantitation of disease. The same imaging approach yields successful disease quantification with other agents to quantitate lesion fluorescence associated with Cathepsin B (IVISense Cat B 750 FAST agent), Cathepsin K (IVISense Cat K 680 FAST agent), or Integrin (IVISense Integrin Receptor 680 agent) levels.

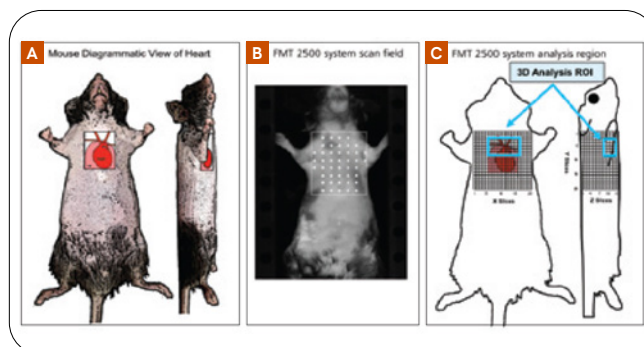


Figure 1: 3D atherosclerosis imaging by FMT.

A. The precise localization of the mouse heart was achieved by careful dissection of animals (following *in vivo* imaging) and is represented in diagrammatic form.

B. The optimal FMT scan field for aortic arch imaging encompasses the entire upper chest region to best gather sufficient tomographic information for depth resolution. Individual dots represent laser positioning sites for the transillumination raster scan.

C. Front and side diagrams of the mouse illustrate the optimal X, Y, and Z coordinates for analysis of the aortic arch and aortic root.

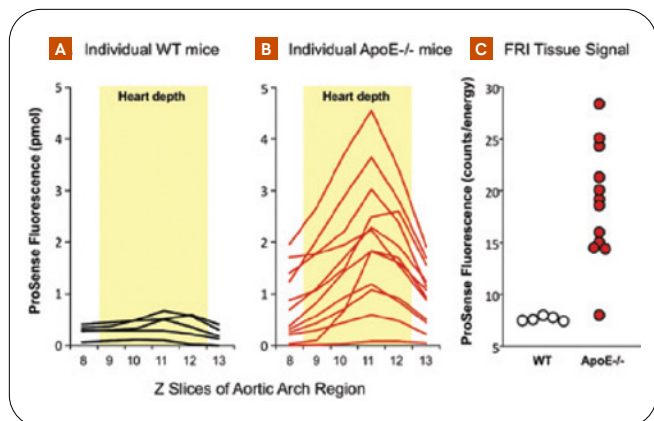


Figure 2: Systematic imaging slice analysis of the FMT through the aortic arch region to optimize 3D quantitation. To best define the optimal depth of ROI placement in atherosclerotic mice, apoE^{-/-} mice were maintained on high-cholesterol diets for 30 weeks and were imaged by the FMT 2500 system 24h after IVISense Pan Cathepsin 750 agent was injected (8 nmol per mouse). Age-matched WT mice on normal chow were used as negative controls. A region encompassing the correct width and height (see Figure 1C) to capture the upper heart and aorta was dissected into tomographic slices on the depth axis for each mouse. The fluorescence signal from individual 1 mM slices of the ROI box (encompassing the front chest region of the mouse, slices 8-13) were separately quantified and graphed as a function of depth for 5 WT **A.** and 12 apoE^{-/-} **B.** mice. Each line represents changes in fluorescence with depth in an individual animal. Slices 9-12 (yellow shading) represented the regions of maximal difference in apoE^{-/-} mice as compared to WT controls. **C.** The heart and aortic tissue was removed from all mice and imaged using the FRI capacity of the FMT 2500 system. Assessment of fluorescence intensity (counts/energy) confirmed the wide distribution of disease severity detected *in vivo* by FMT

Materials and methods

Experimental animals. Specific pathogen-free female apoE-deficient mice (6-8 weeks of age, 18-20 g) were obtained from The Jackson Labs (Bangor, ME) and housed in a controlled environment (72 °F; 12:12-h light-dark cycle) under specific pathogen-free conditions. Mice homozygous for the *ApoE*^{tm1Unc} mutation show a marked increase in total plasma cholesterol levels that are unaffected by age or sex. Fatty streaks in the proximal aorta are found at 3 months of age, and the lesions increase with age. To accelerate the onset of disease, these mice were fed a 0.2% high cholesterol diet (Western type No. 88137, Harlan Laboratories, Madison WI) for assessment at different timepoints relative to the start of feeding (from 15 to 30 weeks on diet).

