In Vivo Nanoparticle Assessment of Pathological Endothelium Predicts the Development of Inflow Stenosis in Murine Arteriovenous Fistula

Jie Cui,* Chase W. Kessinger,* Jason R. McCarthy, David E. Sosnovik, Peter Libby, Ravi I. Thadhani, Farouc A. Jaffer

Objective—In vivo assessment of pathological endothelium within arteriovenous fistula (AVF) could provide new insights into inflow stenosis, a common cause of AVF primary failure in end-stage renal disease patients. Here we developed nanoparticle-based imaging strategies to assess pathological endothelium in vivo and elucidate its relationship to neointimal hyperplasia formation in AVF.

Approach and Results—Jugular-carotid AVFs were created in C57BL/6 mice (n=38). Pathological endothelium in the AVF was visualized and quantified in vivo using dextranated magnetofluorescent nanoparticles (CLIO-VT680 [cross-linked iron oxide-VivoTag680]). At day 14, CLIO-VT680 was deposited in AVF, but only minimally in sham-operated arteries. Transmission electron microscopy revealed that CLIO-VT680 resided within endothelial cells and in the intimal extracellular space. Endothelial cells of AVF, but not control arteries, expressed vascular cell adhesion molecule-1 and showed augmented endothelial permeability near the anastomosis. Intravital microscopy demonstrated that CLIO-VT680 deposited most intensely near the AVF anastomosis (P<0.0001). The day 14 intravital microscopy CLIO-VT680 signal predicted the subsequent site and magnitude of AVF neointimal hyperplasia at day 42 (r=0.58, P<0.05). CLIO-VT680 deposition in AVF was further visualized by ex vivo MRI.

Conclusions—AVF develop a pathological endothelial response that can be assessed in vivo via nanoparticle-enhanced imaging. AVF endothelium is activated and exhibits augmented permeability, offering a targeting mechanism for nanoparticle deposition and retention in pathological endothelium. The in vivo AVF nanoparticle signal identified and predicted subsequent inflow neointimal hyperplasia. This approach could be used to test therapeutic interventions aiming to restore endothelial health and to decrease early AVF failure caused by inflow stenosis. (Arterioscler Thromb Vasc Biol. 2015;35:189-196. DOI: 10.1161/ATVBAHA.114.304483.)

Key Words: arteriovenous fistula ▪ endothelium ▪ imaging ▪ stenosis

Hemodialysis is a life-sustaining treatment for 1.5 million end-stage renal disease patients across the world. Effective hemodialysis treatment requires reliable large caliber vascular access. Dysfunctional dialysis access disrupts scheduled dialysis treatment and associates with higher mortality rates. Thus, preservation of patent dialysis access is essential to the care of hemodialysis patients. Arteriovenous fistulae (AVFs), the preferred access route for hemodialysis, are an essential to the care of hemodialysis patients. Arteriovenous fistulas (AVFs), the preferred access route for hemodialysis, have an unacceptably high failure rate. After surgical creation, ≤30% to 50% of AVFs are unsuitable for hemodialysis as a result of a failure to mature. In contrast to late AVF failures, which are usually caused by thrombosis and venous neointimal hyperplasia (NH), early AVF failures primarily result from juxta-anastomotic inflow stenosis.

Pathologically altered endothelium is implicated in AVF failure. At present, however, there is minimal understanding of the role of pathological endothelium in AVF failure. The ability to assess AVF endothelium in vivo could furnish new insights into the topography of pathological endothelium and its site-specific relationship to the subsequent development of inflow stenosis, a common cause of AVF failure. This study tested the hypothesis that fluorescence and MRI imaging of dextran-coated nanoparticle deposition could assess dysfunctional endothelium within AVF. After demonstrating anastomosis-based nanoparticle uptake by AVF, but not sham-operated control vessels, this study explored mechanisms underlying nanoparticle retention in AVF. We further tested the hypothesis that the intensity and location of the pathological
endothelial signal on AVF imaging would predict the subsequent extent and location of NH within the maturing AVF.

**Materials and Methods**

A study flowchart (Figure I) and detailed Methods are available in the online-only Data Supplement.

