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Intravital Fluorescence Microscopic Molecular Imaging of Atherosclerosis

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Abstract

Atherosclerosis is a lipid deposition and inflammatory disease that results in considerable morbidity and mortality worldwide. Advances in molecular imaging, particularly near-infrared fluorescence imaging, are now enabling the in vivo study of fundamental biological processes that govern atherogenesis and its complications. Here we describe applications of near-infrared fluorescence reporter technology and intravital fluorescence microscopy to elucidate important biological processes in atherosclerosis in vivo.

Keywords

Atherosclerosis; near-infrared fluorescence; molecular imaging; intravital microscopy; inflammation

1. Introduction

Progression and complication of atherosclerotic plaques may produce unheralded sudden cardiac death, myocardial infarction, stroke, and ischemic limbs. Unraveling of the underlying biology of atherosclerosis has provided new opportunities to limit these devastating complications, however improved understanding and treatment of atherosclerosis remains urgently needed.

Molecular imaging, specifically optical molecular imaging, is well positioned to provide novel insight into atheroma initiation, progression, and complications in vivo (1–3). The development of multichannel intravital fluorescence imaging systems in concert with versatile fluorescence reporter agents is permitting a high-resolution window into atherosclerosis biology in vivo. In particular, a multitude of new near-infrared fluorescence (NIRF) imaging agents has significantly expanded the capabilities of intravital microscopy. Imaging in the NIR window offers several advantages compared to visible light fluorochromes, including increased penetration depth of NIR photons due to markedly reduced blood photon absorption, as well as reduced tissue background autofluorescence (4,5). Recent studies employing NIRF reporters have shed light in vivo insight on macrophage phagocytic activity, cysteine protease activity, and osteogenesis in atherosclerosis(6–8). Substantial growth in intravital fluorescence imaging studies is

anticipated in the next several years as NIRF imaging agents and intravital systems become routine components of vascular biology research centers.

2. Materials

2.1 Intravital laser scanning microscope

1. For acquisition of optical sections through atheromata in vivo, a commercially available system is the IV100 (Olympus, Japan). The system is capable of rapid, interleaved detection of 3 fluorochromes for multicolor fluorescence detection. The system has laser excitations at 488nm, 561nm, 633nm, and 748 nm (the latter two channels allowing detection of NIRF agents).(9) Objectives (standard and “stick”) allow imaging from 1x-50x optical zoom. Optical sections (similar to confocal microscopy) are obtained using a motorized platform. Anesthesia for small animals may be delivered via a gas regulator with isoflurane.
2. Digital camera – the Olympus IV 100 does not have white light reflectance based imaging capability. Digital photography of the imaged atheroma, particularly in serial studies, is helpful.
3. ImageJ Software Analysis Program (version 1.40, freeware, NIH, <http://rsbweb.nih.gov/ij/download.html>)

2.2 Near-infrared fluorescence imaging agents

1. Selection depends on the specific molecular/cellular target(s) to be investigated:
2. Phosphate buffered saline (1x PBS) or 0.9% (normal) saline for dilution of imaging agents
3. Mouse tail vein injection setup (Narrow diameter plastic intravenous tubing that can snugly house a 30 gauge needle; 0.5–1.0 ml syringe)

2.3 Angiographic fluorescence imaging agents

1. Fluorescein isothiocyanate (FITC)-dextran (MW 2,000,000, Sigma catalog FD2000s). Excitation/emission 490nm/520nm. Stable for 2–3 years at 4°C.
2. Alternatively, if a near-infrared channel is available, a long half-life NIRF intravascular agent may be used (Angiosense680(10) or Angiosense750, Visen Medical) or shorter half-life agent indocyanine green (ICG, 5 mg/kg, excitation 780 nm)

2.4 Murine atherosclerosis model

1. Apolipoprotein E deficient (ApoE^{-/-}, females, B6.129P2-Apoe<tm1Unc>/J Jackson Labs, 8–9 weeks on arrival, see Note 1)
2. High-cholesterol diet (0.2% commonly used)
3. Optional: cholesterol measurement kit (cholesterol kit,; colorimetric assay, spectrophotometer for absorption measurement)

2.5 Surgical setup for carotid arterial dissection

1. Stereomicroscope, standard rodent animal surgical equipment for vascular dissection (surgical instruments; ketamine/xylazine for anesthesia (see Note 2)).

¹In addition to ApoE^{-/-} mice, LDL receptor deficient mice are also commonly used but develop atherosclerosis more slowly.

