

Detection of dual-gene expression in arteries using an optical imaging method

Hunter H. Chen

Johns Hopkins University
Department of Biomedical Engineering
Baltimore, Maryland 21205

Xiangcan Zhan

Johns Hopkins University School of Medicine
Department of Radiology
and
Department of Oncology
Baltimore, Maryland 21205

Ananda Kumar

Xiangying Du

Johns Hopkins University School of Medicine
Department of Radiology
Baltimore, Maryland 21205

Holly Hammond

Linzhao Cheng

Johns Hopkins University School of Medicine
Department of Oncology
Baltimore, Maryland 21205

Xiaoming Yang

Johns Hopkins University School of Medicine
Department of Radiology
Traylor Building, #330
720 Rutland Avenue
Baltimore, Maryland 21205
E-mail: xyang@mri.jhu.edu

Abstract. We evaluate the *in vivo* use of an optical imaging method to detect the vascular expression of green fluorescent protein (GFP) or red fluorescent protein (RFP), and to detect the simultaneous expression of GFP and RFP after transduction into arteries by a dual-promoter lentiviral vector driving their concurrent expression. This method involves using a charge-coupled device camera to detect fluorescence, a fiber optic probe to transmit light, and optical filters to distinguish each marker. In animal models, these vectors are locally delivered to target arteries, whereas the gene for a nonfluorescent cell-surface protein is transduced into contralateral arteries as the sham control. The images show distinct areas of bright fluorescence from GFP and RFP along the target arteries on excitation; no exogenous fluorescence is observed in the controls. Measured signal intensities from arteries transduced with the single- and dual-promoter vectors exceed the autofluorescence signal from the controls. Transgene expression of GFP and RFP *in vivo* is confirmed with confocal microscopy. We demonstrate the use of an optical imaging method to concurrently detect two distinct fluorescent proteins, potentially permitting the expression of multiple transgenes and their localization in the vasculature to be monitored. © 2004 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.1803842]

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1 Introduction

Gene therapy has shown great promise for the treatment of cardiovascular disease such as atherosclerosis. Strategies for vascular gene therapy include preventing thrombosis, reducing postangioplasty restenosis, and promoting angiogenesis.^{1–4} The success of gene therapy is critically dependent on high gene transfer efficiency and sufficient gene expression.^{5,6} The choice of gene delivery vectors is dependent on the specific target cells and the period of time the gene is to be actively expressed. Replication-incompetent viruses use their native machinery to infect cells and transfer exogenous genes, but cannot produce additional progeny viruses. They can be produced at high concentrations and have generally high transduction rates.¹ Lentiviruses that integrate exogenous genes into the host genome are advantageous if sustained transgene expression is desired.

Proteins that emit fluorescence on excitation have proven to be useful in detecting gene expression. Specifically, the fluorescent properties and formation of green fluorescent protein (GFP) exhibited no dependence on other gene products.^{7,8} This inherent fluorescence has been an important advantage of GFP and other fluorescent proteins, which have been widely

used as a biological marker for the detection of gene expression and for protein localization.^{6,8–12} Furthermore, the emission wavelength is independent of the excitation wavelength,¹³ allowing the emission from fluorescent proteins to be isolated by optical filtering. The absorption and emission spectra of various fluorescent proteins allow for selective excitation and detection by using appropriate optical filters.

Several imaging modalities including optical imaging have been used for detecting gene expression, but most have focused on nonvascular systems. Currently, experiments that have used optical imaging and GFP for monitoring gene expression have been primarily focused on the eye, tumors, and respiratory tract.^{10,11,14} A previous study demonstrated the feasibility of using optical imaging to detect GFP expression in vascular smooth muscle cells *in vitro* and in arterial tissue *ex vivo*.⁹ A recent study also reported success in creating a lentiviral vector, with independent promoters driving the simultaneous expression of two distinct genes, GFP and red fluorescent protein (RFP, encoded by the DsRed2 gene).¹⁵ Hence, the combination optical imaging and a dual-promoter vector encoding separate fluorophores may yield a method that addresses the need for *in vivo* detection and localization of gene expression in the vasculature. The objective of this current study was to evaluate the *in vivo* use of an optical imaging method to detect the vascular expression of GFP or RFP after