**Results**

**Measurement of Blood Flow After AVF Creation**

Murine AVF were created using an established common carotid artery-jugular vein end-to-side anastomosis approach (Figure IIA in the online-only Data Supplement).10–13 The anastomosis was defined as the surgical connection between the carotid artery and the jugular vein. AVFs exhibited a 7-fold increase in carotid arterial blood flow compared with baseline (pre, 0.35±0.03 mL/min, versus post, 2.56±0.28 mL/min; \( P < 0.0001 \); Figure IIB in the online-only Data Supplement). Mice that developed acute venous thrombosis immediately after AVF creation were excluded from further study (n=9 of 47, blood flow <0.1 mL/min).

**Histological Assessment of Inflow Neointimal Hyperplasia in Murine AVF**

To examine whether these experimental AVFs could recapitulate human inflow AVF stenosis, a group of mice were euthanized 42 days after AVF surgery (n=7). Modified Verhoeff Van Gieson histological stain visualized elastin layers within the AVF. NH was evident in the juxta-anastomotic arterial limb. The area of NH, calculated as the area between the lumen and the internal elastic lamina, steadily decreased with increasing distance away from the anastomosis (\( r = -0.89; P = 0.0067 \); Figure 1). Sham-operated contralateral vessels did not develop NH.

**CLIO-VT680 Nanoparticles Report on Pathological Endothelium in AVF**

To assess the in vivo vascular endothelial response after AVF creation, dextranated magnetofluorescent nanoparticles (CLIO-VT680 [cross-linked iron oxide-VivoTag680], 10 mg Fe/kg) were intravenously injected into day 13 post-AVF surgery mice (n=9). CLIO-VT680 nanoparticles have a hydrodynamic diameter of 49 nm with a 5 nm iron oxide core and can be phagocytosed by murine macrophages, endothelial cells, and smooth muscle cells in vascular disease states.14,15 After 24 hours, mice receiving CLIO-VT680 were euthanized and AVF were resected. Fluorescence microscopy revealed that CLIO-VT680 nanoparticles deposited in the juxta-anastomotic arterial segment of the AVF, but minimally in contralateral sham-operated vessels (Figure 2A). CLIO-VT680 mainly colocalized with CD31-positive endothelial cells. Minor areas of nanoparticle deposition were also evident in the smooth muscle cell–rich superficial medial layer (Figure 2A). Endothelial cells of AVF, but not sham-operated arteries, expressed vascular cell adhesion molecule-1, a marker of endothelial cell activation (Figure 2B).16 En face confocal analysis of the AVF segment also demonstrated CLIO-VT680 deposition near the anastomosis (Figure 2C).

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**Nonstandard Abbreviations and Acronyms**

<table>
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<th>Abbreviation</th>
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<tr>
<td>AVF</td>
<td>arteriovenous fistula</td>
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<tr>
<td>CLIO-VT680</td>
<td>cross-linked iron oxide-VivoTag680</td>
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<td>IVM</td>
<td>intravital microscopy</td>
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<td>NH</td>
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**Figure 1.** Representative arteriovenous fistulae (AVF) pathology at day 42 after AVF creation. A, Neointimal hyperplasia was evident in the inflow arterial limb of the AVF (left column, hematoxylin and eosin (H&E); right column, Van Gieson’s stain (VVG); Scale bar, 100 μm). B, Correlation between the neointimal area in day 42 AVF as a function of increasing distance from the anastomosis (\( r = -0.89; P = 0.0067 \); n=7 mice). Error bar, SEM.
Transmission electron microscopy of AVF tissue sections sampled at day 14 revealed the precise localization of the nanoparticles within AVF (Figure 3A). The electron dense iron oxide core (5 nm) of the CLIO-VT680 nanoparticles were identified as hypointense signal areas on transmission electron microscopy and localized inside endothelial cells, as well as in the subendothelial intimal space bordered by the internal elastic lamina. Sham-operated control arteries showed minimal nanoparticle deposition.

As the transmission electron microscopy images revealed subendothelial deposition of CLIO-VT680 nanoparticles in AVFs, we hypothesized that AVF would exhibit abnormal endothelial barrier function. To test this hypothesis, Evans blue was intravenously injected in day 14 animals (n=3), 30 minutes before euthanization to access endothelial permeability. Light microscopy revealed Evans blue deposition in the juxta-anastomotic region of the AVF, indicating increased permeability. Sham-operated contralateral arteries showed minimal Evans blue staining. Fluorescence reflectance imaging and fluorescence microscopy of Evans blue fluorescence similarly revealed augmented signal in the juxta-anastomotic region of the AVF compared with sham-operated arteries (Figure 3B).

