Percutaneous optical imaging system to track reporter gene expression from vasculatures *in vivo*

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Abstract. This study develops a percutaneous optical imaging system for tracking fluorescent reporter gene expression in vasculatures. We build a percutaneous optical imaging system that primarily comprised a 1.5-mm, semi-rigid, two-port optical probe. The performance of the optical probe is first tested *in vitro* with cell phantoms, and then the feasibility of the percutaneous optical imaging system is validated *in vivo* in eight femoral artery segments of two pigs. The green fluorescent protein (GFP) gene is locally delivered into four arterial segments, while saline is delivered to the four contralateral arterial segments as controls. The targeted arteries are localized using color Doppler, and thereafter the optical probe is positioned to the target arterial segments under ultrasound guidance. Optical imaging captures are obtained using different exposure times from 10 to 60 s. Subsequently, the GFP- and saline-targeted arteries are harvested for fluorescent microscopy confirmation. The percutaneous optical probe is successfully positioned at a distance approximately 2 mm from the targets in all eight arteries. The *in-vivo* imaging shows higher average signal intensity in GFP-treated arteries than in saline-treated arteries. This study demonstrates the potential using the percutaneous optical imaging system to monitor, *in vivo*, reporter gene expression from vasculatures.
[DOI: 10.1117/1.2209559]

Keywords: gene; gene therapy; optical imaging; green fluorescent protein; cardiovascular disease.

Paper 05242RR received Aug. 18, 2005; revised manuscript received Jan. 1, 2006; accepted for publication Jan. 6, 2006; published online Jun. 7, 2006.

1 Introduction

Atherosclerotic cardiovascular disease remains the leading cause of death in the United States and other developed countries. Vascular gene therapy holds promise as an exciting alternative strategy to the existing therapeutic methods for dealing with the causes of atherosclerotic cardiovascular disease.[1] Many genes have been either experimentally or clinically shown to be useful to inhibit postangioplasty/in-stent restenosis, promote angiogenesis, reduce thrombogenesis, and prevent transplantation allograft vasculopathy.[2] A catheter-based local gene delivery approach has become a promising tool to localize a high dose of the transgene at a target site while minimizing undesirable systemic toxicity. To date, different imaging techniques, such as magnetic resonance (MR) imaging and nuclear imaging, as well as optical imaging, have been developed to monitor gene therapy.[3] An *in-vivo* imaging modality can help answer several issues pertinent to the success of vascular gene therapy, including assessments of 1. desirable distribution and localization of transgene delivery at the target vessel wall; 2. whether there is a sufficient level of transgene expression for efficient therapeutic effect on the targets; and 3. the functional period of therapeutic genes at the targets.[4]

Optical imaging, which is based on the detection of fluorescence or emitted light from the luciferase-luciferin reaction, has shown some prominent advantages, including relative technical simplicity, portability, cost effectiveness, the ability to provide real-time imaging, and the lack of radiation. A previous *in-vitro*/*ex-vivo* study proved the principle of using optical imaging to detect vascular reporter gene expression.[5] A subsequent study developed a surface optical imaging system, which offers the potential to detect green fluorescent protein (GFP) gene expression from superficially seated arteries.[6]

However, to detect reporter gene expression from deep-seated vessels, the surface optical imaging is limited by light signal loss due to problems of penetration depth of illumination light in tissue, and forward scattering of incident and emitted light. To circumvent these problems, an alternative strategy is to position the optical imager close to the gene-targeted vessels via a minimally invasive approach. This encouraged us to develop a percutaneous optical imaging system, which was specifically designed for *in-vivo* detection of imaging reporter gene expression from deep-seated arteries or organs.