Age-matched C57BL/6 mice were used as wild-type (WT) controls for all studies and were maintained on standard chow and water. All experiments were performed in accordance with Revvity IACUC guidelines for animal care and use.

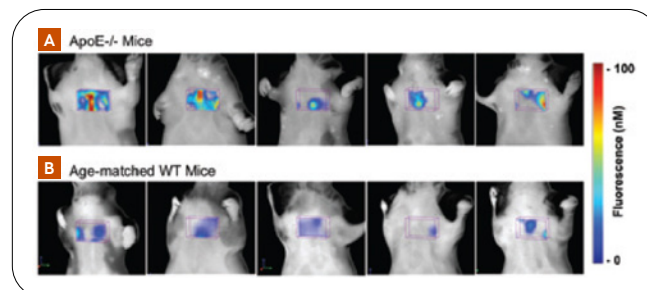


Figure 3: FMT images of IVISense Pan Cathepsin 750 agent in apoE^{-/-} and WT mice.

A. Images from 5 representative apoE^{-/-} mice maintained on high-cholesterol diets for 30 weeks (from study represented in Figure 2) were generated by FMT 24h after IVISense Pan Cathepsin 750 agent was injected (8 nmol per mouse). **B.** Images from control WT mice on normal chow. Fluorescence represented is from the aortic arch ROI only, with other non-heart region sources of fluorescence excluded for clarity. No fluorescence thresholding was applied to the tomographic datasets.

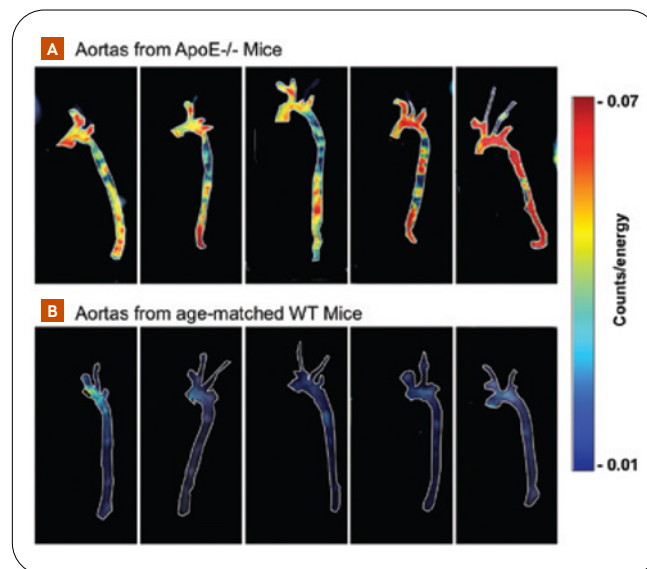


Figure 4: FMT FRI images of aortas from apoE^{-/-} and WT mice injected with IVISense Pan Cathepsin 750 agent. Aortic tissues from WT and apoE^{-/-} mice injected with IVISense Pan Cathepsin 750 agent were removed and imaged by FRI.

A. FRI images from 5 representative apoE^{-/-} maintained on HCD for 30 weeks were generated by FMT 24h after probe injection (8 nmol per mouse). **B.** Images from control WT mice on normal chow. Fluorescence signal in apoE^{-/-} mouse tissues is distributed throughout the aortic arch and descending aorta. White outlines were added to better visualize the tissues.

