Tyrosinase as a dual reporter gene for both photoacoustic and magnetic resonance imaging

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Abstract: Reporter genes are useful scientific tools for analyzing promoter activity, transfection efficiency, and cell migration. The current study has validated the use of tyrosinase (involved in melanin production) as a dual reporter gene for magnetic resonance and photoacoustic imaging. MCF-7 cells expressing tyrosinase appear brown due to melanin. Magnetic resonance imaging of tyrosinase-expressing MCF-7 cells in 300 μL plastic tubes displayed a 34 to 40% reduction in T1 compared to normal MCF-7 cells when cells were incubated with 250 μM ferric citrate. Photoacoustic imaging of tyrosinase-expressing MCF-7 cells in 700 μm plastic tubes displayed a 20 to 57-fold increase in photoacoustic signal compared to normal MCF-7 cells. The photoacoustic signal from tyrosinase-expressing MCF-7 cells was significantly greater than blood at 650 nm, suggesting that tyrosinase-expressing cells can be differentiated from the vasculature with in vivo photoacoustic imaging. The imaging results suggest that tyrosinase is a useful reporter gene for both magnetic resonance and photoacoustic imaging.

OCIS codes: (170.5120) Photoacoustic imaging; (170.3880) Medical and biological imaging; (170.2655) Functional monitoring and imaging; (000.1430) Biology and medicine.

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1. Introduction

Reporter genes are useful tools allowing identification of specific cells within a population of cells. Green fluorescent protein (GFP) is a commonly used reporter gene that allows identification of transfected cells as well as subcellular localization of proteins when GFP is tagged to proteins of interest [1]. In vivo applications are challenged by strong light-scattering and poor penetration in tissue. Luciferase is another commonly used reporter gene that can be used in vivo although the light produced by most luciferases has a relatively short wavelength (<600 nm) causing poor tissue penetration and significant scattering [2].

Tyrosinase (TYR) is the key enzyme regulating melanin production in hair and skin in humans [3]. Although melanin displays maximal optical absorption at ultraviolet wavelengths, melanin has a broad spectrum of optical absorption with significant absorption at some near infrared wavelengths which have strong tissue penetrating properties [4]. TYR has previously been shown to be a reporter gene for magnetic resonance imaging (MRI) due to the binding of melanin with heavy metals which can provide contrast in MRI [5,6]. The current study tests whether TYR is also a reporter gene for photoacoustic imaging since melanomas which produce melanin can easily be detected with photoacoustic imaging [7–10].

Photoacoustic imaging involves exposing a sample with a pulsed laser and detection of the emitted ultrasound produced by the thermo-elastic expansion caused by the absorption of the laser light in the sample. Combining light delivery and ultrasound detection in one imaging modality allows imaging with ultrasonic resolution and is not limited by optical scattering [11]. LacZ, encoding the lactose metabolizing enzyme β-galactosidase, has previously been demonstrated as a reporter gene for photoacoustic imaging although imaging LacZ expression requires the injection of X-gal in a subject [12–14]. X-gal can occasionally cause skin irritation and was not efficiently metabolized in tumors when X-gal was injected systemically through the tail vein of rats [12]. TYR as a photoacoustic reporter gene does not require co-administration of an enzymatic substrate and, in theory, should produce a very strong photoacoustic signal since each molecule of expressed TYR will aid in the production of a photoacoustic signal.

2. Methods

2.1 Cell culture

MCF-7 Tet-On® cells (also called MCF-7–TYR in this manuscript), which stably expresses the reverse tetracycline-controlled transactivator (rtTA), were purchased from Clontech Laboratories, Inc. (Mountain View, CA). MCF-7 cells were maintained in Dulbecco’s modified Eagle’s growth medium with 10% fetal bovine serum and 200 μg/mL G-418 (A.G. Scientific Inc, San Diego, CA).

For MRI and photoacoustic imaging experiments, T-150 cm² tissue culture flask were inoculated with approximately 3.5 x 10⁶ cells. The next day, flask had growth medium replaced with medium that may have contained 1 μg/mL doxycycline (Dox) and/or 250 μM ferric citrate (only for MRI experiments). Three days after the medium change, cells were washed once with phosphate buffered saline (PBS), trypsinized from the flasks, and 10 mL growth medium was added to each flask to inhibit trypsin. Cells were centrifuged at...
approximately 250 x g and cells were washed once with PBS. For MRI experiments, $10^7$ cells were centrifuged in 300 μL tubes and PBS above pelleted cells was removed before imaging. For photoacoustic experiments, cells were resuspended at $10^6$ cells/mL in PBS and cell suspensions were injected in 700 μm transparent plastic tubes which were used for imaging.

