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Quantifying receptor density *in vivo* using a dual-probe approach with fluorescence molecular imaging

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Abstract

Molecular imaging technologies are advancing rapidly and optical techniques in particular are set to play a large role in preclinical pharmaceutical testing. These approaches, however, are generally unable to quantify the level of expression of imaging probe reporters. In this study a novel method of quantification is presented using dual-probe fluorescence imaging, where an endothelial growth factor receptor (EGFR) fluorescent probe was paired with a non-targeted probe before being injected, and tracer kinetic compartmental modeling was used to determine EGFR expression in a region of interest from the uptake curves of the two drugs in that region. The approach was tested out in a simulation experiment and then applied in an *in vivo* study in one mouse to investigate EGFR expression in various tissue types (pancreas, pancreas tumor, and leg). The binding potentials (a unitless correlate of target availability) of EGFR expression in the various tissue types were 8.57, 25.64, and 0.11 for the pancreas, pancreas tumor, respectively. For the pancreas and leg, these results correlate well with expected levels of EGFR expression, with the pancreas demonstrating a much higher expression than the skin and also as expected, the tumor expressed much more EGFR than either healthy tissue.

Keywords

fluorescence molecular imaging; tracer kinetic modeling; dual probe imaging

1. INTRODUCTION

New advances in biomedical optics engineering and fluorescent molecular probe design have made it possible to carry out whole-body optical molecular imaging in small animals and deep tissue imaging in clinical applications (*e.g.* whole breast imaging). These advances have led to an eruption of commercial systems designed specifically for small animal optical molecular imaging all vying to capture the massive market of preclinical pharmaceutical trials [1]. To this point, the major selling point of these systems has been lower cost compared to nuclear medicine or magnetic resonance molecular imaging approaches; however, a real advantage of optical systems that has not fully been realized is the potential to image more than one molecular signal concurrently. In this study, a molecular imaging approach is demonstrated that employs the multi-probe resolution property of optical systems to its advantage for quantifying receptor expression. This is an extension of earlier work using a dual-probe injection [2], but here is matched with accurate modeling to quantify the observed binding potential of the tissue of interest.

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Molecular imaging is used to investigate biological processes and cell signaling expression *in vivo*; however, most of the imaging approaches have offered only a qualitative measure of receptor expression. As such, researchers generally go on the assumption that the level of probe uptake in a region of interest is directly proportional to the expression of the specific receptor. Owing to variability in hemodynamics and vasculature, each of which affect the uptake of a probe, between different tissue types and between individuals, it is nearly impossible to use this approach of single molecule imaging to characterize levels of receptor expression definitively. In particular, this is a problem for cancer imaging because tumors have often have poorly formed vasculature and lymphatic drainage, which predisposes molecular imaging agent to leak into and accumulate in tumors even in the absence of probe-specific receptor expression [3].

Our approach to quantifying receptor binding employs a reference probe (*i.e.*, a fluorescent probe that does not bind to any specific receptor) to account for the non-specific uptake of a targeted probe in any tissue. The idea is to use a reference probe and the targeted probe labeled with different fluorophores that emit and/or absorb at different wavelengths. This enables uptake of both probes to be measured simultaneously with fluorescent imaging devices that provide spectral unmixing.

As a first step to testing out this dual-probe approach for quantifying receptor expression, the stability of the model was investigated by fitting the model to noise-added simulated data. Next, we tested out the model in an *in vivo* study, in an attempt to image epithelial growth factor receptor (EGFR) expression, a target for cancer therapy that is overexpressed in some tumor lines [4], in nude mice inoculated with either a human pancreas tumor (ASPC-1) or a human glioma (U251) using the fluorophore, IRdye-800CW (LI-COR Biosciences, Lincoln, NE) tagged with EGF as a targeted probe and free IRdye-700CW as the reference probe.

2. MATERIALS AND METHODS

2.1 The dual-probe model

The idea behind the dual-probe approach was to use a reference probe as a substitute for the blood and unbound concentrations of the targeted probe (see Fig. 1). Then, by using the uptake of the reference probe in the same region-of-interest as the uptake of the targeted probe, it could be insured that the hemodynamics and vasulature for the two tracers were the same because they were collected from the same tissue.

The dual-probe model is based on solving differential rate equations from a pair of compartment models: a one-tissue compartment model for the reference probe (Fig. 1a) and a two-tissue compartment model for the targeted probe (Fig. 1b).