Address all correspondence to Xiaoming Yang, Johns Hopkins Univ. School of Medicine, Department of Radiology, Traylor Building, #330, 720 Rutland Ave., Baltimore, MD 21205. Tel: 410-502-6960; Fax: 443-287-6730; E-mail: xyang@mri.jhu.edu

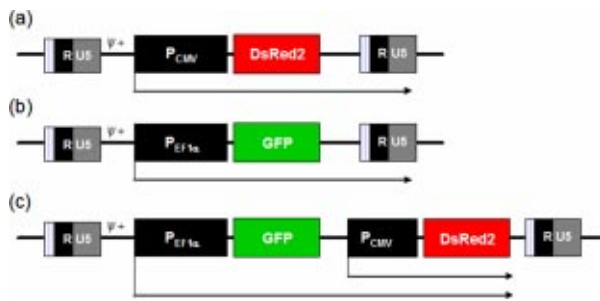


Fig. 1 Schematic diagram of the single- and dual-promoter vectors used in this study. (a) Expression of RFP, encoded by the DsRed2 gene, was controlled by P_{CMV}, the CMV immediate early promoter. (b) Expression of GFP was controlled by the internal promoter P_{EF1α}, which is the promoter for the human EF1α housekeeping gene. (c) Dual-promoter vector having P_{EF1α} driving expression of GFP, followed by P_{CMV} driving expression of RFP. R and U5 are the repeat and U5 regions of the viral long-terminal repeat, respectively. Transcripts from these vectors are denoted by arrows.

gene transfer with a single-promoter vector, and to detect the simultaneous expression of GFP and RFP after transduction into arteries by a dual-promoter lentiviral vector driving their concurrent expression.

2 Materials and Methods

2.1 Gene Vector Preparation

A replication-incompetent recombinant lentivirus was generated using the three-plasmid system by cotransfection of 293T cells through a lipofectamine method. In addition to the transducing vectors for GFP (EF.GFP) only, RFP (CMV.DsRed2) only, or the dual-gene GFP/RFP vector (EF.GFP-CMV.DsRed2) (Fig. 1), two help vectors were used in the viral production. GFP and RFP expression were controlled by the promoter of the human elongation factor (EF) 1α housekeeping gene and the human cytomegalovirus (CMV) promoter, respectively. One was pMD.G, expressing the vesicular stomatitis virus, G-envelope protein, and another was pCMVΔR8.91, containing the HIV-1 gag/pol, tat, and rev genes required for efficient lentivirus production.¹⁶ The ratio of EF.GFP-CMV.DsRed2 or EF.GFP or CMV.DsRed2, pMD.G, and pCMVΔR8.91 was fixed at 1.5:0.5:2 μg for 10⁶ 293T cells plated in a 35-mm well.¹⁵ Viruses were harvested at 48 and 72 h after transfection and titered based on the percentage of GFP⁺ cells after transduction with serially diluted viral supernatant. The titers calculated as transduction units (TUs) per milliliter of supernatant were usually in the range of 1 × 10⁶ to 10 × 10⁶ TUs per ml. The evaluation of transduction efficacy was carried out by adding 2 ml of virus supernatant to 10⁵ vascular endothelial cells (ECs) in a six-well plate in the presence of polybrene (8 μg/ml). To quantify the transduction efficacy of the dual-promoter lentivirus, the transduced and wild-type ECs were analyzed with flow cytometry (CellQuest, Becton Dickinson, San Jose, California) as previously described.¹⁵ Fluorescent microscopy of GFP and RFP expression was also performed using a Nikon (TE300) microscope fitted with excitation and emission filter sets for GFP (excitation 450 to 490 nm; emission 500 to 550 nm) and RFP (excitation 515 to 575 nm; emission 535 to 685 nm).¹⁵

2.2 Gene Delivery

2.2.1 Surgery-based approach in a rabbit

To test the utility of the optical imaging system for detecting RFP from vessel walls, we used a New Zealand white rabbit, approximately 4 kg in weight. Under general anesthesia, a 1.5-cm-long segment of the right carotid artery was isolated proximally and distally with two Sentinel loops (Sherwood Medical, Saint Louis, Missouri) via an arteriotomy approach. Blood in the arterial lumen was removed by inserting a 24-gauge catheter (Quik-Cath, Baxter, Marion, North Carolina) into the isolated segment. Then, approximately 0.5 ml of the single promoter RFP-plasmid solution was injected through the catheter and allowed to remain in the arterial lumen for 1 h. The left carotid artery was not transferred to serve as the negative control. The animal was kept alive for five days afterward to allow for peak RFP expression.