**In Vivo Assessment of Pathological Endothelium in AVF by Intravital Fluorescence Microscopy**

Intravital microscopy (IVM) simultaneously illuminates the molecular and structural details of vascular disease at high resolution in vivo. To determine whether nanoparticle-delineated pathological endothelium in AVF could be imaged in vivo, mice underwent confocal IVM at day 14 (n=9) and day 21 (n=4) post-AVF surgery, 24 hours after CLIO-VT680 administration. AVFs were surgically exposed to allow IVM biological–structural imaging of the anastomosis and the adjacent...
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Areas of pathological endothelium displayed near-infrared fluorescence emission from CLIO-VT680. The vessel wall and lumen were detected and identified jointly by second harmonic generation and fluorescein isothiocyanate-dextran signals, respectively (Figure III in the online-only Data Supplement). IVM coronal Z-stacks were obtained and regions-of-interest were defined as CLIO-VT680-positive areas inside the vessel wall on resliced axial micrographs (Figure 4A). The anastomosis was identified by the presence of the AVF sutures. The inability of light to penetrate the nylon sutures precluded analysis of near-infrared fluorescence signals at the site of proximal anastomosis (distance=0 μm).

Analysis of the day 14 IVM data revealed that the highest nanoparticle signal occurred in the juxta-anastomosis arterial segment (Figure 4A). In vivo CLIO-VT680 signals decreased linearly as the distance away from the anastomosis increased ($r=−0.98; P<0.0001$; Figure 4C). A similar trend was evident in day 21 AVF ($r=−0.98; P<0.001$; Figure 4C), with higher CLIO-VT680 target-to-background ratios compared with day 14 AVF, indicating progressive endothelial abnormality in developing AVF. Using the AVF anastomosis as a fiducial marker, matched ex vivo arterial sections similarly demonstrated that day 14 pathological endothelial signal (higher nanoparticle target-to-background ratio) diminished steadily as the distance from the anastomosis increased ($r=−0.96, P<0.0001$; Figure 4B and 4D).

In vivo and ex vivo nanoparticle target-to-background ratios correlated on matched sections ($r=0.59, P<0.0001$; Figure 4E).

Nanoparticle Fluorescence in Pathological Endothelium of AVF Predicts the Development of Arterial Neointimal Hyperplasia Formation

Ex vivo studies link impaired endothelial function to NH and AVF failure.9,11 It remains unknown, however, whether imaging of pathological endothelium can quantitatively predict sites of future inflow stenosis. We therefore performed survival IVM imaging of the AVF arterial circuit in day 14 AVF using CLIO-VT680 (n=3), followed by NH assessment at day 42. On day 42, mice were euthanized and the arterial NH was measured using Verhoeff Van Gieson staining. Anastomosis sutures were used as the 0 μm reference point to match day 14 IVM and day 42 histology (Figure 5A and 5B). Using the AVF anastomosis as a fiducial marker, we found that both the day 14 in vivo CLIO-VT680 signal and day 42 NH area steadily decreased with the distance from the anastomosis (n=3 mice, $r=−0.98, P=0.0009$; $r=−0.92, P=0.0089$; Figure 5C). The individual day 14 CLIO-VT680 target-to-background ratio values (reflecting the degree of pathological endothelium) predicted the site and magnitude of NH on day 42 ($r=0.58, P=0.016$; Figure 5D). A similar correlation was found in an additional group of mice (n=2) using a different batch of CLIO-VT680 ($r=0.67, P=0.01$, data not shown). The pattern of day 42 NH distribution was similar to the day 14 CLIO signal distribution (Figure IV in the online-only Data Supplement).