The differential rate equation for the one-tissue compartment model (Fig. 1a) is

$$\frac{dC_r}{dt} = K_1' C_p(t) - k_2' C_r(t) \quad (1)$$

and for the two-tissue compartment model (Fig. 1b) are,

$$\frac{dC_f}{dt} = K_1 C_p(t) - k_2 C_f(t) - k_3 C_f(t) + k_4 C_b(t) \quad (2)$$

and

$$\frac{dC_b}{dt} = k_3 C_f(t) - k_4 C_b(t). \quad (3)$$

Regions-of-interest measured by any molecular imaging device will then be a weighted sum of the various compartments and blood volume presented in Fig. 1, where the reference probe uptake curve, $REF(t)$, can be written as,

$$REF(t) = v_p C_p(t) + C_r(t) \quad (4)$$

and the targeted probe uptake curve, $ROI(t)$, can be written as,

$$ROI(t) = v_p C_p(t) + C_f(t) + C_b(t). \quad (5)$$

Equations (1)-(5) can be rearranged and solved to get the following parameters: k_2 (related to the vascular permeability), and BP, the “binding potential”, is equal to k_3/k_4 . For *in vivo* studies, this BP is proportional to the receptor density multiplied by the affinity of the probe to the receptor [5]. Since the affinity of the probe for the receptor should not change from one tissue or one individual to the next, the BP measured in this study is directly proportional to the level of receptor expression.

2.2 Simulation

To test out the stability of the dual-probe model for determining k_2 and BP, simulated ROI curves were produced using a random reference probe uptake curve from the animal experiment described in Section 2.3. Typical values of k_2 and BP in normal tissue were chosen (0.1 min^{-1} , and 3, respectively). Stochastic Gaussian noise was then added to the simulated ROI curve at 1 and 10% of the peak curve intensity, before fitting model back to the simulated data to extract the three input parameters. This step was repeated 1000 times and the average values of each parameter, plus or minus the standard deviation were calculated. Other values of k_2 and BP were tested out; however, the results are not presented here, but discussed briefly.

2.3 Animal experiment

The dual-probe model was further studied in a six-week-old SCID male nude mice (Charles River, Wilmington, MA), inoculated orthotopically with a xenograft pancreatic cancer cell line (ASPC-1). The cancer cells were implanted using an approach discussed in detail in a previous publication [6]. Tumors were allowed to grow for approximately two weeks before imaging. Just prior to imaging, the mouse was anesthetized with an intra-peritoneal injection of ketaminexylazine, the superficial tissue was removed from about the tumor, and the mouse was placed tumor-side down on a glass slide and a tail vein was catheterized for probe injection. Once plated, the mouse was positioned in an Odyssey Scanner (LI-COR Biosciences, Lincoln, NE). The Odyssey Scanner has two lasers at 685 and 785 nm wavelengths and is designed to decouple fluorescence from the LI-COR 700 nm fluorescent dye and the LI-COR 800 nm fluorescent dye by raster scanning the lasers over the sample and decoupling the fluorescence signals with a set of dichroic mirrors.

The scanning procedure included collecting a pre-injection scan to determine background fluorescence followed by an injection of an EGFR-targeted probe (IRDye-800CW-EGF) mixed with a non-targeted reference probe (free IRDye-700CW). Follow up scans were repeated every 90 seconds up to 20 minutes and then at intervals of 6 minutes up to 1 h post-injection. Following the scan, the animals were sacrificed and the tumors and pancreas were harvested for immunohistochemistry (the results of which are not presented here). The basic scanning procedure is summarized in Figure 2.

A custom Matlab (MathWorks, Natick, MA) program was written to select regions-of-interest from the pancreas, tumor and leg of each mouse. From each of these regions-of-interest, the uptake curve of both the targeted and reference probes were calculated as the mean fluorescence at 800 and 700 nm, respectively, over time. The dual-probe model was then applied to each of these curve pairs to calculate the physiological parameters, k_2 and BP using the non-linear least squares fitting algorithm, `fminsearch` in Matlab. To be thorough, these parameters were also determined in the tumor using the targeted probe uptake curve from the leg as a possible reference tissue input, as opposed to using the reference probe. To account for differences in quantum efficiency and system sensitivity between the two probes, the ratio of the two probe uptake curves in the leg (where EGFR expression was expected to be very low) was used as a factor for scaling the reference probe fluorescence to be comparable to the targeted probe.