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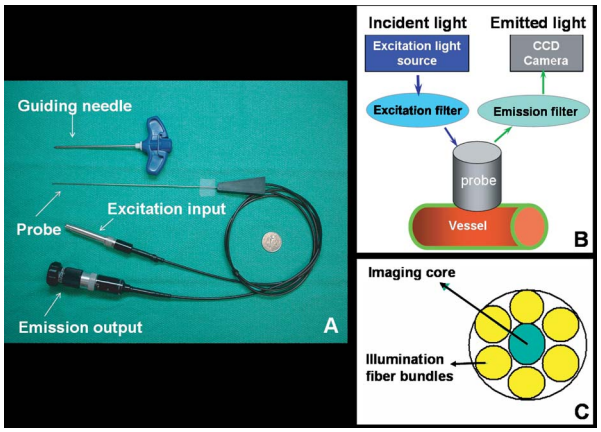

Fig. 1 (a) The dual-port percutaneous optical imaging probe, which can be positioned via a guiding needle. (b) The working mechanism of the percutaneous optical imaging system. (c) Schematic diagrams of the optical imaging probe, demonstrating the inner imaging core (green) surrounded by loosely bound illumination fiber bundles (yellow) (color online only).

2 Materials and Methods

2.1 *Study Design*

This study included four components. First, we tested, *in vitro*, the possibility of using the percutaneous optical imaging probe to detect GFP-positive cells through human tissue-like phantoms. Second, we evaluated, *ex vivo*, the feasibility of using ultrasound imaging to guide the percutaneous positioning of the optical imaging probe close to the target. Third, we validated, *in vivo*, the feasibility of performing the entire procedure of percutaneous optical imaging to detect green fluorescence emitted from the GFP/lentivirus-transduced arteries of living pigs. Fourth, we confirmed the performance of the percutaneous optical imaging system by correlating the optical images with fluorescence microscopy.

2.2 *Percutaneous Optical Imaging System*

The percutaneous optical imaging system consisted of a dual-port fiber optic probe (Zibra Corporation, Westport, Massachusetts) [Fig. 1(a)], a 250-W broadband cold halogen light source (KL 2500 LCD, Schott, Germany), a thermally cooled charge-coupled device (CCD) camera (Sensi-Cam QE, Cooke Corporation, Michigan), and an image grabber installed on a personal computer. The fiber optic imaging probe was primarily modeled on a Y-shape milliendoscope, with one of the proximal ports connected to the cold halogen light source, which was equipped with a filter wheel containing an excitation filter [475 to 488-nm (XF1072), Omega Opticals Incorporated, Brattleboro, Vermont) appropriate for excitation light for green fluorescence. The other proximal port was connected to the CCD camera through a bandpass emission filter [480 to 530 nm, (D505/40), Chroma Technology Corporation, Rockingham, Vermont), and a system of video relay lenses. A schematic showing the operation and layout of the optical imaging system is presented in Fig. 1(b). The distal end of the probe was semi-rigid, 5 cm in length, and 1.5 mm in outer diameter. The probe consisted of an inner imaging core made of silica fiber bundles (10,000-pixel bundle) surrounded by loosely bound illumination fiber bundles made up of soft glass, and a series of objective lenses at the tip for imaging [Fig. 1(c)].

A previous study using Monte Carlo simulation has analyzed the basic light transport of the percutaneous optical probe.[7] Monte Carlo simulations showed that the optimal optical signal detection was at a probe tip distance of around 1.5 to 2 mm from the target vessel. At this distance, the incident fluorophore distribution was sufficient to produce a detectable amount of emitted fluence.[7]

2.3 *In-Vitro Experiments*

2.3.1 *Phantoms*

To investigate the performance of the percutaneous optical imaging probe, it was necessary to closely simulate the actual tissue properties, such as absorption and scattering, which modulate the incident illumination on the embedded fluorophores as well as the emitted fluorescence. The optical imaging setup for the *in-vitro* phantom studies is as shown in Fig. 2(a). The inhomogeneous phantom was based on the model proposed by Durkin, Jaikumar, and Richards-Kortum.[8] To