2.2.2 Catheter-based approach in pigs

To validate the feasibility of using the optical imaging system to detect the expression of GFP ($n=2$) from the single-promoter vector, as well as the simultaneous expression of GFP and RFP from the dual-promoter vector ($n=3$), we used five domestic pigs, 15 to 20 kg in weight. To minimize the invasiveness of the gene transfer, we used a catheter-based approach to locally deliver GFP- or GFP/RFP-lentiviral vectors to the femoral arteries of the pigs. The animals were sedated with an intramuscular injection of ketamine (22 mg/kg body weight; Fort Dodge Animal Health, Fort Dodge, Iowa), acepromazine (1.1 mg/kg; Fermenta Animal Health, Kansas City, Missouri), and atropine (0.05 mg/kg; American Regent Laboratory, Shirley, New York). A vein in the ear was cannulated to permit hydration with sterile saline. Pentobarbital (20mg/kg; Abbott Laboratory, North Chicago, Illinois) was later administered intravenously to bring the animal to a surgical plane of anesthesia. The animals were intubated and mechanically ventilated with 1.5 to 2.0% isoflurane (Ohmeda Incorporated, Liberty Corner, New Jersey). The animals were also heparinized (100 IU/kg) to achieve an activated partial thromboplastin time greater than 80 s. A 9F introducer was cannulated into the aorta through the right carotid artery. To obtain an angiogram of the pelvic and femoral arteries, a 4F pigtail angiography catheter was then positioned into the abdominal aorta, and 20 ml of 60% diatrizoate meglumine (Hypaque, Nycomed Incorporated, Princeton, New Jersey) was injected at a flow rate of 10 ml/s [Fig. 2(a)].

An appropriate segment (2 cm in length and 3.0 to 3.5 mm in diameter) of the left femoral artery was selected for gene transduction. A balloon dilatation infusion gene delivery catheter (Remedy, Boston Scientific, Boston, Massachusetts) [Fig. 2(c)] was placed at the target segment where 1.5 ml of supernatant containing GFP- or GFP/RFP-lentiviral vectors were infused into the arterial wall at a flow rate of 10 ml/s [Fig. 2(b)]. The diameter of the inflated balloon ranged from 3.5 to 4.0 mm, and the ratios (diameter of the target artery:diameter of the inflated balloon) were 3.0:3.5 mm and 3.5:4.0 mm. Choosing a balloon with a slightly greater diameter than the artery ensured proper infusion of the gene vectors into the arterial wall while minimizing arterial wall damage. The corresponding segment of the contralateral femoral artery served

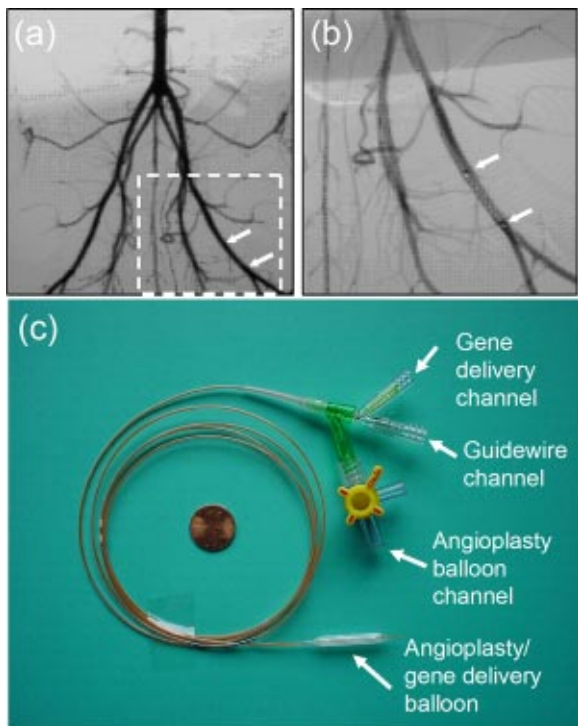


Fig. 2 (a) A digital subtraction angiogram of the bilateral iliac and femoral arteries of a pig. (b) Corresponding to the dashed box in panel (a), the arrows indicate the location of the two metal markers delineating the length of the balloon of the gene delivery catheter and the 2-cm segment of the left femoral artery to be transduced with the single- or dual-promoter lentivirus. The corresponding segment of the contralateral artery was transduced with lentivirus carrying the gene for Surfin, a nonfluorescent surface protein gene, as the sham control. (c) A balloon dilatation infusion gene delivery catheter was used to locally deliver lentiviral vectors into the femoral artery. Separate channels in the catheter were used for the guidewire, balloon angioplasty, and gene delivery.

as the sham control and was transduced with a lentiviral vector containing an irrelevant gene encoding a nonfluorescent cell surface receptor (Surfin) developed in our laboratory.