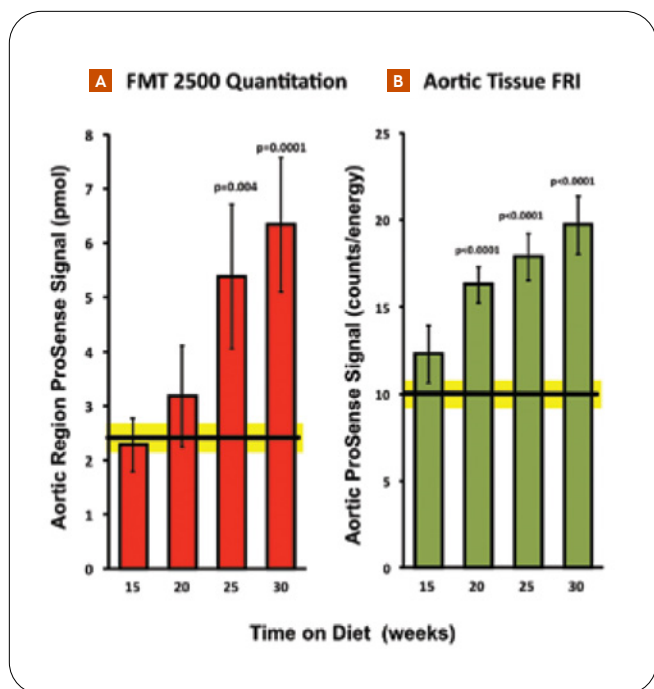


Figure 5: FMT 2500 quantitation of IVISense Pan Cathepsin 750 agent in atherosclerosis progression. The utility of FMT and IVISense Pan Cathepsin 750 agent (8 nmol/mouse) to quantify atherosclerosis progression was assessed by imaging apoE^{-/-} mice fed a high-cholesterol diet for different lengths of time. **A.** FMT quantitation of aortic arch region IVISense Pan Cathepsin signal in apoE^{-/-} mice maintained on high-cholesterol diets for 15 (n = 20), 20 (n = 12), 25 (n = 12), and 30 (n = 12) weeks. The black line represents the corresponding quantitation (with standard error in yellow) from a pooled cohort of 32 WT mice (aged 20-40 weeks) on normal chow. **B.** Corresponding FRI analysis from the same mice, focusing the quantitation on the aortic arch region only. A similar pattern of progression was seen by both non-invasive FMT imaging and terminal tissue fluorescence measurement.

NIR fluorescent imaging agents for the detection of atherosclerosis.

A suite of biomarker-specific Revvity imaging agents (Table 1) were used to detect atherosclerosis severity, progression, and response to therapy. Individual agents targeted at specific biomarkers of atherosclerotic disease were imaged using FMT to quantitate protease activity or tissue integrin levels, *in vivo*.

Table 1:

Fluorescent pre-clinical imaging agent	Excitation/emission (nm)	Molecular weight (g/mol)	Agent specificity
IVISense Pan Cathepsin 750	750/780	~ 400,000	Pan-cathepsin activated
IVISense Cat K 680 FAST	675/693	~ 8,500	Cathepsin K activated
IVISense Cat B 750 FAST	755/775	~ 8,500	Cathepsin B activated
IVISense Integrin Receptor 680	675/693	1,432	$\alpha_v\beta_3$ integrin targeted

Each of the agents were administered intravenously in all imaging studies. The typical adult mouse dose is 2 nmoles per 150 μ L saline, but for these studies a higher dose of 8 nmoles/mouse was used to enhance signal within the very small anatomical region of the aortic arch. Imaging of ProSense agent and IVISense Integrin Receptor agent was performed 24h following injection of the agent, whereas optimal imaging timepoints for the FAST agents (IVISense Cat K and Cat B FAST agents) was 6h.

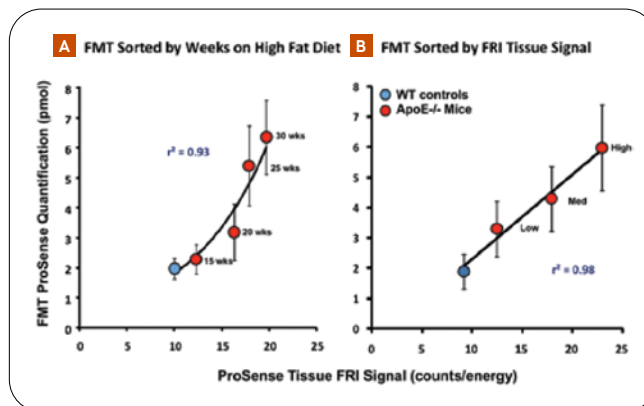


Figure 6: FMT correlations to ex vivo tissue fluorescence. To objectively corroborate the validity of non-invasive FMT quantitation, correlation analysis was performed in comparison to fluorescence analysis of excised aortic tissue. **A.** Timecourse FMT data from the study in Figure 5 was graphed in comparison to tissue FRI analysis. An excellent curvilinear correlation was seen, with FRI tissue analysis showing greater sensitivity at 15 weeks. **B.** Sorting the pooled timecourse data into groups with high, medium, and low tissue fluorescence intensity (independent of time), shows that FMT quantitative imaging can accurately, and non-invasively, rank groups of animals according to their severity of disease.