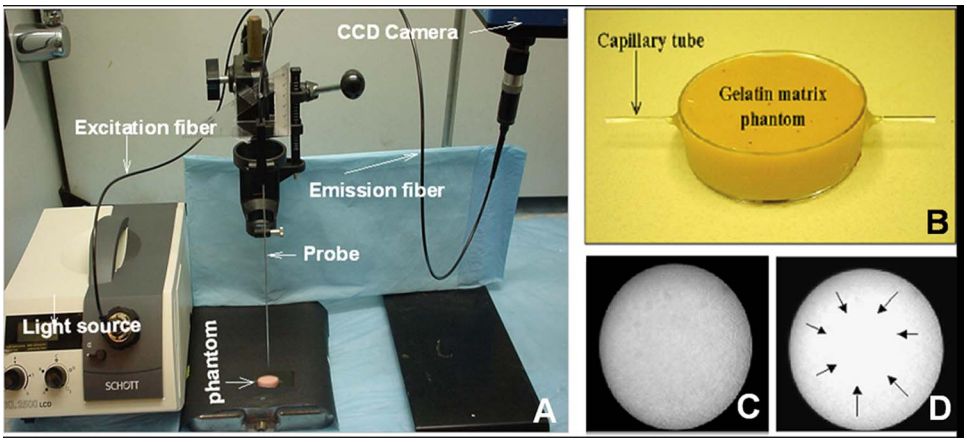

Fig. 2 (a) *In-vitro* experimental setup. The optical imaging probe vertically contacts the pink-colored phantom. (b) The human tissue-like phantom, through which a capillary tube is placed to mimic the vessel. (c) *In-vitro* optical images of the capillary tube filled with saline compared to (d) GFP-positive cells. Brighter light in the center of the capillary tube (arrows) is visualized with GFP/cells than with saline.

model the fluorescence spectra of inhomogeneous tissue, solid phantoms were prepared with unflavored gelatin (Knox) as the matrix, because it served as a substrate with minimal absorption, scattering, and fluorescence [Fig. 2(b)]. Human hemoglobin containing 2% red blood cells (RBC) by volume (Sigma Chemical Company, Saint Louis, Missouri) was used as the absorber to mimic the double absorption windows of blood in this wavelength range. Polystyrene microspheres (Polysciences Incorporated, Warrington, Pennsylvania) with high diffuse reflectance and minimal fluorescence and absorption were used as the scattering media (0.625% by volume in sample solution). Initially, gelatin was dissolved in heated phosphate buffered saline (PBS), and then the hemoglobin and microspheres were stirred in. No other fluorophores were added to the phantom to prevent interference with fluorescence emitted from GFP. The phantom was allowed to cool and solidify overnight. A polyethylene terephthalate capillary tube (0.7-mm inner diameter and 0.3-mm wall thickness), embedded 6 mm below the surface, was used to mimic the blood vessel geometry. The optical images of the control, saline, and the GFP-positive cells are shown in Figs. 2(c) and 2(d), respectively.

2.3.2 *Cell lines*

A third-generation GFP-carrying lentivirus was used to carry and express GFP genes.[9] It was used to transduce Hu-293 T cells. The Hu-293 T cells were cultured in high glucose Dulbecco's modified Eagle's medium (Invitrogen Corporation, California) containing 10% by volume of fetal bovine serum (FBS) (Sigma-Aldrich Corporation, Saint Louis, Missouri). On the day of the experiment, the adherent cells were washed with Trypsin and PBS, counted, and then washed and resuspended in PBS. Initially, the capillary tube was filled with PBS, and baseline data were obtained using the optical imaging system. The image parameters were an incident excitation power=8 mW/cm^2, an exposure time=0.3 s, and a probe distance of 1 mm above the sample. Then, the PBS solution was replaced with a GFP-positive cell suspension (7.5 million cells/ml) and was imaged using the same image capture parameters.

2.4 *Ex-Vivo Experiments*

The purpose of carrying out an *ex-vivo* experiment was to test the possibility of using ultrasound imaging to precisely guide positioning of the percutaneous optical probe close to the target vessels. Cubical volumes of porcine meat were used as phantoms, across which a 3-mm-diam polyethylene capillary tube was inserted transversely to mimic the "vessel" geometry. We first used color Doppler (SonoSite, Incorporated, Bothell, Washington) to localize the "vessel" that was flushed with saline. Then, under ultrasound imaging guidance, we percutaneously inserted a guiding needle (Cook Incorporation, Bloomington, Indiana) close to the vessel, replaced the core of the guiding needle with the optical probe, and positioned the probe tip at a distance of 1 to 2 mm from the vessel that was measured on ultrasound images.