MRI of Pathological Endothelium in AVF

To determine whether a clinical imaging modality could assess pathological endothelial function in AVF, ex vivo MRI was performed on resected day 14 AVF (n=5). Day 14 mice received...
CLIO-VT680 nanoparticles or PBS before euthanization and tissue resection. CLIO-VT680 nanoparticles are derived from ultrasmall superparamagnetic iron oxide nanoparticles that can induce signal loss in T2-weighted magnetic resonance images. In mice that received the magnetofluorescent CLIO-VT680 nanoparticles, strong near-infrared fluorescence signal emanated from the juxta-anastomotic region (arrow head, Figure 6A), consistent with the in vivo IVM results. Minimal near-infrared fluorescence signal enhancement occurred in PBS-injected mice. T2-weighted rapid acquisition with refocused echoes images demonstrated signal hypointensity around the AVF anastomosis in CLIO-VT680-injected mice, consistent with the IVM and FRI data. Iron oxide deposition in the arterial AVF segment generated lower MRI signal on T2-weighted images compared with the PBS group (signal-to-noise ratio, 41.5±1.8 CLIO versus 64.9±1.8 PBS; P=0.003; Figure 6B).

Discussion
This study reports the development of a novel nanoparticle-based imaging approach to map and quantify murine AVF-induced pathological endothelial function in vivo. It showed that in the early stage, the in vivo AVF endothelial injury response predicted the development of later stage AVF arterial NH and inflow stenosis. The greatest degree of endothelial injury and NH occurred in the juxta-anastomotic arterial segment of the AVF.

Endothelial cells near the AVF anastomosis exhibited greater retention of CLIO nanoparticles and showed increased permeability and vascular cell adhesion molecule-1 expression. After AVF creation in humans, arterial blood flow usually increases 40-fold to 1000 mL/min. The response to augmented blood flow as well to the initial surgical procedure can induce endothelial dysfunction, characterized by an imbalance between endothelium-derived vasodilators and vasoconstrictors, abnormal leukocyte–endothelial interactions, and an increased expression of adhesion molecules, as well as impaired vascular integrity and increased permeability. Indeed, we found heightened vascular cell adhesion molecule-1 expression and Evans blue–delineated endothelial permeability near the AVF anastomosis (Figure 3B), the area that ultimately developed the greatest degree of NH and...
stenosis. Augmented endothelial cell activation and endothelial permeability in AVF further provide mechanisms for the deposition of CLIO-VT680 nanoparticles in endothelial cells and the subendothelial space, respectively, as revealed by transmission electron microscopy (Figure 3A). Although smooth muscle cells (SMCs) within atheroma may phagocyte CLIO nanoparticles,14 we found little nanoparticle deposition in SMCs within AVF. This observation might relate to differential phagocytic profiles of SMCs in differing arterial disease states,23 as well as reduced access of CLIO nanoparticles to the deeper medial layers.

Although many studies have elucidated mechanisms underlying late AVF failure, such as venous NH and thrombosis, only a few studies have studied in vivo mechanisms underlying primary AVF failure, a major clinical problem. The primary failure rate of AVF reaches 30% to 50%.24,25 Inflow stenosis as a subset of juxta-anastomotic stenosis accounts up to one-third of AVF cases referred to interventional centers.26–28 At present, scant data shed light on in vivo biological mechanisms underlying inflow stenosis. This study provides new knowledge into the relationship between in vivo regional AVF-related endothelial injury and subsequent inflow neointimal formation. We found that the degree and location of pathological endothelium could predict the development of NH at a later stage. The validated experimental methodology here provides a new approach to investigate genetic or pharmacological manipulations of endothelial function in vivo and to assess its subsequent effects on the development of NH and inflow stenosis in AVF. In addition, future studies can also investigate the possible contributions of comorbidities relevant to renal patients, such as hyperlipidemia, diabetes mellitus, and hypertension to mechanisms of AVF failure.

The most commonly used clinical imaging method to assess AVF patency is duplex ultrasound.29 Multidetector computed tomography is another noninvasive tool that can evaluate the entire AVF circuit.30 X-ray angiography provides structural information and guides AVF treatment, but is invasive. Additional limitations of CT and invasive angiography include the inability to image the biological processes underlying AVF failure, as well as radiation exposure and the risk of contrast-induced nephropathy. Furthermore, none of the current AVF imaging methods accurately predicts inflow stenosis, nor leads to improved AVF patency rates.31–33 In this study, the initial results with MRI of iron oxide nanoparticle deposition illustrate a possible path to image pathological endothelium in AVF in humans. Clinical magnetic nanoparticles, such as ferumoxytol, are similar to the CLIO-VT680 nanoparticles used here and also induce MRI signal loss on T2-weighted images.34 Although our feasibility study was limited to ex vivo MRI at high-field strength in mice, the larger size and more superficial location of arm AVFs in human subjects might enable noninvasive ferumoxytol-enhanced MRI of pathological endothelial function. New in vivo imaging strategies that can identify AVFs at risk for inflow stenosis offer the opportunity to decrease the early fistula failure rate and improve dialysis patients’ outcomes. Such high-risk patients might be candidates for surgical AVF revision, endovascular treatment, or novel proendothelial healing approaches to reduce AVF failure.