All animals were treated according to the “Principles of Laboratory Animal Care” of the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* (NIH, number 80-23, revised 1985). All experimental protocols were approved by our institution’s Animal Care and Use Committee.

2.3 Optical Imaging

The optical imaging system consisted of a charge-coupled device (CCD) camera (SensiCam QE, Cooke Corporation, Auburn Hills, Michigan), with a detection area of 1376×1040 pixels and a pixel size of $6.45 \times 6.45 \mu\text{m}^2$, coupled to a series of lenses (25 mm f/0.95, Fuji Photo Optical, Japan, and 50 mm f/1.8, Nikon, Japan), and a fiber optic ring-light probe (TransLite, Sugar Land, Texas) [Fig. 3(a)]. The ring-light probe was comprised of an open cylindrical housing that encased 1-mm-diam fibers oriented at a 45-deg angle toward the central axis of the probe from normal. These fibers transmitted excitation light from a 250-W broadband halogen source (Schott Group, Germany) that was passed through a

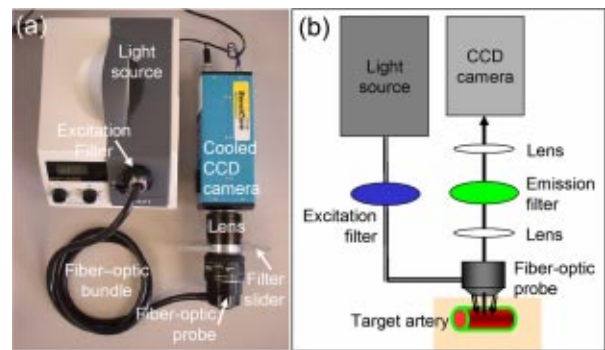


Fig. 3 (a) The optical imaging system consisted of a halogen light source, cooled CCD camera, optical lenses, excitation and emission filters, filter slider, fiber optic bundle, and fiber optic ring-light probe. (b) Diagram of the optical imaging system. Broadband light was passed through an excitation filter before being transmitted through the probe, which was fixed in a position in direct contact with the artery during image acquisition. Fluorescence was passed through an emission filter and detected by the CCD camera. The choice of excitation and emission filters was dependent on the fluorophore of interest.

bandpass filter appropriate for GFP excitation (460 to 490 nm; XF1072, Omega Optical Incorporated, Brattleboro, Vermont) or RFP excitation (530 to 560 nm; HQ545/30, Chroma Technology Corporation, Brattleboro, Vermont). The incident irradiance rate reaching the 1.2-mm-diam field of view was measured by a power meter and determined to be 40 mW/cm^2 . Fluorescence was collected through the open cylindrical center of the probe and passed through a separate bandpass filter selective for GFP emission (500 to 530 nm; XF3080, Omega Optical Incorporated) or RFP emission (570 to 640 nm; HQ610/75, Chroma Technology Corporation) before reaching the CCD camera. The excitation and emission filters were selected so that the excitation maxima (λ_x) and emission maxima (λ_m) of GFP ($\lambda_x = 489 \text{ nm}$; $\lambda_m = 508 \text{ nm}$) and RFP ($\lambda_x = 558 \text{ nm}$; $\lambda_m = 583 \text{ nm}$) fell within the narrow range of wavelengths passed by the filters. Images were digitally captured using software (IPLab, Scanalytics Incorporated, Fairfax, Virginia) with scripts written in our laboratory to acquire images at user-specified time intervals.

Optical imaging of the target and control arteries was performed when the expression of GFP and RFP reached its peak at 5 days posttransduction. To precisely locate the target arterial segments for imaging, we previously established two distinct markers. First, on conventional angiography, we transduced a 2-cm unbranched portion of the femoral artery, just distal to a branch of the artery. According to the location of this branch, we could easily localize the target segment of the artery when exposed surgically. Second, we made two surface markers with sutures on the skin. The sutured spots were marked along the target arteries under x-ray fluoroscopy and served as a surface guide for exposing the vessel.