2. Place intravenous tubing (0.3mm diameter) to be placed underneath the vessel, to aid localization of atheromata (will absorb light)
3. Tail vein catheter (intravenous tubing with 30G needle on the front end)

2.6 NIRF-enabled fluorescence microscope for fluorescence microscopy

1. A commercially available NIRF-capable system is the Eclipse 80i epifluorescence microscope (Nikon, Japan, see Note 3).
2. Standard histopathological setup for obtaining frozen sections: optical cutting temperature (OCT) compound (Sakura Finetek), cryotome, glass slides.
3. Microscopy image acquisition and analysis software

3. Methods

Carotid atheromata of ApoE^{-/-} mice are surgically exposed for intravital microscopy.^(6–8) Multichannel intravital fluorescence microscopy is next performed using near-infrared and visible light fluorochromes. Compared to visible light fluorochromes, near-infrared fluorescence (NIRF) reporters offer greater depth penetration and signal-to-noise detection due to reduced photon absorption and less tissue autofluorescence, respectively. Multichannel high-resolution optical sections (10x10x10 micrometer resolution) are next obtained allowing multiplexing of various biological targets/processes in vivo. The following step-by-step method plan details the experimental procedure.

3.1 Development of atherosclerosis

1. Obtain institutional animal committee approval for planned experiments.
2. Initiate ApoE^{-/-} mice on high-cholesterol diet from age 10 weeks to 24 weeks. (see Note 4)
3. Confirm the effect of high-cholesterol diet by cholesterol measurement (see Note 5)

3.2 Injection of Imaging Agents

1. Inject (via i.v. tail vein) long-circulating atherosclerosis-targeted agents 24 hours prior to imaging: magnetic nanoparticles (e.g. iron oxide)-based agents typically at a dose of 5–10 mg Fe/kg; protease-activatable agents typically at a dose of 80–200 nmol/kg.
2. Inject vascular angiographic agents immediately prior to imaging: FITC-dextran 0.5 mg per mouse (excitation 488nm) or Angiosense 2 nmol/mouse (excitation 680nm or 750nm), or indocyanine green 5 mg/kg (excitation 780nm).

²Isoflurane gas anesthesia may be used, but compared to ketamine/xylazine, we have that isoflurane increases carotid arterial pulsation artifacts presumably due to its vasodilatory properties.

³Fluorescence microscopy is an excellent molecular research tool that naturally supports intravital fluorescence imaging studies. Of note is that most commercial fluorescence microscopes do not have the routine filters to detect NIR channels; this requires customized NIR filters. The Nikon 80i epifluorescence microscope can resolve two NIR channels (e.g. 680nm and 750nm) using appropriate bandpass excitation and emission filters as well as dichroic mirrors. As an alternative, the IV100 system itself, while not optimized for confocal microscopy of slides, could also be investigated for this purpose (n.b. routine confocal microscopes are not able to detect far red fluorochromes (>700 nm).)

⁴This high cholesterol diet protocol produces animals with visible carotid atheromata approximately 85%–90% of the time; dermatitis and stopped posture may occur in animals > 30 weeks of age.

⁵Cholesterol values for 20–50 week ApoE^{-/-} mice on chow diet is 275 mg/dL, and increases to >450 mg/dL on the above 0.2% cholesterol supplemented diet⁽¹¹⁾.

3.3 Preparation of mouse for IVFM

1. Induce anesthesia with ketamine (IP 100 mg/kg) and xylazine (IP 5 mg/kg); give maintenance doses every 30–45 minutes (see Note 2).
2. Place tail vein catheter (see Note 6).
3. Make a vertical surgical neck incision just off the midline on the side of the carotid of interest; typically the right carotid artery is selected.
4. Bluntly dissect and expose the distal common carotid artery (see Note 7). Plaques are typically at the distal vessel (towards the head) and usually extend into the bifurcation/trifurcation of this vessel.
5. Place a small piece of plastic tubing gently underneath the artery to facilitate vessel localization on IVFM.
6. Obtain high-magnification digital photograph of exposed surgical field / carotid plaque – particularly helpful for serial imaging studies where precise registration of the initial imaging field is necessary.
7. Transfer surgically exposed animal to the intravital imaging system.