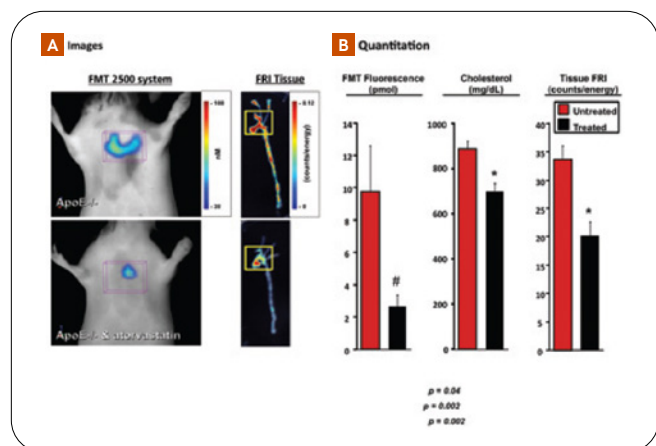


Figure 7: **Prophylactic atorvastatin treatment.** The efficacy of FMT and IVISense Pan Cathepsin 750 agent imaging to detect therapeutic efficacy was assessed in apoE^{-/-} mice fed high-cholesterol diets with and without 0.01% wt/wt of atorvastatin.

A. Representative FMT images from untreated and treated apoE^{-/-} mice on high-cholesterol diets imaged by non-invasive FMT and corresponding FRI tissue images.

B. Quantitation of FMT aortic arch fluorescence, serum cholesterol, and aortic arch tissue fluorescence decreases with atorvastatin treatment.

Tomographic imaging and analysis. Prior to imaging, mice were anesthetized using an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg), and they were depilated to minimize fur interference with fluorescent signal. Nair® lotion (Church & Dwight Co., Inc., Princeton, NJ) was applied thickly on skin over the upper torso (front, back, and sides) of each mouse, rinsed off with warm water, and re-applied until all fur had been removed. ApoE^{-/-} and WT mice were then imaged using FMT. Briefly, the anesthetized mice were carefully positioned in the imaging cassette to immobilize the animal during imaging, which was then placed into the FMT imaging chamber. A NIR laser diode transilluminated (*i.e.*, passed light through the body of the animal to be collected on the opposite side) the thorax region, with signal detection occurring via a thermo-electrically-cooled CCD camera placed on the opposite side of the imaged animal. Appropriate optical filters allowed collection of both fluorescence and excitation datasets, and the multiple source-detector fluorescence projections were normalized to the paired collection of laser excitation data. The entire 3D image acquisition sequence took approximately 4-5 minutes per mouse. FMT FRI (reflectance) imaging was routinely performed prior to each tomographic imaging session using built-in LED front illuminators and collection of single camera images.

3D reconstruction and analysis. The collected 3D fluorescence data was reconstructed by TrueQuant™ software v1.1 for the quantitation of three-dimensional fluorescence signal within the aortic arch region. Three-dimensional regions of interest (ROI) were drawn in the upper torso encompassing the upper chest region, excluding 1 mM ventrally and 8 mM dorsally. For analysis of IVISense Pan Cathepsin agent imaging datasets, background signal in WT mice was sufficiently low allowing analysis to be performed without removal of background fluorescence. For the other agents, 30-200 nM thresholding was applied to minimize the quantitation of low-intensity background within the aortic arch region. Thresholding was agent-dependent and determined as 2X the mean (in nM) of the fluorescence quantitated in WT mice. The total amount of lung fluorescence (in pmoles) was automatically calculated relative to internal standards generated with known concentrations of appropriate NIR dyes. For visualization and analysis purposes, TrueQuant software provided 3D images.

Ex vivo tissue imaging. To confirm that the fluorescent signal in atherosclerotic mice originates in the aortic arches, NIR fluorescent images of excised heart and aortic tissues were captured using the reflectance imaging function of the FMT 2500 system. Tissue signal intensity and distribution was established by subtracting background image field signal from tissue fluorescence. Fluorescent signal was normalized to the power of the illuminating LED light sources (counts/energy).

Atorvastatin treatment. To assess the ability of the ProSense agent to detect therapeutic changes in atherosclerosis inflammation, some mice received 20 weeks of high cholesterol diet enriched with 0.01% wt/wt of atorvastatin. Treated and untreated mice were imaged at 20 weeks. At the time of sacrifice, blood was obtained by retroorbital bleeding. Serum was obtained by centrifugation at 16 g for 15 minutes at 37 °C, and total serum cholesterol levels were measured by Charles River Labs using a colorimetric cholesterol.

Statistical analysis. The statistical significance of differences between experimental groups was analyzed using one-tailed analysis of variance. Group means were compared using the Scheffe multiple comparison test, with p values <0.05 considered significant.