2.5 *In-Vivo Experiments*

2.5.1 *Animals*

We used eight femoral artery segments from two domestic pigs, approximately 29 to 30 lbs in weight. The 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* (National Institutes of Health publication number 80-23, revised 1985). The Animal Care and Use Committee at our institution approved the experimental protocol.

2.5.2 *Anesthesia*

Animals were sedated with an intramuscular injection of a mixture of ketamine (22 mg/kg body wt; Fort Dodge Animal Health, Fort Dodge, Iowa), acepromazine (1.1 mg/kg body wt; Fermenta Animal Health, Kansas City, Missouri), and atropine (0.05 mg/kg body wt; American Regent Laboratories, Shirley, New York). An ear vein was cannulated, which permitted maintenance of hydration using sterile saline. Intravenous pentobarbital (20 mg/kg body wt; Abbott Laboratories, North Chicago, Illinois) was administered later to bring the animal to a surgical plane of anesthesia. Animals were intubated and mechanically ventilated using an anesthesia machine breathing 1.5 to 2.0% isoflurane (Ohmeda Incorporated, Liberty Corner, New Jersey), and maintained at 37°C by use of a water-jacketed heating pad. The animals were also heparinized (100 IU/kg). Anesthesia was monitored during the experiment using regular tests of the eyelid reflex.

2.5.3 *Catheterization*

Using arteriotomy, a 9F introducer was inserted into the aorta through the right carotid artery. Then, a 4F pigtail angiography catheter was positioned into the abdominal aorta. By injection of 20-mL 60% Hypaque (Diatrizoate meglumine; Nycomed Incorporated, Princeton, New Jersey) at an injection flow of 10 mL/s, we obtained a conventional angiogram that included the pelvic arteries and the femoral arteries at both sides. We then selected the proximal and distal portions of the left femoral artery, 3.0 to 3.5 mm in diameter and 2 cm in length, as the gene-targeted vessels. Subsequently, we identified corresponding proximal and distal portions in the right femoral artery to inject with saline, and thus, serve as the control. A total of eight segments of the femoral arteries of the two pigs were selected for experimentation.

The method for preparation of GFP/lentivirus has been described elsewhere.[10] For local GFP-lentivirus delivery, we positioned a Remedy gene delivery catheter (Boston Scientific, Boston, Massachusetts), 3.5 to 4.0 mm in diameter, into the selected distal gene-targeted portion of the left femoral artery via an 0.014-in. guidewire. The catheter has a centrally located angioplasty balloon that is surrounded by multiple gene delivery/infusion channels with 30-μm micropores on the surfaces. The diameter ratios between the gene-targeted artery and the gene delivery balloon were approximate 3.0/3.5 mm or 3.5/4.0 mm. We inflated the balloon with saline to prevent blood flow in the region, and then transferred GFP/lentivirus [at titer range of 1×10^6 to 6×10^6 transducing units (TUs)/mL of supernatant] to the targeted artery wall at an infusion rate of 5 or 10 ml/hr using a syringe pump (Harvard,

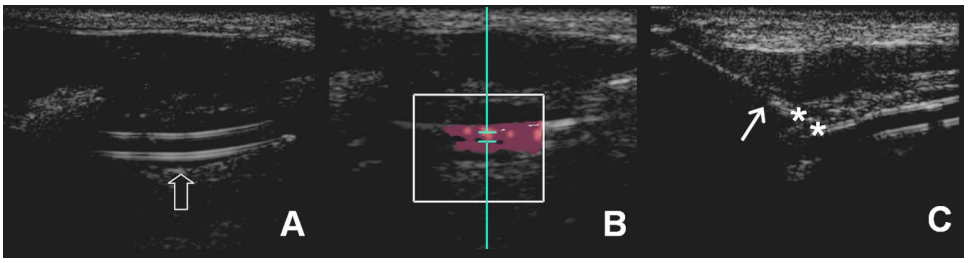

Fig. 3 Ultrasound-guided positioning of the optical imaging probe *ex vivo*. (a) The vessel [arrow on (a)] is localized by real-time ultrasound imaging and confirmed (b) by color Doppler. (c) Subsequently, the optical imaging probe (arrow) was positioned with its tip 2 mm from the vessel (the distance between the stars).