2.2 Generation of MCF-7 cells stably expressing TYR

cDNA encoding human TYR (accession number NM_000372.1) was purchased from Genecopoeia, Inc. (Rockville, MD) in the pReceiverM02 vector. This vector was used in a polymerase chain reaction (PCR) with primers flanking the TYR open reading frame with KpnI and NotI restriction enzyme sequences within the 5′ and 3′ primers, respectively. The PCR reaction yielded primarily one DNA species at approximately 1.7 Kbp which was agarose-gel purified using a Qiagen Gel Extraction kit (Qiagen, Mississauga, Ontario). The purified TYR encoding insert cDNA and the pTRE-Tight inducible expression vector (2.6 Kbp; Clontech Laboratories, Inc, Mountain View, CA), were both digested with KpnI and NotI restriction enzymes (Invitrogen, Carlsbad, CA). Cut vector and insert were ligated together with T4 DNA ligase (New England Biolabs, Pickering, Ontario) and the ligation mixture was used to transform Fusion Blue competent cells (Clontech Laboratories, Inc, Mountain View, CA) which were plated on Lysogeny broth plates supplemented with 50 μg/mL ampicillin. Bacterial colonies were isolated and plasmid DNA of approximately 4.3 Kbp was isolated from the resulting colonies. This DNA was co-transfected with DNA containing a hygromycin resistance marker in MCF-7 Tet-On® cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). One day after transfection, cells were trypsinized and transferred to 10 cm² plates. The next day plates had a medium change containing DMEM with 10% FBS, 200 μg/mL G-418, and 300 μg/mL hygromycin. Cells in plates were grown for several weeks until large cell colonies were visible. Colonies were individually trypsinized from the plates using cloning disks (Invitrogen, Carlsbad, CA) and each colony was tested for the ability to produce TYR in the presence of Dox by light microscopy. One colony producing brown cells in the presence of 1 μg/mL Dox was designated MCF-7 + TYR cells and these cells were used for subsequent experiments (see Fig. 1).

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**Fig. 1.** Tet-On® inducible system for TYR expression. The pTRE-Tight vector contains a promoter composed of the minimal cytomegalovirus (CMV) promoter as well as a modified tetracycline responsive element (TREMOD). The tetracycline-responsive transactivator (rTetR), containing three minimal transcription activation domains from the Herpes Simplex Virus VP16 protein, will only bind to the TREMOD sequence and induce expression of TYR in the presence of Doxycycline (Dox).
2.3 MR imaging: instrumentation and protocols

The vials of cells as prepared above were imaged on a 9.4 Tesla animal MR imaging system (Magnex Scientific, Oxford, UK). This is a horizontal bore unit with an inner bore diameter of 12.0 cm, and a 200 mT/m gradient set. All radio frequency (RF) transmission and reception for image acquisition was performed by a 44 mm inner diameter birdcage coil. The unit is controlled by a TMX console (National Research Council of Canada Institute for Biodiagnostics—West, Calgary, AB, Canada).

The vials in groups of four were oriented vertically and clustered in a square array at the most sensitive region of the RF coil. With the samples present, the coil was tuned and matched at 400 MHz and was then positioned at the magnet isocentre. Calibration of a 90 degree pulse was accomplished by maximizing the signal resulting from a one-pulse experiment (utilizing the same selective RF pulse to be used in subsequent imaging) with a lengthy 8 second repetition time (TR). The RF magnetic field within the birdcage coil (B1) was mapped to ensure uniformity within the region of interest. A double-angle technique [15] was utilized for this purpose, revealing a B1 field with less than 5 percent deviations from uniformity over the vials. The quantification of T1 in this work was accomplished through a method similar to that used by Ponce et al. [16] Images were acquired using a single-slice spoiled gradient-echo sequence with a TR of 250 ms, and a TE at 5 ms. The fields-of-view for these images were 20 by 20 mm encoded over a 128 by 128 matrix, yielding pixels with dimensions of 0.16 mm. The coronal image had a slice thickness of 1.0 mm and cut a cross-section mid-way through the cell populations in the vials. Multiple versions of these images were acquired over a series of flip-angles ranging between 5 and 90 degrees in 5 degree increments.