Figure 5. Pathological endothelium in vivo and the development of subsequent arteriovenous fistulae (AVF) inflow stenosis. A and B, Representative day 14 survival IVM axial images and subsequent matched day 42 histological images. The anastomosis was identified by suture ligatures and served as the fiducial zero reference point for matching. Red, CLIO-VT680; Blue, second harmonic generation (SHG) signal. IVM images were processed and windowed identically. Scale bar, 50 μm. C, In vivo day 14 CLIO-VT680 TBR measured by IVM and day 42 neointimal hyperplasia in the arterial segment, as a function of the distance from the anastomosis. Error bar, SEM. D, Correlation between the in vivo IVM CLIO-VT680 signal at day 14 and the degree of subsequent neointimal hyperplasia at day 42 (17 AVF sections from 3 animals). CLIO-VT680 indicates cross-linked iron oxide-VivoTag680; and TBR, target-to-background ratio.
This study has limitations. Although the used murine AVF approach of carotid artery mobilization differs from the clinical practice of vein mobilization, this murine model is well established experimentally and recapitulates many of the features of human AVF, including inflow stenosis. Venous side juxta-anastomotic stenosis, another common cause of AVF failure, could not be imaged in vivo in this study because of limited light penetration through surgery-induced scar tissue. In this first proof-of-concept study, AVF function was investigated in normal mice without uremia. Evaluation of the AVF endothelial injury response in uremic mice will be of substantial interest in future investigations. Confocal IVM has limited depth sensing of ≤200 μm, restricting in vivo insights into AVF to the top arc of murine AVF. In contrast, ex vivo fluorescence microscopy can image the full depth of the fluorescence signals in the vessel, which may explain a moderate rather than strong correlation between in vivo and ex vivo CLIO-VT680 signal intensities. Multiphoton IVM approaches may allow deeper high-resolution imaging and could help address this issue and also allow venous circuit imaging. Although this study did not probe directly possible mechanisms of modified endothelial function, altered endothelial shear stress influenced by AVF geometry could contribute to the development of dysfunctional endothelium after AVF creation.

In conclusion, pathological endothelium in murine AVF can be assayed in vivo by fluorescence and MRI of dextranated nanoparticle deposition. The extent and location of abnormal AVF endothelium predicted the local degree of NH formation. Such imaging approaches offer the ability to predict the likelihood and even the specific location of inflow stenosis and further provide a theranostic framework to assess therapies aiming to restore endothelial health and reduce primary AVF failure.

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Disclosures
None.

References
Arteriovenous fistulae (AVFs) or grafts are the lifelines for 1.5 million end-stage renal disease patients receiving dialysis. Dysfunctional vascular access disrupts dialysis treatment and is associated with higher mortality rates. Notably, the primary failure rate of AVF reaches 30% to 50%, and current clinical modalities do not accurately predict access failure. Hence, we developed nanoparticle-based fluorescence and molecular imaging approaches to image pathological endothelium, a key driver of AVF failure. We found that the intensity of nanoparticle deposition further predicted the degree of endothelial pathology and was highest at the AVF anastomosis. Nanoparticle deposition further predicted the failure of local neointimal formation and inflow stenosis within maturing AVF. The overall results provide a framework to assess genetic and pharmacological manipulations of endothelial biology and their in vivo effects of neointimal formation in AVF. Nanoparticle imaging of pathological endothelium also offers a translational MRI strategy to identify human AVF prone to early failure.
In Vivo Nanoparticle Assessment of Pathological Endothelium Predicts the Development of Inflow Stenosis in Murine Arteriovenous Fistula