Holliston, Massachusetts). A total of 1.5-mL GFP/lentivirus was transferred to the distal segment of the left femoral arteries. Then, the same catheter was withdrawn and positioned in the selected proximal segment of the same blood vessel, and then GFP lentivirus was delivered again in the same manner as explained before. Thereafter, the catheter was introduced into the right femoral artery and 1.5-mL saline (as a control) was delivered to the distal segment, first followed by the proximal segment using the same flow rate and other delivery parameters as used at the left side. Thus, using the catheter-based gene delivery approach, 1.5-mL GFP/lentivirus was locally delivered into each of the femoral artery segments ($n=4$), while 1.5-mL saline was delivered into each of the contralateral femoral artery segments ($n=4$) to serve as a control.

2.5.4 *Optical imaging*

To precisely identify each of the targeted artery segments for subsequent optical imaging and tissue harvesting, we made two skin markers with sutures under x-ray fluoroscopy, which indicated the location and length of the targeted artery segments. The pigs were then kept alive for six days to allow sufficient GFP gene expression. On day 7, we began to optically image the targeted arteries using two different approaches: surgery-based imaging and percutaneous-based imaging.

Surgery-based imaging was used to confirm the performance of the optical probe in the direct detection of vascular GFP *in vivo*. We surgically exposed four GFP-targeted and four control femoral arteries of two pigs, and then positioned the optical probe close to them for imaging in a dark room. For the eight artery segments, we acquired optical images randomly using an incident excitation power=13 mW/cm^2 with five different exposure times of 10, 20, 30, 45, and 60 s. A total of 125 images were obtained from the eight exposed arterial segments [(13 images from four GFP-arteries+12 images from four saline-arteries) ×5 exposures times=125 images].

A percutaneous-based imaging approach was used to validate the feasibility of performing the entire process of *in-vivo* percutaneous optical imaging to detect vascular GFP. The percutaneous-based optical imaging was performed before the surgery-based optical imaging in the second pig. We first localized two GFP-targeted and two control artery segments of the pig using color Doppler with a 10-MHz linear transducer (Sonosite). Then, under ultrasound guidance, we positioned percutaneously the optical imaging probe at the target arteries. After the probe tip was placed 1 to 2 mm from the targets that were measured on ultrasound images, we obtained optical imaging of the target arteries using the same capture parameters as those used for the surgery-based imaging approach. Of the two control arteries, 20 images were captured (2 segments ×5 exposure times/segment ×2 images/segment=20 images), while of the two GFP-targeted arteries, 10 images were captured (2 segments ×5 exposure times/segment=10 images).

In addition, before the surgery- and percutaneous-based optical imaging described before, we captured 38 images using different parameters to test and establish the optical imaging protocol. These data of tested images/measurements were not included in the subsequent imaging and statistical analysis.

2.6 *Imaging Analysis*

The CCD-captured optical images were analyzed using National Institutes of Health (NIH) ImageJ software. A rectangular region of interest (ROI) was selected on the images. The average signal intensity was measured over the rectangular ROI. All data were presented as mean±SD. Of each exposure time in the surgery-based optical imaging group, a student's t-test was used to compare the average optical signal intensities between the control arteries and the GFP-targeted arteries. A p-value less than 0.05 was considered statistically significant.

After obtaining satisfactory optical imaging of the gene-transfected and control arterial segments, the animals were euthanized with a dose of 100 mg/kg of pentobarbital. The GFP-transfected and control vessel tissue segments were harvested and fixed in 4% paraformaldehyde solution for 30 min. The tissue was then sectioned into 10-μm-thick unstained frozen sections. The frozen sections were analyzed using fluorescence microscopy (Nikon Eclipse TS100, Tokyo, Japan) to confirm GFP expression in the target vessels.