2.4 MR image analysis

Images were exported offline and processed using Matlab for T1 quantification. The signal variation pattern seen over each pixel as the flip angle was changed was fitted with a non-linear unconstrained optimization scheme (Matlab’s fminsearch function) to the analytic function below:

\[
S = S_0 \cdot \sin(\theta) \cdot \frac{1 - \exp \left( \frac{-TR}{T_1} \right)}{1 - \cos(\theta) \cdot \exp \left( \frac{-TR}{T_1} \right)},
\]

where \(\theta\) was the flip angle and \(S_0\) was the maximum signal available. With the flip angles and the TR known, the only variables to be assigned by the optimization were \(T_1\) and \(S_0\). In this way, a pixel-by-pixel T1 time was calculated over all the vials.

2.5 Photoacoustic imaging system

A 25 MHz ultrasound transducer (V324-SM, Panametrics, Waltham, MA, USA), with a focal length of 12.7 mm and a –6 dB fractional bandwidth of approximately 50%, was mounted onto a motor controller to obtain interlaced ultrasound-photoacoustic B-scans images. The transducer was connected to a pulser-receiver unit (Olympus Panametrics-NDT Model 5073PR) which excited the transducer and amplified received signals by 39 dB. Photoacoustic signals were generated by an Optical Parametric Oscillator (OPO; Continuum, Surelite OPO Plus) pumped by a 10 Hz, 532 nm, Q-switched laser (Continuum Surelite, SLIII-10). The OPO produced tunable-wavelength 8-ns pulses range from 410 to 710 nm. The laser pulse was steered around the ultrasound transducer by a conical mirror custom light-delivery optics and focused at a point 12.7 mm below the transducer using a previously described setup [17]. The system provided a photoacoustic lateral spatial resolution of 180 \(\mu\)m, while the axial spatial resolution was ~40 \(\mu\)m. A digital input/output card (National Instruments, NI CB/SCB-
2162) was used to trigger both ultrasound pulses and laser pulses to enable interlaced ultrasound and photoacoustic imaging. The voltage levels were digitized by a 125 megasample/s data acquisition card (Gage, CompuScope 8289) with 12-bit accuracy and stored for post-processing by Matlab software. A photodiode measured the amount of light from each laser pulse and was used to normalize the photoacoustic signals to account for laser pulse-to-pulse instability. At all wavelengths used, the laser fluence at the ultrasound focal location, measured in air was <20 mJ/cm². The actual fluence reaching the targets will be much less in a scattering medium.

2.6 Photoacoustic imaging protocol

Five 700-µm (inner-diameter) transparent plastic tubes were aligned on an acrylic frame. One of the tubes was loaded with whole rat blood (blood was taken from the rat and placed in a 10 ml BD Vacutainer containing 143 USP units of sodium heparin). The remaining four test tubes were loaded with one of the four combinations of ± Dox and MCF-7 ± TYR cells in PBS, hereby named −/−, +/-, +/−, +/+ tubes ( ± Dox/MCF-7 ± TYR), where + represents the presence of either Dox or MCF-7 + TYR cells and - represents the absence of either Dox or MCF-7–TYR cells. The tubes were submerged in water with or without Intralipid to mimic optical scattering which is present in biological tissue. Using a voice-coil actuator and motor controller to laterally move the ultrasound transducer at a rate of 0.5 mm/s, a one-to-one, interlaced ultrasound-photoacoustic B-scan image was formed of all five test tubes. Ultrasound imaging was used to determine the position of the tubes and ensure only photoacoustic signals within the plastic tubes were analyzed. The speed of sound was assumed to be constant throughout the ultrasound propagation at approximately 1500 m/s. The motor position was reset before each B-scan was initiated. Five B-scan images were taken at five points, spaced 1 mm apart, along the tubes. The B-scan image sets were taken for four different wavelengths; 576 nm, 594 nm, 604 nm, and 650 nm. Finally, the experiment was repeated for several Intralipid (Intralipid 20%, Fresenius Kabi, Uppsala, Sweden) concentrations which correspond to a reduced scattering coefficient (µs) of 0 (in pure water), through 3.10 cm⁻¹ at a depth of 12.7 mm. The reduced scattering coefficient of the intralipid stock-solution was measured independently via an oblique-incidence-diffuse-reflectance technique using a 532-nm continuous-wave diode laser. The methodology used for measuring the reduced scattering coefficient of the Intralipid stock demonstrated accurate results (R² = 0.99) in a previous study [18]. Since the imaging depth of high-resolution photoacoustic microscopy is typically 2-3 mm, light penetration over the ultrasonic focal distance of 12.7 mm in a solution with µs = 1.7 cm⁻¹ (the value used for Fig. 5 below) is roughly equivalent to light penetration over ~2.2 mm in a µs = 10 cm⁻¹ turbid medium. Hence experimental conditions are designed to mimic typical penetration distances in biological tissues. For the highest µs examined, penetration in a µs = 10 cm⁻¹ turbid medium would be equivalent to ~4 mm.