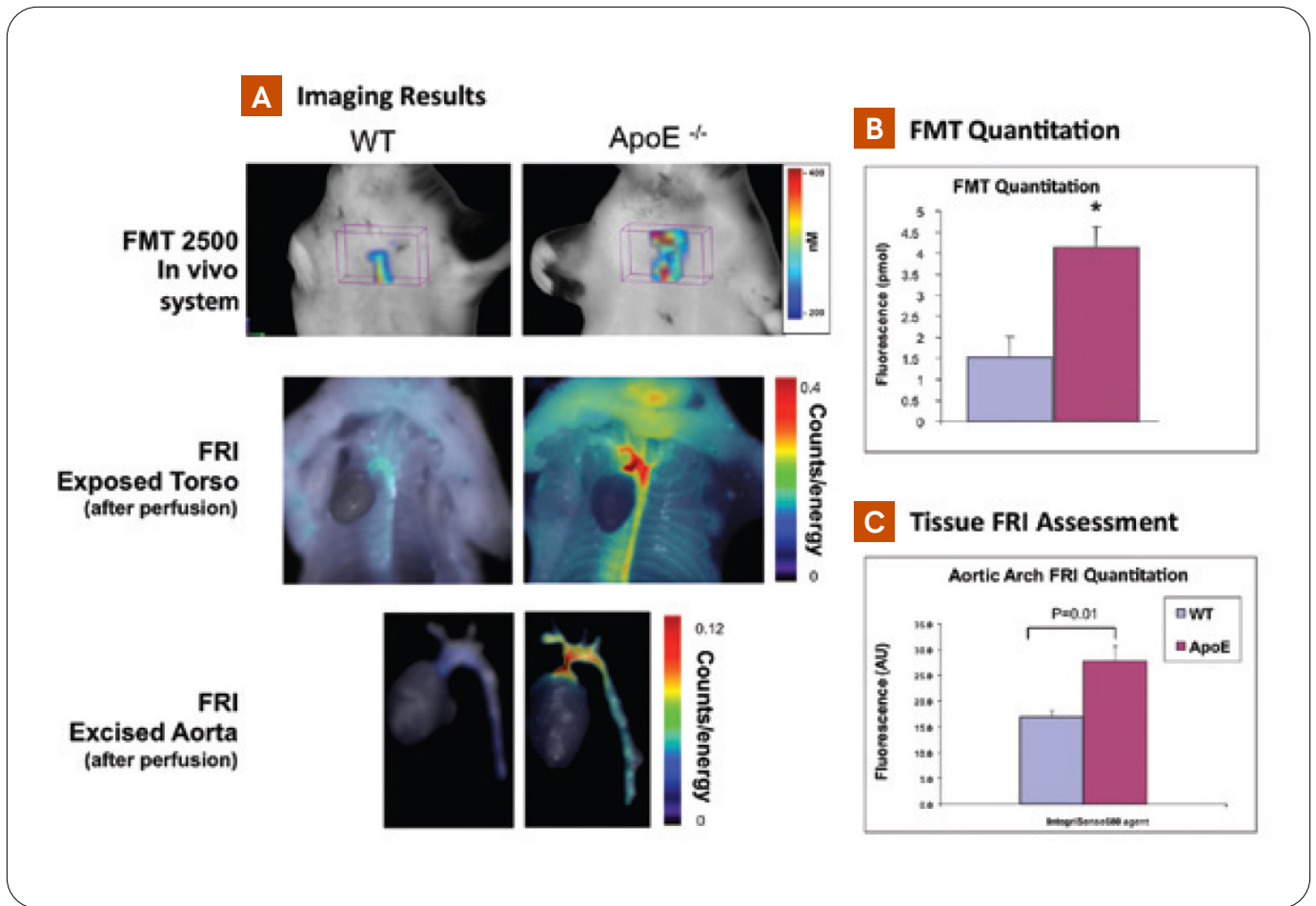


Figure 8: **Imaging atherosclerosis with IntegriSense680 agent.** ApoE^{-/-} mice on high-cholesterol diet for 16-18 weeks, and control mice, (n=5 mice per group) and were imaged by the FMT using different imaging agents to detect integrin upregulation potentially attributed to foam cells, vascular endothelium, and smooth muscle cells in the neointima.

A. Representative FMT images from WT and apoE^{-/-} mice using 8 nmols/mouse of IVISense Integrin Receptor 680 ($\alpha_v\beta_3$ expression). Imaging was performed 24h after injection in living mice (upper panel), *ex vivo* dissections (middle panel), and in excised aortic tissue (bottom panel).