3 Results

Of the *in-vitro* experiments, optical imaging demonstrated brighter light detected in the GFP-positive cell-containing tube than in the saline-containing tube [Figs. 2(c) and 2(d)]. Of the *ex-vivo* experiments, color Doppler allowed us to confirm vessel location, and the probe could be precisely positioned at a distance of 1 to 2 mm from the vessel under ultrasound imaging guidance (Fig. 3).

Of the *in-vivo* experiments using the surgery-based ap-

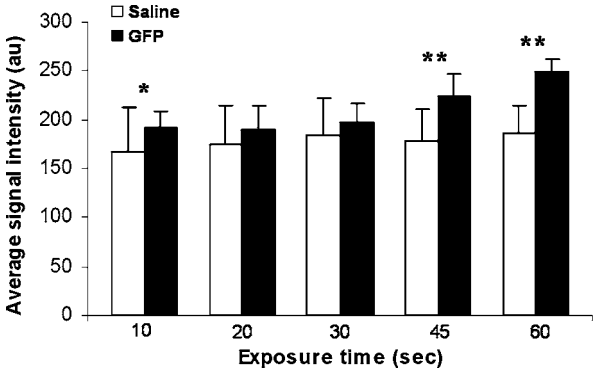

Fig. 4 Data from the surgery-based optical imaging approach, presenting higher average signal intensities for GFP-treated arteries than for saline-treated arteries for all exposure times. $*p<0.05$; $**p<0.01$.

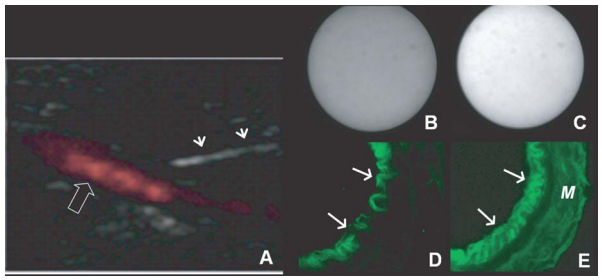

Fig. 5 *In-vivo* percutaneous optical imaging of pig arteries. (a) Ultrasound imaging, demonstrating that the target femoral artery (open arrow) is imaged by the optical imaging probe (arrowheads). (b) and (c) Direct view of *in-vivo* optical images obtained from a percutaneous-based approach. The emission light is brighter in the (c) GFP-treated artery than in (b) the saline-treated artery. (d) and (e) Fluorescent microscopy examination of percutaneously imaged artery tissues, showing higher green fluorescence from both intima [arrows on (e)] and media (M) of (e) the GFP-treated artery compared to autofluorescence from internal elastic lamina [arrows on (d)] of (d) the saline-treated artery. (Magnification 20×.)

proach, the optical imaging of the exposed arteries showed higher average signal intensity from GFP-treated arteries than from saline-treated arteries for each of five different exposure times (Fig. 4). At exposure times of 10, 45, and 60 s, the average signal intensities were significantly higher in GFP-treated arteries than in saline-treated arteries ($p<0.05$).

Of the *in-vivo* experiments using the percutaneous-based approach, the optical probe was successfully positioned with its tip 1 to 2 mm from the targeted arteries under ultrasound guidance (Fig. 5). The difference in emission light signals between GFP-treated arteries and saline-treated arteries could be differentiated under optical imaging (Fig. 5). Fluorescent microscopy examination confirmed that higher fluorescence in the media layer was detected in GFP-treated arteries than in saline-treated arteries.

4 Discussion

Clinical imaging of vascular gene therapy includes two components: 1. assessing the success of the primary gene delivery procedure; and 2. tracking the functional period of vascular gene expression after the primary gene delivery.[4] The current study outlines a potential method of exploring the second component, tracking vascular gene expression. In the scientific field of gene therapy, colored fluorescent protein genes, such as GFP genes, have been widely used as imaging reporters for *in-vivo* optical imaging.[9,11] GFP does not require the injection of any secondary substrates and does not appear to interfere with cell growth and function.[12]