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Materials and Methods

Murine arteriovenous fistula (AVF) creation
All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Massachusetts General Hospital and conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. Male C57BL/6 mice used in this study were 12–16 weeks old (Jackson Laboratory, Bar Harbor, ME). AVF were created by end-to-side anastomosis of the ipsilateral carotid artery (ICA) to the jugular vein (Supplemental Figure S1).\textsuperscript{1,2} Briefly, on day 0, mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (12 mg/kg). A midline skin incision was made, and the jugular vein and carotid artery were exposed by blunt dissection. The side branches of the inferior jugular vein were ligated using 7-0 silk sutures. The carotid artery was mobilized from surrounding tissue and fascia, and carotid blood flow was measured (Pre-AVF) by Doppler ultrasound (Transonic System Inc. TS420, Ithaca, NY). The carotid artery was then ligated at the level of bifurcation using 7-0 silk suture. Next, a clamp was placed on the proximal portion of the carotid artery and the carotid artery was cut below the ligation. The jugular vein was clamped proximally and distally to perform a venotomy of 0.70 mm in length.\textsuperscript{1} Then the vein and arterial segments were flushed with heparinized saline, and an end-to-side anastomosis was performed with 10-0 interrupted sutures. AVF blood flow was measured at baseline and then at 10 minutes post-AVF creation (Supplemental Figure 1A). Then the sham surgery was done on the left side using blunt dissection of the left carotid artery and jugular vein. The surgical incision was then closed using 6-0 interrupted sutures. The mouse was then allowed to recover and then returned to the animal facility.

Molecular imaging of pathological endothelium
Pathological endothelium in AVF was imaged using magnetofluorescent dextranated iron oxide nanoparticles, detectable by both fluorescence and MR imaging modalities.\textsuperscript{3-7} Cross-linked iron oxide nanoparticles (“CLIO”) were obtained from the Center for Systems Biology Chemistry Core at Massachusetts General Hospital. CLIO nanoparticles were rendered fluorescent after conjugation with VivoTag 680 (\(\lambda_{\text{max}}\) absorption=670 nm; \(\lambda_{\text{max}}\) emission=688 nm; VT680, PerkinElmer, Waltham, MA)\textsuperscript{7}. The final nanoparticle, termed CLIO-VT680 (10 mg/kg) was injected intravenously 24 hours before intravital microscopy or MRI.

En face confocal microscopy imaging of endothelial morphology
At day 14 post-AVF creation, three mice that received CLIO-VT680 were sacrificed. AVF vessels and their respective contralateral carotid artery were dissected and incubated in 2 \(\mu\)g /ml Hoechst for 10 minutes (Supplemental Figure 1B). Tissues were then washed in sterile PBS for 1 minute at room temperature. Then tissue samples were mounted on microscope glass slides, and images were taken by confocal microscopy (Nikon A1R, Japan).

Assessment of endothelial permeability
To investigate the integrity of the endothelium, 50µl of 0.5% Evan’s blue was injected into three mice (n=3) with day 14 AVF (Supplemental Figure 1C). Mice were sacrificed 30 minutes after Evans blue injection. Light images were taken after AVF and contralateral carotid artery were resected. Epifluorescence microscopy (Eclipse 90i, Nikon) of cut axial sections of the AVF and contralateral carotid artery were performed in the Evans Blue (ex/em 650/710nm) and FITC channels (ex/em 490/520nm), as previously.

Transmission electron microscopy imaging
To determine the cellular localization of CLIO-VT680 within the AVF intima, day 14 AVF mice were sacrificed 24 hours after CLIO-VT680 injection (Supplemental Figure 1D). Tissues were fixed in 2.0% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 (Electron Microscopy Sciences, Hatfield, PA) overnight at 4 C. They were rinsed in buffer, post-fixed in 1.0% osmium tetroxide in cacodylate buffer for one hour at room temperature, rinsed in buffer again and dehydrated through a graded series of ethanol to 100%, followed by 100% propylene oxide. Tissues were then infiltrated with Epon resin (Ted Pella, Redding, CA) in a 1:1 solution of Epon:propylene oxide overnight at room temperature on a rocker. The following day they were placed in fresh Epon overnight at 60 C. Sections of 70 nm thickness were cut on a Leica EM UC7 ultramicrotome, collected onto formvar-coated grids, stained with uranyl acetate and lead citrate and examined in a JEOL JEM 1011 transmission electron microscope at 80 kV. Images were collected using an AMT digital imaging system (Advanced Microscopy Techniques, Danvers, MA).