2.7 Photoacoustic image analysis

Signal levels were assessed by examining the maximum signal generated within each tube (tube lumen positions determined by ultrasound images) and the average absolute signal within a 5 by 7 pixel area (0.25 mm laterally by 0.11 mm depth). The maximum and average signals were averaged over all five B-scan images and recorded along with the standard deviation. Combined ultrasound-photoacoustic B-scan images were formed by first separating the two signals, adjusting the depth of the photoacoustic signal, and combining them by thresholding the photoacoustic signal. The threshold level was adjusted manually for best visual contrast while still maintaining the form of the raw B-scan image. Further image processing was used to smooth the image, by interpolating the image so that its size quadrupled in both dimensions, and series of median filters were used to remove large amplitude noise artifacts. Depending on the signal to noise ratio, a sequence of either one or three median filters was used. For the images with tubes submerged in pure water, a single
median filter with dimensions of [3x3] was used to eliminate the speckle. Only one median filter was chosen in these cases because abnormal spikes in noise were still negligible compared to the signal generate from the TYR+/Dox + and blood tubes. For images with tubes submerged in water containing intralipid, a series of three median filters were used, with each subsequent median filter using a larger area (square matrix of 3, then 5, then 7). This was necessary because the signal from the tubes was only slightly greater (although consistently greater) than abnormal spikes in noise and the resulting image contrast was worsened because of speckle. Statistical differences between signals from different samples were determined using two-tailed student t-tests.

3. Melanin expression in MCF-7 cells

MCF-7–TYR and MCF-7 + TYR cells were inoculated in T-25 cm² flasks containing growth medium with or without 1 μg/mL Dox. After three days of cell growth, flasks were imaged with an Olympus IX70 microscope (200× magnification) to visualize melanin expression (Fig. 2). Only MCF-7 + TYR cells incubated with Dox contained melanin which appeared as a dark brown pigment in the cells, suggesting that TYR expression was inducible with Dox.

![Fig. 2. Microscopic analysis of TYR expression in MCF-7 cells. MCF-7-TYR (a + b) and MCF-7 + TYR cells (c + d) were incubated in growth medium with (b + d) or without (a + c) 1 μg/mL Dox. MCF-7 + TYR cells incubated with DOX appear dark brown due to melanin production. Scale bars represent 100 μm lengths.](image)

4. MR imaging of melanin-producing cells

A T₁ map and a corresponding T₁-weighted image of a four-vial cell group are displayed in Fig. 3. The mean T₁ times of the vials in msec, starting with the top right and moving clockwise are 756 +/- 50 (MCF-7 + TYR, + DTX), 1243 +/- 96 (MCF-7 + TYR, -Dox), 1269 +/- 128 (MCF-7-TYR,-Dox), and 1146 +/- 89 (MCF-7-TYR + Dox). This represents a 34 to 40 percent reduction in T1 when cells are capable of producing TYR in the presence of Dox.
5. Photoacoustic imaging of melanin-producing cells

B-scan images show tubes containing, from left to right, −/−, +/-, +/-, (+ Dox/MCF-7 ± TYR) and blood. The photoacoustic signal from the −/−, +/-, and +/- tubes were negligible in all images — any signal from the tube primarily originated from the tube walls – 50 mV. In water, μ\_s of 0 cm\(^{-1}\), the signal from these tubes at all wavelengths were less than 60 mV and were generally 5-10 times lower than the signal from either blood (p < 0.0005) and TYR (p < 0.003). For wavelengths around 576 nm the blood demonstrated a much more intense signal than the +/- tubes (2.1-fold greater; p < 0.003), while at 650 nm the blood demonstrated a much smaller signal intensity than the +/- tubes (2.2-fold smaller; p < 0.02). Image quality decreased rapidly as the amount of Intralipid increased; however, signal levels from both the blood and +/- tubes consistently demonstrated higher intensities compared with −/−, +/-, and +/- tubes. The characteristic shape provided in Fig. 4 is consistent for μ\_s of up to 2.5 cm\(^{-1}\) at a depth of 12.5 cm; however, signal intensities decrease. Finally, the image processing described above showed that the photoacoustic signal from blood was much greater than the +/- tube at 576 nm while the signal from blood was small compared with +/- tube at 650 nm (Fig. 5).
Fig. 5. Combined ultrasound (grayscale) and photoacoustic (orange colormap) B-scans of tubes containing our test samples. White circles are manually drawn to further demarcate tube locations. From left to right the tubes are −/-, +/-, -/+ and blood (−/- = -Dox/MCF-7-TYR). Only the cells in the +/+ tube expressed melanin. (a) B-scan image at a wavelength of 576 nm. A threshold of 50% was used. Blood is seen best at this wavelength. (b) B-scan image at a wavelength of 650 nm in the same turbid medium as (a). A threshold of 50% maximum signal was used. Compared to the other samples, the melanin-expressing cells displayed the highest photoacoustic signal at 650 nm.