B. Quantitation of FMT aortic arch region fluorescence from non-invasive imaging datasets using optimal thresholding to minimize background fluorescence.

C. Corresponding FRI analysis from the same mice, focusing the quantitation on the aortic arch region only.

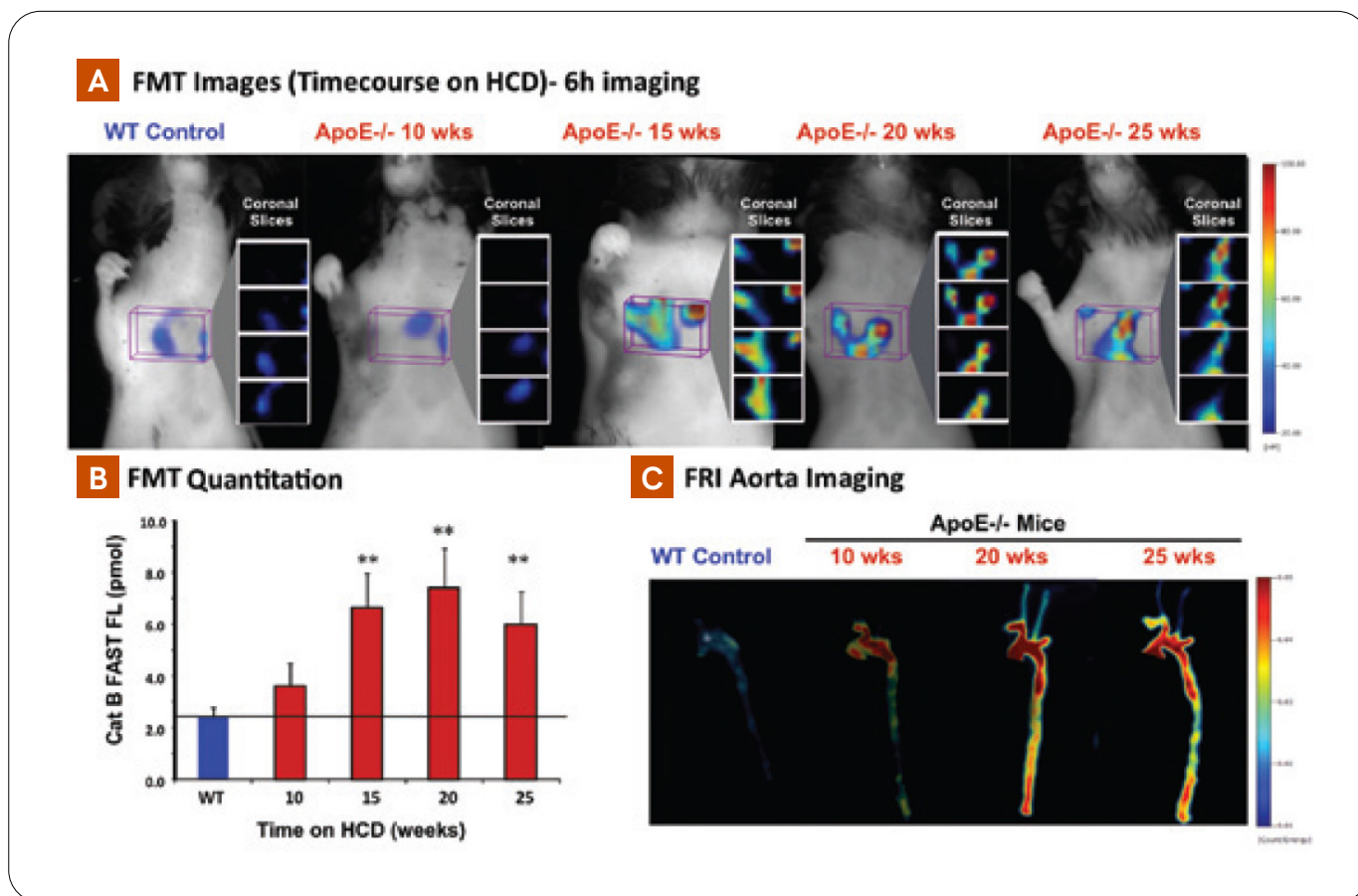


Figure 9: **Early atherosclerosis detection by IVISense Cat B FAST 750 agent.** ApoE^{-/-} mice on high-cholesterol diet for 10, 15, 20, or 25 weeks (n =13, 22, 16, and 8, respectively), and control mice (n = 31), and were imaged by the FMT using IVISense Cat B 750 FAST agent (selective for cathepsin B activity).