Histology, immunohistochemistry, and fluorescence microscopy
At the time of sacrifice, tissues were perfused with PBS, and AVFs were harvested. Samples were immediately flash frozen in optimal cutting temperature (OCT, Tissue-Tek) medium. Serial cryosections (6 µm thick) were obtained from the AVF anastomosis towards the proximal of the carotid artery to generate axial sections. Hematoxylin and eosin (H&E) staining was performed, and elastin was visualized via modified Verhoeff-Van Gieson Elastin stain. The neointimal hyperplasia area was defined as the area between the lumen and the internal elastic lamina. Additional sections were double stained for endothelium marker CD31 (1:100, BD Pharmigen) and the smooth muscle cell (1:400, Abcam) by immunofluorescence. The secondary fluorescent-labeled antibody Cy3-donkey anti-rat (1:800, Jackson ImmunoResearch Laboratories, INC) and Dylight 405-donkey anti-rabbit (1:400, Jackson ImmunoResearch Laboratories, INC) were applied. Then fluorescence images were obtained by confocal microscopy (Nikon A1R, Japan). For immunohistochemistry, sections were stained for the macrophage marker CD68 (1:1000, BD Pharmigen), and VCAM-1 (1:200, Abcam). The appropriate biotinylated secondary antibodies were applied, and Vectastain ABC kit (Vector Laboratories, INC., Burlingame, CA) was used for color development. All sections were counterstained with hematoxylin.

The intensity and distribution of CLIO-VT680 was assessed by whole section micrographs capture via epifluorescence microscopy (Nikon 90i, Japan) as performed previously. ROIs were used to calculate mean signal intensity (MSI) of CLIO-VT680.
signals, and were defined as an area 20 µm inside and 20 µm outside the internal elastin lamina. The MSI of CLIO-VT680 of each section was measured after encompassing the ROI. The TBR was calculated as MSI of the AVF divided by the MSI of the contralateral artery.

**IVM Study protocol**
Two IVM studies were performed (n=18 mice in total). CLIO-VT680 was injected 24 hours prior to the IVM study. In the first IVM imaging study, mice underwent imaging at day 14 (n=9) or day 21 (n=4) after AVF creation, followed by euthanasia and histological assessment (Supplemental Figure 1E). In a second IVM study to determine how pathological endothelium preceded the development of neointimal hyperplasia, mice underwent survival IVM imaging on day 14 (n=5, three with one batch of CLIO-VT680, two with a second batch of CLIO-VT680), and then were euthanized on day 42 for matched histological assessment of neointimal hyperplasia (Supplemental Figure 1F).

**IVM imaging of pathological endothelial cells**
On day 14 or 21 after AVF creation, mice were imaged with a custom-built confocal multichannel intravital microscope. All mice were intravenous injected with CLIO-VT680 24 hours before imaging. After anesthesia with ketamine and xylazine, AVFs were exposed. Fluorescein-labeled dextran (FITC-dextran, 0.25 mg in 50µl PBS, molecular weight 2,000,000, Sigma Chemical, St. Louis, MO), employed for angiography, was excited with a 491nm solid-state laser (Dual Calypso, Cobolt AB, Solna, Sweden) and detected with a photomultiplier tube (PMT) through a 528±19 nm bandpass filter (FF01-528/38-25, Semrock, Rochester, New York). CLIO-VT680 was excited with 635 nm generated by a Helium-Neon laser (Radius, Coherent Inc, Santa Clara, CA), and detected through a 695 ± 27.5 nm bandpass filter (XF 3076695AF55, Omega Optical, Brattleboro, Vermont). Collagen in the arterial wall was imaged by second harmonic generation (SHG) signals generated by a femtosecond pulsed laser source at 840nm (200mW) (Mai Tai HP, Spectra-Physics, Irvine, California). The AVF was imaged with a 30x, N.A.=0.9, water-immersion microscope objective lens (EAF-30-1, LOMO, Northbrook, IL), giving a field of view of 714 µm by 714 µm. The contralateral sham-operated carotid artery was also imaged and utilized as the background in TBR calculations.