A surgical incision was carefully made to expose the target vessel such that the fiber optic probe could be placed in direct contact with the transferred portion of the vessel. Once the probe was placed in the desired position, a tripod stand fixed the position of the imaging system during image acquisition. The probe was placed directly onto the target vessel, and using the excitation and emission filters selective for GFP or

RFP, a series of images was captured for exposure times ranging from 1 to 5 s. The custom filter slider, fabricated in our laboratory, allowed us to change the emission filter while keeping the position of the camera fixed and the field of view constant. This surgical and imaging procedure was repeated with the control artery. On completion of imaging, the animals were immediately euthanized. The target arteries and corresponding control arteries were harvested and imaged using confocal microscopy to confirm the expression of GFP and RFP. All animals survived without abnormal clinical manifestations, such as ischemia or necrosis in the extremities.

From the images obtained *in vivo*, the mean signal intensity within a selected region of interest (ROI) along the vessel wall was measured. Three investigators selected ROIs independently to cover the target vessel segment, and efforts were made to maximize the size of the ROIs while avoiding extraneous structures. Images collected at 3- and 5-s exposures were used as representative images of the single-fluorophore and dual-fluorophore experiments, respectively, and used for comparison. These time points were chosen because fluorescence was clearly detected, and the boundaries of the arterial wall were easily identifiable, allowing the placement of the ROI and measurement of signal intensities to be more accurate and consistent. Image analysis was performed using IP-Lab software (Scanalytics Incorporated, Fairfax, Virginia). Values of signal intensity are means \pm standard deviation and in arbitrary units (au).

2.4 Confocal Microscopy

Confocal microscopy was used to confirm the expression of GFP and RFP. Paired segments of the transduced and control arteries from each experiment were harvested and immediately mounted onto slides with the intimal layer exposed for imaging with a confocal microscope (LSM 410, Zeiss Group, Thornwood, New York). Briefly, GFP and RFP emissions at 510 to 560 nm and >570 nm, respectively, were filtered and detected by cooled photomultiplier tubes (PMT) in response to excitation by a 488-nm argon laser for GFP and a 543-nm HeNe laser for RFP. The excitation beam for GFP was passed through a short-pass 510-nm dichroic beamsplitter, while for RFP, a short-pass 560-nm dichroic beamsplitter was used. Images were collected through an inverted microscope with a C-Apo X40 water-immersion objective lens (NA=1.2). Eight line averages were used to reduce noise.

We performed a total of 18 (nine paired) optical imaging and confocal microscopy examinations, including: one pair from a rabbit transferred with RFP plasmid, two pairs from pigs transduced with the single-promoter GFP lentivirus, and six pairs from pigs transduced with the dual-promoter GFP/RFP-lentivirus.

3 Results

The animal studies involving only a single fluorophore, either GFP or RFP, were used to evaluate the gene transfer and optical imaging methodology. From the images obtained *in vivo* by the optical imaging system, the measured signal intensities for the GFP- or RFP-transferred artery were greater than their respective controls [Fig. 4(a)]. The measured signal intensity value was 31.3 ± 12.2 for GFP-transduced arteries

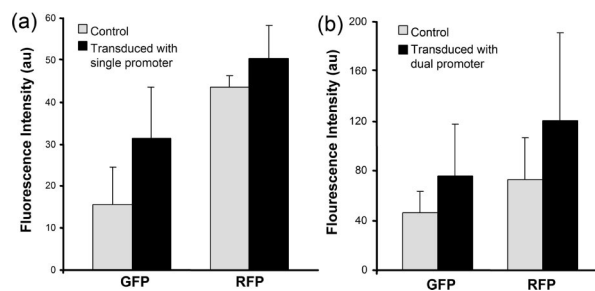


Fig. 4 (a) Measured signal intensities from images obtained *in vivo* by the optical imaging system. The mean values for GFP and RFP fluorescence from the transduced arteries were greater than their respective controls. The arteries were transferred with single-promoter vectors encoding the GFP or RFP gene only. The signal intensities for GFP-transduced and RFP-transferred arteries were higher than their respective control arteries. This increased intensity indicated a positive expression of GFP and RFP and successful detection by the optical imaging system. (b) Similarly, the mean values for GFP and RFP fluorescence measured from arteries transduced with the dual-promoter lentivirus exceeded their respective controls. In these experiments, fluorescence from GFP and RFP were detected from the same arteries. Efficient gene transfer and simultaneous expression of both fluorophores with the dual-promoter lentivirus were evident from these results.