3.4 IVFM data acquisition (Figures 1 and 2)

1. Choose 4x objective for imaging (see Note 8).
2. Rapidly localize carotid atheromata on fluorescence images by an absorbing or fluorescent phantom under the vessel (see Note 9)
3. Set laser power and gains for target channels. This takes some individualization based on the anticipated signal-to-noise in vivo. The goal is to maximize dynamic range without saturating (“clipping”) the data. A helpful strategy is to have 2 power settings, a low power and high power setting for each subject / each study. Using the same power settings thought study facilitates comparison between subjects. (see Note 10)
4. Set imaging boundaries in the z-direction – this defines the IVFM stack. In practice NIR fluorochromes can be detected up to 500 micrometers below the external surface (adventitia) of the plaque. Typical parameters thus utilize 50 slices with a slice thickness of 10 micrometers.
5. Inject vascular angiographic agent (e.g. FITC-dextran, Angiosense, ICG) via tail vein. Begin live imaging (choose a slice in the middle of the imaging stack containing the vessel) until a vascular angiogram is apparent.
6. Acquire multichannel image stack, and repeat as indicated if temporal data is required, as well as varying power settings and zooms. In the usual multichannel acquisition, one channel will be for the angiogram, and the other one or two channels will be used for molecular/cellular targets of interest.

⁶It may be easier to place tail vein under isoflurane which is vasodilatory, then switch over to ketamine/xylazine for the remainder of the experiment.

⁷Avoid excessive trauma to vagus nerve running along side the carotid artery or the mouse may suffer a respiratory arrest.

⁸Higher magnification objectives (10x and above) often reduce image quality because of pulsation motion artifact sensitivity. Of note is that the IV100 system allows a 2-fold zoom feature without changing objectives, so that the user can acquire 8x magnification images with the 4x objective.

⁹As PE-10 tubing is absorptive in many fluorescent channels, the associated signal void can be used to localize the carotid artery, obviating the need to fill the PE-10 phantom with a fluorochrome.

¹⁰Fluorescence image artifact, particularly in the 750 channel, may occasionally be seen. Adding saline to the field may help to reduce this artifact.

7. For survival experiments, remove phantom and surgically close the incision, and provide analgesia per animal committee-approved protocol.

3.5 Image analysis

1. Obtain and analyze image stacks from the IV100 system. Images are typically stored as multi-layer tiff files. In a 3 channel experiment, if 50 slices were acquired, there will be $50 \times 3 = 150$ images. The first 50 would be from channel 1 (typically the 488nm channel), the next 50 would be from channel 2 (typically the 633 nm channel), and last 50 would be from channel 3 (typically the 748 nm channel).
2. Analyze images with NIH ImageJ: Choose “Z-Project” from the menu. Input the start slice (e.g. 51) and the end slice (e.g. 100). Next choose Projection type: Sum slices (not maximal intensity or other choices). An example summation projection image is provided in Figure 2 (see Note 11).
3. Use the manual ROI tool (either freehand or polygon tool) to circle the fluorescent region of interest. It is imperative to recognize that the fluorescent signals may define only part of the atheroma. It is therefore very helpful to review the digital photographic images and angiograms obtained to looking for plaque, vascular filling defects, and side branches. This information increases the accuracy/confidence of these measurements.
4. After drawing the ROI of the target area, select Measure from the menu. Record the area (check header for units, typically in cm^2) and mean (average signal intensity = total signal in ROI / # pixels in ROI).
5. Repeat ROI measurement for a background fluorescence signal in the vessel (non plaque region as confirmed by digital photography and angiogram image). The target-to-background ratio is calculated as average signal intensity(plaque) / average signal intensity(background). This can be repeated for each channel of interest. An example is provided in Figure 2.

3.6 Fluorescence microscopy

1. Fluorescence microscopy of plaque sections provides critical corroborative data for intravital imaging results (i.e. Figure 1).
2. At the end of the study, sacrifice and perfuse the animal with saline to clear residual fluorochromes from the vasculature (see Note 12).
3. Resect carotid artery and embed fresh in OCT compound and freeze at -80°C
4. Perform fluorescence microscopy of cryocut 5 micrometer fresh frozen sections. The typical exposure times on the Nikon 80i 680 channel: 1–5 seconds; 750 channel 5–30 seconds (see Note 13).
5. Obtain 488 channel fluorescence (exposure time 0.5–1 sec) to obtain tissue autofluorescence and confirm unique fluorescence distribution of targeted NIR fluorochromes (see Note 14).

¹¹While time consuming, it may be helpful to analyze each slice within the image stack, as the individual images are higher resolution than projection image.