**IVM image acquisition and analysis**
IVM datasets were collected as coronal z-stacks of the AVF anastomosis area with each single z-plane comprised of the mean of 6 frames, collected at 30 frames per second (fps). For each z-stack, a stepsize of 2 µm was utilized with a maximum of 130 slices being acquired. For image analysis, coronal Z-stacks were re-sliced into the axial plane. ROIs were identified as the area of positive CLIO-VT680 signals in the vessel wall as demarcated by SHG collagen signals. The MSI for a given distance away from the anastomosis (MSI_{avf}) was calculated as the average MSI across 7 images centered at the given distance, representing a 10 µm length segment. The TBR was measured on reconstructed axial images every 60 µm starting from the anastomotic site towards the proximal aspect of the carotid artery. The
contralateral carotid artery served as the control. All image analysis was performed using NIH ImageJ software.

**Fluorescence reflectance imaging (FRI) and magnetic resonance imaging (MRI)**

Mice underwent ex vivo FRI studies. 24 hours after CLIO-VT680 (n=3) or PBS injection (n=2) (Supplemental Figure 1G). Resected day 14 AVF tissues were rinsed in sterile PBS, and placed on the stage of an FRI system (Kodak Image Station 4000MMPro, Carestream Health Inc, New Haven, CT). Optical images at 630/700nm excitation/emission were collected with 8-second exposure time. Then the same AVF tissues were placed on top of an agar plate, immersed in 4% paraformaldehyde and imaged by a 7.0T high-resolution MRI scanner (Bruker, Billerica, MA). After a series of scout images, T2 weighted rapid acquisition with refocused echoes (RARE) T2 images were obtained using the following parameters: slice thickness 0.5 mm, in-plane resolution 0.21 X 0.15mm; TE 12 msec (effective TE 36msec), TR 2000 msec. The total imaging time was <1 hour. MRI images were analyzed with Image J software (V1.cc, NIH). Signal intensities were measured by using a manual ROI tracing tool. The ROI for MRI data was defined by a 400 µm arterial segment adjacent to the anastomosis. The signal-to-noise ratio (SNR) was calculated as follow: mean signal intensity of ROI / standard deviation of the noise.

**Statistical analysis**

All results are reported as mean ±SEM. Correlation coefficients were determined by calculating the Pearson correlation coefficient (r). A p value < 0.05 was considered statistically significant. All statistical analyses were performed using Prism (V 5.0c, GraphPad, La Jolla, CA).
References


Supplemental Figure I. Study protocol for all in vivo and histological studies.
SUPPLEMENTAL FIGURE II

**Supplemental Figure II.** Creation of AVF in mice. **A.** AVFs were created in C57BL/6 mice by mobilizing the carotid artery and then anastomosing it end-to-side to the ipsilateral jugular vein (SJV: superior jugular vein; IJV: inferior jugular vein; CA: carotid artery. The dotted box shows the centered imaging field during IVM studies. **B.** The carotid artery blood flow measured by Doppler ultrasound in mice (n=8) increased 7-fold after AVF creation (*p < 0.01). Scale bar, 1 mm. Error bars depict the S.E.M.
Supplemental Figure III. Representative axial intravital microscopy (IVM) images of day 14 murine AVF. A. The section was 120µm away from the anastomosis. CLIO-VT680 (red) localized below adventitial arterial wall collagen (SHG, Blue). B. FITC-dextran, a blood pool agent outlined the arterial lumen (green). C. Fusion image demonstrates that CLIO-VT680 signals are within the imaging illumination field on confocal microscopy. Pulsatile motion artifact likely explains why CLIO-VT680 appears to colocalize with the lumen (green), while in reality localizing to the intima as shown in figure 2. Scale bar, 100µm. SHG, second harmonic generation.
Supplemental Figure IV. Additional examples of week 2 CLIO endothelial IVM signals and the corresponding week 6 neointimal hyperplasia (NH) from matched sections. The confocal IVM images assess approximately the top 1/3rd of the vessel. (A, B) AVF examples show that the week 2 IVM CLIO signals and week 6 neointimal patterns are roughly similarly asymmetric. (C) An AVF example with more symmetric week 2 IVM CLIO signals and week 6 neointimal hyperplasia. Red=CLIO-VT680 (pathological endothelium); blue=second harmonic generation signal (SHG, type I collagen). IVM images were processed and windowed identically. IVM Scale Bar: 100µm.