compared to 15.5 ± 9.1 for the control. Similarly, the signal intensity for the RFP-transferred artery was 50.3 ± 8.1 compared to 43.6 ± 2.8 . This increased intensity indicated a positive gene transfer and expression as well as successful detection by the optical imaging system. Confocal imaging also confirmed the expression of GFP and RFP in harvested sections of the transferred artery. Furthermore, the results of the GFP transduction showed approximately twice the measured signal compared to the control, while RFP generated an enhancement of 15%. This difference in performance may be due to the different animal models or gene vectors used. The lentivirus may have been more effective than plasmid in transferring genes into the cells.^{1,17} Further, the catheter-based gene delivery was more efficient and less invasive, which permitted us to deliver higher titers of lentivirus to the target artery in comparison with the surgery-based approach. This catheter-based method was also more reproducible and less surgically complex.

With this delivery strategy in mind, we developed a lentiviral vector carrying both GFP and RFP genes. To quantify the transduction efficacy of the dual-promoter lentivirus, transduced and wild-type ECs were analyzed with flow cytometry. *In vitro* studies with flow cytometry showed that 82.7 and 77.8% of the gated population of cells were positive for GFP and RFP expression, respectively, 48 h after transduction [Fig. 5(a)]. The cells were successfully transduced, and both promoters were stably driving expressions of both genes. To further assess the concurrent expression of these fluorescent proteins, a monolayer of the transduced cells (2.5 to 3.5×10^6 cells/ml) was imaged with a fluorescence microscope. The images showed that fluorescence from both GFP and RFP were originating from the same cells [Fig. 5(b)]. These data suggest that the dual-promoter vector was being efficiently transferred and that both fluorescent proteins were functional and being expressed concurrently from individual cells.

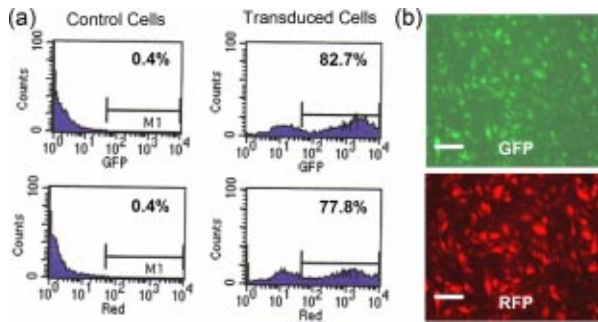


Fig. 5 (a) Flow cytometry results of vascular endothelial cells transduced with the dual-promoter lentiviral vector encoding both GFP and RFP. 82.7 and 77.8% of the gated population of cells were positive for GFP and RFP expression, respectively, but not for control cells in the M1 region, which was selected on the basis of the negative control fluorescence ($\leq 1\%$ GFP- or RFP-positive cells). (b) Fluorescence microscopy of a monolayer of transduced cells (2.5 to 3.5×10^6 cells/ml) showed that both GFP and RFP were being concurrently and stably expressed in the same cells. Fluorescence from GFP and RFP expressed within cells was clearly observed, illustrating the functionality of the dual-promoter lentiviral vector construct. (Scale bar: $20 \mu\text{m}$.)

For the application of the dual-promoter vector *in vivo*, porcine models were used, since higher titers of lentivirus could be delivered through the gene delivery catheter. Five days after gene delivery, the transduced and control arteries were imaged using the optical imaging system. While the probe was fixed in direct contact with the target artery, images of the same artery were collected using the excitation/emission filter set for GFP, and then followed by the filter set for RFP. The *in vivo* images showed that fluorescence from both GFP and RFP were detected in the target arteries by optical imaging and observed as distinctly bright areas along the artery (Fig. 6). The measured signal intensities from the arteries transduced with the dual-promoter vector were 75.8 ± 41.8 for GFP and 120.9 ± 70.7 for RFP [Fig. 4(b)]. These