¹²Perfusion with harsh fixatives such as formaldehyde may damage bound fluorochromes. Fresh frozen tissue is the safest way to ensure the integrity of tissue fluorochromes.

¹³Typical broadband halogen light sources have relatively fewer excitatory photons in the NIR window, thus requiring relatively longer exposure times compared to visible light fluorochromes.

6. Perform microscopy image data analysis – one can obtain merged/blended fluorescence images and perform ROI threshold analysis for quantification of the fluorescence signal distribution in plaque sections.
7. Perform correlative immunohistochemistry / histopathology to confirm specificity of NIRF signal obtained on fluorescence microscopy.

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¹⁴In the 488nm channel, atheroma sections usually show substantial signal in the media of the vessel due to elastin fiber autofluorescence, as well as plaque autofluorescence from collagen and macrophages (hence the rationale for using NIR fluorochromes for targeted imaging).

Figure 1.

In vivo detection of macrophage phagocytic activity in atherosclerosis using a macrophage-targeted near-infrared fluorescent nanoparticle (CLIO-Cy5.5). **A.** Following CLIO-Cy5.5 (ex/em 673nm/694nm, dose 10 mg iron / kg) injection 24 hours beforehand, the right carotid artery of an ApoE^{-/-} mouse is surgically exposed in preparation for intravital laser scanning fluorescence microscopy. A typical yellowish-white atherosclerotic plaque is present at the distal common carotid artery bifurcation. A fluorescent phantom is used as reference during imaging (placed adjacent to the plaque). The dashed box represents the region displayed in laser scanning fluorescence microscopy imaged in **B.** **B.** Following indocyanine green (ICG) injection, a vascular angiogram becomes evident, along with an intravascular filling defect due to the plaque. In the Cy5.5 NIR channel, strong focal plaque signal is evident. Merged image of the ICG and Cy5.5 signal confirms colocalization of the macrophage Cy5.5 signal to the filling defect seen on the angiogram. **C.** Fluorescence reflectance imaging of an excised carotid atheroma from an ApoE^{-/-} CLIO-Cy5.5-injected animal shows bright enhancement in the Cy5.5 channel, consistent with NIR nanoparticle uptake by plaque macrophages, and distinct from FITC-channel autofluorescence. **D.** Correlative immunohistochemistry confirms that the NIRF signal colocalizes with immunoreactive plaque macrophages (right). Modified by permission from Pande et al. (6)

Figure 2.

Two-channel intravital fluorescence microscopy (IVFM) of cathepsin K protease activity in carotid plaques of ApoE^{-/-} mice. At 24 hours prior to imaging, a cathepsin K NIRF agent (ex/em 670/690nm, dose 200 nmol/kg) was injected intravenously. During IVFM, a second spectrally-distinct NIRF agent (Angiosense750, ex/em 750/770 nm, dose 2 nmol) was co-injected to provide a vascular angiogram. **A.** Fusion images of cathepsin K protease activity (green) and Angiosense750 signal (red angiogram) reveal focal NIRF signal (arrowhead) indicating abundant cathepsin K activity. **B.** A control NIRF agent (the - amino acid analog of the cathepsin K agent) shows relatively scant NIRF signal, indicating that cathepsin K cleavage of the peptide substrate is required to generate substantial NIRF signal. **C, D.** Summation projection images (obtained by ImageJ analysis) demonstrate greater plaque signal with the cathepsin K agent compared to the control, uncleavable D-analog agent. **E.** Quantification of plaque target-to-background ratio distinguishes the cathepsin K NIRF agent from the control NIRF agent. Reproduced by permission from Jaffer et al.(7)

Table 1

NIRF agents utilized in intravital microscopy of atherosclerosis

Target/Process	Source
Multicathepsin enzyme activity	MGH-CMIR; Visen medical (Prosense)
Cathepsin K activity	MGH-CMIR
Macrophages	MGH-CMIR (CLIO-VT680)
VCAM-I	MGH-CMIR (VINP-28)
Osteogenesis / Calcification	Beth Israel Deaconess Medical Center (Frangioni Lab); Visen medical
Matrix metalloproteinase activity	MGH-CMIR; Visen medical
Cell trafficking	MGH-CMIR (CLIO-VT680); Visen medical (Vivotag)
Angiography	FITC-Dextran (Sigma), Visen medical (Angiosense), Indocyanine Green (Akorn)