A. Representative FMT images from WT and apoE^{-/-} mice using 8 nmols/mouse agent at each timepoint. Imaging was performed 6h following agent injection, and tissues were collected from perfused animals immediately thereafter. Insets represent the 4 coronal slices within the ROI.

B. Quantitation of FMT aortic arch region fluorescence from non-invasive imaging datasets using optimal thresholding to minimize background fluorescence.

C. Corresponding FRI images of aortic tissue from the same mice.

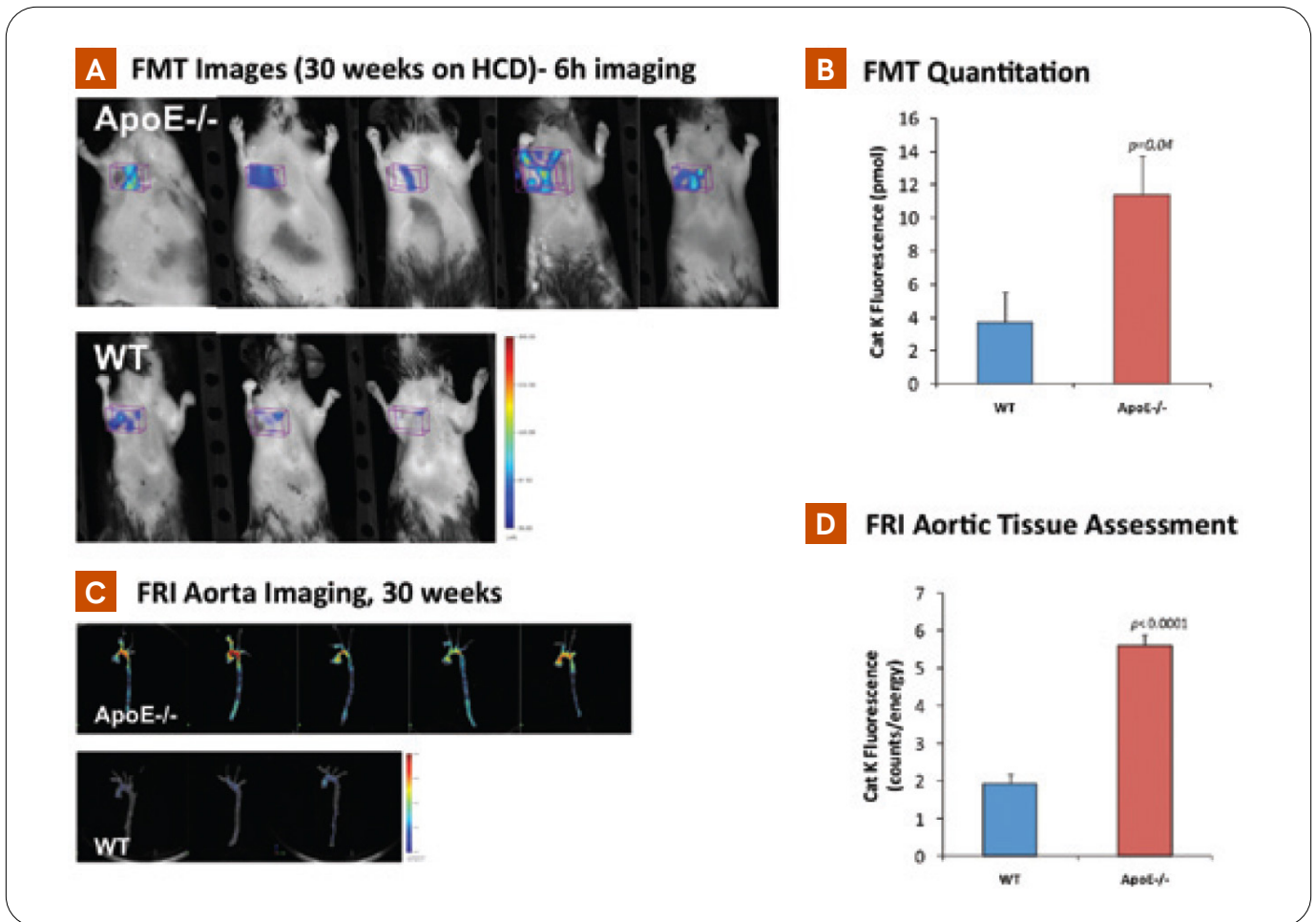


Figure 10: **Early atherosclerosis detection by IVISense Cat K FAST 680 agent.** ApoE^{-/-} mice on high-cholesterol diet for 30 weeks (n =5), and control mice (n = 3) were imaged by the FMT 2500 system using IVISense Cat K 680 FAST agent (high selectivity for cathepsin K activity).