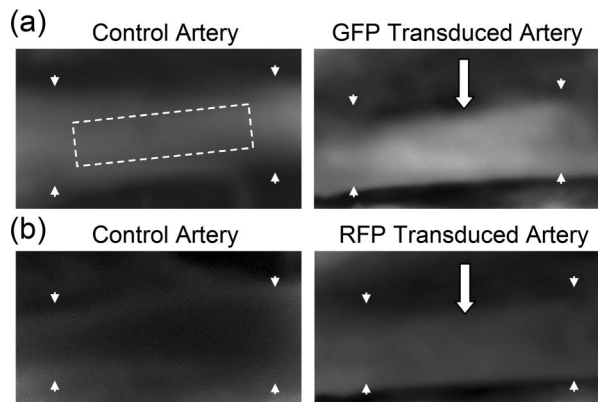


Fig. 6 Images taken by the optical imaging system of the control artery transduced with the Surfin gene and the corresponding artery transduced with the dual-promoter vector. The distinctly bright areas of fluorescence (block arrows) along the target artery was representative of (a) GFP and (b) RFP expression. The same artery was imaged using filters specific for GFP and followed by filters for RFP. Autofluorescence was detected in the control artery. Mean signal intensity values were measured within the region of interest (dashed box).

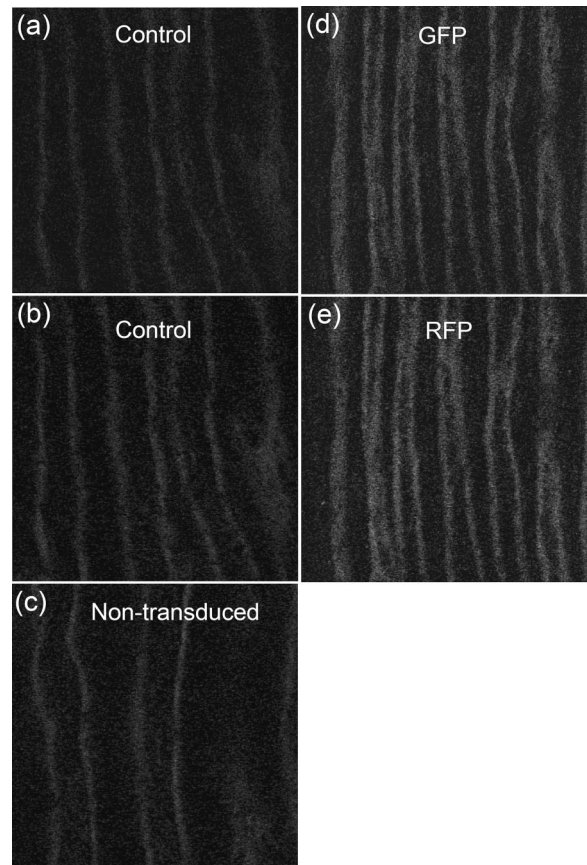


Fig. 7 Confocal microscopy images of (a) and (b) control arteries and (c) and (d) arteries transduced with the dual-promoter lentivirus encoding GFP and RFP. In (d) and (e), fluorescence from GFP and RFP were detected, indicating a functional gene transfer and the subsequent expression of both fluorophores mediated by the dual-promoter lentivirus. No exogenous fluorescence was observed with the Surfin-transduced control tissues (a) and (b) compared to (c) the negative or nontransduced tissue, confirming that Surfin did not affect the autofluorescence signal of normal arterial tissue.

values were greater than the signal intensities of autofluorescence from their corresponding controls, which were 46.5 ± 17.2 and 72.7 ± 34.1 , respectively. Hence, transduction with the dual-promoter lentivirus increased the mean signal by 63 and 66% for GFP and RFP, respectively. This indicated that the catheter-based delivery was an efficient gene transfer technique, and that the promoters of the vector were stably expressing both fluorophores *in vivo*. To ensure that the sham control, where Surfin was transduced, did not affect autofluorescence levels, an untreated or normal segment of arterial tissue proximal to the Surfin-transduced segment was harvested to serve as the negative control. Confocal images of the sham control and negative control showed no discernable differences between them (Fig. 7). Thus, the expression of the Surfin gene did not affect the autofluorescence characteristics of the control.