Optical imaging is a promising molecular imaging tool to characterize and measure biologic processes at the cellular and molecular level *in vivo*.[3] However, the primary disadvantage of *in-vivo* optical imaging is the limited ability to either send the excitation light into or detect the emission light from deep-seated tissues and organs in the body. Thus, the penetration depth of light in tissue prevents it from being an ideal imaging tool for whole body imaging of large animals and humans. This disadvantage is more obvious when using surface optical imaging to noninvasively detect fluorescent signals from deep-seated arteries, such as the iliac and renal arteries, as well as the aorta, which are the primary targets of gene therapy of atherosclerosis. To deal with this problem, we attempted to develop a percutaneous optical imaging system, expecting to detect vascular reporter gene expression using a minimally invasive approach. Our study presents encouraging evidence that a percutaneous optical imaging approach may help overcome the limited light penetration of optical imaging, which presents the potential to provide a useful, minimally invasive molecular imaging method to monitor reporter gene expression in vessels or deep-seated organs.

For percutaneous imaging, accuracy in positioning the probe with respect to the blood vessel is of paramount importance, as it affects the optimal probe distance from the region of maximum gene expression and the best focus point. Our *ex-vivo* study demonstrated that under ultrasound imaging guidance, the optical imaging probe could be precisely positioned within a distance of 1 to 2 mm of the target vessel. Subsequent surgery-based experiments further confirmed the performance of the percutaneous optical imaging probe *in vivo*. Finally, we successfully performed the entire procedure of percutaneously imaging fluorescent gene expression from vasculatures using an ultrasound-guided, minimally invasive approach in a preclinical setting, the living pig.

In current practice, the confirmation of successful gene transfection/expression depends primarily on different laboratory tests of harvested tissues obtained from either autopsy or biopsy. In its present state, our percutaneous optical imaging system may provide a useful minimally invasive imaging tool for basic science on cardiovascular gene therapy. The ultimate goal of developing *in-vivo* imaging techniques for gene therapy is to track the expression of therapeutic genes. Recent studies have presented the possibility of simultaneously encoding two different genes from the same vector.[13] This kind of dual gene vector can be used to carry and express two genes simultaneously, e.g., an optical imaging reporter gene (such as the GFP gene) and a therapeutic gene [such as the vascular endothelial growth factor (VEGF) gene]. Thus, direct detection of the GFP gene by optical imaging should indirectly assess the functionality of VEGF, because the two genes are simultaneously expressed with the same vector.

The current report focused only on establishing the "proof of principle" of a new concept or a new technical develop-

ment, using a minimally invasive and cost-effective percutaneous optical imaging method to detect fluorescent reporter gene expression *in vivo*. Further work is required to optimize the probe design with higher-grade optical fibers to reduce transmission losses, improve numerical aperture for higher signal capture efficiency, refine the cross section construction of the probe for simultaneous optimization of excitation and emission fluence, and apply a laser light source that would greatly enhance the incident illumination compared to a halogen light source because of higher coupling efficiency. Limitation of this study is the lack of sham transduced controls. It is essential to optimize the parameters for both local gene delivery and optical imaging, which requires the comparison between different gene/vector groups with controls.

Fluorescence microscopy identifies a more pronounced difference among the images, as it detects the fluorescence emitted from processed tissue sections embedded on slides, with no interfering media. The signal intensity obtained using the percutaneous probe does not reach a similar level of clarity because of interfering tissue and blood, and its own system limitations because of the sensitivity of the optical components, as mentioned before. Fluorophores with a higher range of excitation and emission wavelengths would reduce the autofluorescence from tissue within the body. Such an optical system may be extended for other methods of optical imaging, including the detection of light signals from luciferase and therapeutic genes labeled with other fluorophores.

In conclusion, currently there are no imaging techniques available for detecting gene expression from vasculatures *in vivo*. We have presented a technical development that uses a percutaneous optical imaging system as an *in-vivo* imaging tool to detect fluorescent reporter gene expression from vasculatures, thus providing a minimally invasive approach with the potential to monitor gene therapy in deep-seated vasculatures and organs *in vivo*.

Acknowledgments

This work was supported by National Institutes of Health grant R01 HL67195.

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