We can estimate the sensitivity of our system by accounting for cell settling and the resolution of our ultrasound transducer. Figure 5 demonstrates that the +Dox/+TYR cells settled away from the top of the tube, presenting signal approximately 150 μm below the top of the tube. Therefore, cells were present in approximately 77% of the tube and a correction factor of 1.3 is needed to calculate the concentration of the settled cells. The volume of cells contributing to signal can be calculated using the lateral and axial resolutions of the transducer – 180 μm and 40 μm, respectively [17]. Hence, the number of cells detected in each voxel is approximately 169 cells. Finally, we can find the number of cells to provide unity SNR by dividing by the SNR – calculated using the average signal in a 5x7 (lateral by axial) area around the maximum divided by the standard deviation of the noise calculated using a 20x20 area below any signals generated by the tubes. Cells in media with reduced scattering coefficients of 0, 1, and 2.5 cm$^{-1}$ at a depth of 12 mm demonstrated an average SNR of 158.5, 5.99, and 3.66 V/V, respectively. Table 1 summarizes the number of cells needed for unity SNR (V/V) for each of the four wavelengths used in this study.

### Table 1. Number of Cells for unity SNR (V/V)

| Reduced Scattering Coefficient, μm$^{-1}$ | 576 nm | 594 nm | 604 nm | 650 nm |
|------------------------------------------|--------|--------|--------|--------|
| 0 cm$^{-1}$                               | 0.93 ± 0.31 | 1.06 ± 0.36 | 1.58 ± 0.55 | 1.27 ± 0.43 |
| 1 cm$^{-1}$                               | 34.1 ± 11.7 | 30.6 ± 10.6 | 36.2 ± 9.22 | 24.0 ± 7.13 |
| 2.5 cm$^{-1}$                             | 44.4 ± 3.41 | 43.8 ± 8.34 | 56.6 ± 10.9 | 46.5 ± 10.7 |

### 6. Discussion and conclusions

The use of TYR expression creates a substantial decrease in $T_1$ that can be exploited as a contrast mechanism in MRI. For example, considering the reduction of $T_1$ from approximately 1200 ms to 756 ms as determined experimentally above, a TR setting of 80 ms in conjunction with a 68 degree flip angle will yield a 100% increase in signal over the background. This signal boost is considerably greater than that noted by Weissleder et al. [6], where a 29 percent signal increase was seen. However, the previous study was conducted at a much lower field strength (1.5 T), and the sequence timings they used may or may not have been fully optimized for contrast visualization. Further, the signal boost may be affected by...
other factors including the magnitude of TYR expression and concentration of iron available to be scavenged by melanin.

For the photoacoustic imaging studies, ultrasound was used to co-register the tube locations to ensure photoacoustic signal analysis is appropriately localized to the tubes of interest. In the ultrasound and photoacoustic images, only the top and bottom of the tube outer and inner walls are seen. This is because ultrasound reflected from the side of the tube will be deflected away from the receive-direction of the ultrasound transducer. Additionally, the ultrasound transducer has a band-pass response, and hence emphasizes only edges (changes of acoustic impedance) along the line-of-sight of the transducer. A similar effect is seen in the photoacoustic images: signals are strongest from the top of the cells, while some signal is also seen from the bottom. While light scattering partially illuminates the tube from all directions the ultrasound transducer acts as a bandpass filter along the depth-direction, emphasizing absorption edges. In the case of the melanin-expressing cells, signal is most likely seen from the middle of the tube because cells have settled within the tube. A transducer with an improved low-frequency response could be used to visualize the internal structure rather than just the top and bottom of the cell suspension.