Confocal microscopy was used to verify the expression and functionality of GFP- and RFP in the transduced arterial wall. Images obtained from the confocal microscope showed that GFP and RFP-derived fluorescence were detected from the same tissue for eight of the nine-paired cases. For the

single exception, no distinguishable signal was detected; this was possibly due to an instrumentation error, such as a malfunction of the filter switch or PMT of the channel. Similar to our fluorescence microscopy results, images from confocal microscopy indicated that both fluorophores were simultaneously present and functional in the transduced artery. These results confirmed a functional *in vivo* gene transfer and subsequent gene expression mediated by the single- and dual-promoter lentiviral vectors that were delivered with our surgery and catheter-based approaches.

4 Discussion

Fluorescent probes such as GFP and RFP inherently generate a detectable signal in response to illumination at specific excitation wavelengths. For gene transfer studies, they may be expressed concurrently with therapeutic genes of interest, enabling the expression of the therapeutic protein to be detected by optical imaging. For *in vivo* applications, achieving high transduction efficiency is critical to the success of gene transfer, leading us to utilize a lentiviral vector having dual independent internal promoters that stably coexpress two separate transgenes.¹⁵

The present study demonstrated a method by which optical imaging was used to localize distinctly bright areas of fluorescence representing transgenic expression of GFP and RFP *in vivo*. The exogenous fluorescence from the transduced artery was clearly detected over autofluorescence. The optical imaging device incorporated a fiber optic probe to transmit excitation light. The circular probe geometry and orientation of the fiber optic fibers allowed the illumination of the transduced tissue to be consistent within the field of view. Our positive results indicated that sufficient excitation energy was provided to GFP and RFP such that their emissions were readily detectable. Increasing the exposure times during imaging *in vivo* increased the signal intensity of fluorescence that was observed. This was reflected in the increased magnitude of signal intensities measured from images collected after 3- and 5-s exposures in the single- and dual-promoter lentiviral experiments, respectively. Among all the images captured, those captured between 3 to 5 s exhibited excellent signal-to-noise ratios such that these images were used for comparing the fluorescence signal among the cases. Similar exposure times have been used for optical imaging of GFP *in vitro*, and although longer imaging times have been reported for detecting GFP *in vivo* in implanted tumor cells,¹² the direct contact between our probe and the arterial tissue eliminated the need for longer exposure times. Another study used a bronchoscope-based system to detect GFP in the mucosal surfaces of the lung treated with adeno-associated viral vector with CMV-driven GFP expression.¹⁰ They reported that GFP fluorescence was observed over the entire field of view of the bronchoscope. With our imaging system, GFP and RFP fluorescence was detected along the arterial wall and not the surrounding skeletal muscle, demonstrating that localization of these proteins could be resolved.

This study also enabled us to establish an animal model for transduction of multiple genes into arteries so that the genes are simultaneously expressed from a dual-promoter lentivirus delivered by a catheter-based approach. In combination with our optical imaging system, we have developed a tool to

evaluate the expression of transgenes and fluorescent proteins that are neither modified nor fused to the protein product of interest. This precludes the possibility that the protein fusion may adversely affect the functionality of either or both fusion partners.¹⁵ For example, vascular endothelial growth factor (VEGF) has been shown to be useful in preventing restenosis and promoting angiogenesis.^{18,19} The substitution of either the GFP or RFP gene by the VEGF gene in the dual-promoter lentiviral vector would allow the expression pattern of VEGF to be evaluated under optical imaging. Further efforts are needed to optimize the efficiency of excitation and detection, as well as minimize the invasiveness of the imaging methods, including the development of either a needle-based transcatheter optical imaging device or an intravascular optical imaging probe.^{20,21} These refinements may facilitate progress toward using these optical imaging techniques to determine the functional period of any given gene, and thereby direct further management of cardiovascular disease with either continuation of the given vascular gene therapy or an alternative treatment.

This study focused on investigating the feasibility of using optical imaging to detect and image fluorescent proteins *in vivo*. Our results indicated that the expression of GFP or RFP alone, and the simultaneous expression of both fluorescent proteins in arteries, were readily detected by optical imaging. Fluorescence was observed to be distinctly bright along the transduced arteries. In its current form, our optical imaging method is an effective tool for studies that require localization of gene expression *in vivo* in the cardiovascular system. This study demonstrated the use of an *in vivo* optical imaging method to simultaneously detect two distinct fluorescent proteins in arteries, transduced with a dual-promoter lentiviral vector driving their concurrent expression, and permit the detection of expression for multiple transgenes and their localization in the vasculature.

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