A. Representative FMT images from WT and apoE^{-/-} mice using 8 nmols/mouse agent. Imaging was performed 6h following agent injection, and tissues were collected from perfused animals immediately thereafter.

B. Quantitation of FMT aortic arch region fluorescence from non-invasive imaging datasets using optimal thresholding to minimize background fluorescence.

C. Corresponding FRI images of aortic tissue from the same mice.

D. Corresponding FRI analysis of aortic arch tissue, focusing the quantification on the aortic arch region only.

Disease progression in ApoE-deficient mice fed high cholesterol diet

Time on HCD	Biological process*	IVISense Imaging Agent							
		Pan Cathepsin	MMP	Cat B FAST	MMP FAST	Integrin Receptor	Annexin-V	Cat K FAST	Osteo
4-6 weeks	<ul style="list-style-type: none"> monocyte adhesion sporadic foam cells 								
8-10 weeks	<ul style="list-style-type: none"> early foam cell lesions subendothelial progression 								
10-15 weeks	<ul style="list-style-type: none"> intermediate lesions smooth muscle cell proliferation fibrous caps 								
15 weeks	<ul style="list-style-type: none"> larger fibrous plaques small necrotic cores 								
20-30 weeks	<ul style="list-style-type: none"> advanced plaques increased necrosis 								
30-40 weeks	<ul style="list-style-type: none"> large, advanced plaques progression to coronary arteries large necrotic cores partial medial destruction calcification 								

*Based on Nakashima et al., 1994

Figure 11: **Summary of agents with potential use in imaging atherosclerosis.** ApoE^{-/-} mice on high-cholesterol diets show a steady progression of disease, beginning at 4-6 weeks with early influx on monocytes and progressing by 30-40 weeks to large, advanced fibrous plaques spread throughout the arterial tree. A variety of agents can be used to detect broad or selective (Cat B, Cat K agents) cathepsin activity, matrix metalloproteases activity, integrin expression ($\alpha_v\beta_3$), apoptosis, and plaque calcification. Expected timepoints for imaging with each agent are represented based upon the known biological mechanism(s) operant at particular timepoints and empirical imaging data with some of the agents.

Conclusion

The common current methods for measuring disease in the apoE-deficient mouse model of atherosclerosis rely on either *ex vivo* assessment of disease tissues post-mortem or non-invasive *in vivo* imaging of structural and anatomic features of lesions. Alternatively, optical imaging has been shown to offer a quick and non-invasive *in vivo* approach that can provide quantitative biological information regarding the levels of a variety of important disease biomarkers. Inflammatory cells (such as foam cell macrophages) present in the lesions are known to upregulate a variety of proteases, including cathepsins B and K,

and smooth muscle cells, vascular endothelium, and foam cells show increased $\alpha_v\beta_3$ integrin levels. By using the well characterized near infrared (NIR) protease-sensing *in vivo* IVISense Pan Cathepsin 750 imaging agent, to broadly detect cathepsin activity, in combination with FMT we were able to image atherosclerosis in apoE-deficient mice. By imaging the progression of disease in large numbers of mice over time, we generated datasets that allowed us to refine analysis procedures to maximize the discrimination of cathepsin activity in diseased versus wild-type mice.

Non-invasive FMT imaging quantitated a progression of disease over time with trends of disease seen as early as 20 weeks on high cholesterol diet and good statistical significance seen at 25 to 30 weeks. Quantitative *in vivo* data showed an excellent correlation to post-mortem tissue *ex vivo*, verifying that non-invasive imaging, even in the absence of anatomical information, identified and quantitated the appropriate disease region. Treatment efficacy could also be detected and quantitated; atorvastatin treatment showed an anticipated modest decrease in plasma cholesterol with a significant decrease in ProSense agent fluorescence. The same imaging approach yielded successful disease quantitation with other agents to detect lesion fluorescence associated with Cathepsin B (IVISense Cat B 750 FAST agent), Cathepsin K (IVISense Cat K 680 FAST agent), or Integrin (IVISense Integrin Receptor 680 agent) levels. Using multiplex imaging approaches with FMT, it is now possible to quantitate multiple biologies simultaneously to reveal the complex biological changes that occur both in disease progression and treatment.



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