Although the minimum absorbance of blood is around 680 nm, we chose to examine the photoacoustic contrast between blood and TYR expressing MCF-7 cells at 650 nm because the stability and waveform of the laser pulse was much better. The 576 nm wavelength was chosen to maximize the signal of the blood. The signal for blood rapidly decays while the signal for the TYR expressing MCF-7 cells demonstrated relatively constant signal amplitudes. There is actually signal from the blood tube in Fig. 5(b), however, we chose a threshold value above this signal level as one way to see only gene expression in the + Tyr/+Dox tube. By examining multiple wavelengths at the same time, one can potentially perform multi-wavelength image analysis to suppress the signal from the blood and enhance the signals from melanin-producing cells from images taken in vivo.

TYR expression in MCF-7 cells caused a significantly larger increase in photoacoustic signal (>20-fold) compared to MRI signal (up to 2-fold), suggesting TYR is a stronger reporter gene for photoacoustic imaging compared to MRI. When using TYR as a reporter gene for photoacoustic imaging, no additional contrast agent is necessary while for MRI, a heavy metal such as iron is needed for increased signal. However, the photoacoustic microscopy system we use presently has limited imaging depths while MRI does not, suggesting that MRI may be more appropriate when using TYR as a reporter gene in deep tissues. Future work will investigate photoacoustic imaging using lower-frequency transducers and near-infrared, rather than far-red light, for improved depth-penetration. Additionally, ongoing work aims to show deeper imaging of photoacoustic-based gene expression in real-time. Presently photoacoustic imaging frame-rates are limited by the 10-Hz laser-pulse-repetition rates and the requirement for one laser-pulse-per photoacoustic A-scan line.

Our photoacoustic system provides single cell resolution with SNR of ~1 V/V with fluence of ~20 mJ/cm² when tubes containing tyrosinase-expressing cells are submerged in pure water. When tubes containing tyrosinase-expressing cells are submerged in optical scattering media, more cells are required to achieve a SNR of 1 V/V. Based on our results, an estimate of the number of cells needed to image in vivo (µs' ~10 cm⁻¹) at a depth of 3 mm is approximately 45 cells.

Whole body imaging with fluorescent proteins and luciferases as reporter genes has been primarily limited to small animals due to significant optical absorption and scattering of the emitted signal. The emitted signal of melanin in photoacoustic imaging is not light but acoustic pressure waves which can propagate through tissue to a much greater extent than visible light. For this reason, photoacoustic imaging should be capable of greater theoretical imaging depth compared to conventional optical and fluorescence imaging. Our preliminary results demonstrate that, when using a linear array photoacoustic system, TYR-expressing cells can be visualized through 4 cm of chicken tissue (data not shown), suggesting that it may be possible to image gene expression at greater imaging depths using TYR and photoacoustic imaging compared to conventional optical-based reporter genes.
The sensitivity of TYR as an \textit{in vivo} reporter gene will depend on various factors including 1) levels of TYR expression and melanin production in target cells, 2) total number of TYR-expressing cells used for imaging, 3) tissue depth of TYR-expressing cells in the animal, 4) tissue composition between TYR-expressing cells and transducer (e.g. degree of vasculature, presence of gas or bone), and 5) the resolution and tissue penetration capabilities of the photoacoustic system used for imaging. Researchers should carefully consider all of these factors if using TYR as an \textit{in vivo} reporter gene for photoacoustic imaging.

Our experiments are in agreement with previous work that suggests that TYR expression exhibits low level toxicity in mammalian cells [6]. TYR expression in MCF-7 cells can cause a significant fraction of cells to detach from the tissue culture surface (data not shown). In the current study, TYR expression was inducible which allowed the growth of large amounts of healthy cells which were induced to express TYR three days before imaging. This inducible TYR expression system was useful to minimize TYR toxicity and will likely become crucial for future \textit{in vivo} experiments including MRI and photoacoustic imaging of xenograft tumors with or without TYR expression as well as quantitative determination of the sensitivity of TYR as a reporter gene for both imaging modalities. Doxycycline is an attractive agent for inducing expression \textit{in vivo} because it can simply be added to the drinking water of animals. Such experiments will aid in the development of TYR as a promising reporter gene for MRI and photoacoustic imaging which may be useful non-invasive methods for analyzing relative transfection rates, tumor growth rates, or cell migration in animals. Furthermore, we hope that other absorption-based reporter genes will emerge that will permit sensitive molecular imaging studies of gene expression \textit{in vivo} with high contrast and ultrasonic spatial resolution.

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