3. RESULTS AND DISCUSSION

3.1 Simulation

The results of the simulation experiment demonstrate that under the physiological and probe-specific conditions used in this study's animal experiment, the dual-probe model is quite robust at returning accurate parameters even in the face of considerable added noise. Table 1 presents the average k_2 and BP values that were inputted into the model, and extracted from fitting after the addition of 1 and 10% noise. The excellent agreement between the parameter results from the model and the inputs at 1% noise (where we believe our collected data falls), suggests that the technique is highly accurate. At 10% noise, the results of k_2 determination are strong; however the determination of BP becomes quite variable and the parameter tends to be overestimated. Figure 3 presents an example of the fit when 10% noise has been added to the simulated data.

Though the results are not shown, we did notice that the model became increasingly sensitive to noise as we decreased k_2 . *I.e.*, this model may not be ideal for probes that have very low vascular permeability.

3.2 Animal Experiment

An example of the differences between the uptakes of the targeted and reference probes in a pancreas tumor can be observed in Fig. 2c. There was a clear dichotomy in the uptake curves: there was a rapid and then gradual increase of the targeted probe concentration and a rapid increase and then gradual decrease in the concentration of the reference probe. The fit of the targeted probe uptake curve in the tumor is presented in Figure 4 and the k_2 and BP values for the leg, pancreas, and ASPC-1 pancreas tumor are presented in Table 2.

The highest binding potential, and therefore the highest level of EGFR expression, was found in the pancreas tumor as expected. With the pancreas tissue expressing more EGFR than the leg. As mentioned in Section 2.3, we also tested out the potential to use the targeted probe uptake curve in the leg as a reference tissue input. As suspected, despite an absence of EGFR expression in the leg, the hemodynamic differences between the leg and tumor were presumably too great for the leg to act as a good reference tissue: for example the average binding potential for the pancreas tumor was greater than 1×10^{12} and k_2 was negative, all values well outside a physiologically reasonable range.

4. CONCLUSIONS

In this proceeding, a dual-probe fluorescence molecular imaging technique was presented to quantify receptor expression *in vivo*. This approach essentially utilizes the uptake curve of a non-targeted reference probe to be used as a surrogate of non-specific probe uptake and

blood plasma probe contamination. It should be noted that there are more straight-forward approaches that utilize only a single targeted probe, while providing quantitative receptor expression; however, these approaches either require knowledge of the probe concentration in the blood over time [7] (which is very difficult to acquire in small animals because of a limited blood volume) or requires many hours of imaging to capture kinetics in time windows where the concentration of the probe in the blood can be assumed to be negligible [8]. The presented approach circumvents both these requirements providing an accurate measure of probe vessel permeability (k_2) and receptor expression (BP) in a 30-minute scan, without the requirement of blood sampling.

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Figure 1.

Compartmental Models for (a) the reference tracer and (b) the specific tracer. C_p is the blood concentration of the tracer, C_r and C_f are the free extravascular compartment concentrations of the two tracers, respectively, and C_b is the concentration of bound specific tracer. The arrows represent the flow of the tracers between the compartments with directional flow rates, k . The dashed lines encapsulate the imaging regions-of-interest for both the reference tracer (REF) and the specific tracer (ROI), each a sum of the constituent compartments taking into account the fraction blood volume v_p .

Figure 2.

(a) Image of mouse with skin resected on a glass slide. (b) Odyssey Scanner image (1:leg, 2:tumor, and 3:pancreas). (c) Uptake curves of both probes in the tumor tissue.

Figure 3. Dual-probe model fit (red line) to a simulated targeted probe uptake curve (blue line with data points) with 10% of peak noise added and a $k_2 = 0.1 \text{ min}^{-1}$ and $\text{BP} = 3$. The predicted k_2 and BP for this particular fit were 0.12 min^{-1} and 2.5, respectively.

Figure 4. Dual-probe model fit to a targeted probe uptake curve in a tumor. The blue points are the raw data collected at 800 nm using the Odyssey Scanner from the ASPC-1 orthotopic pancreas tumor. The red line is the fit of the data using the dual-probe model.

Table 1

Physiological parameter results from simulation study

	Inputs	Outputs with 1% noise	Outputs with 10% noise
k_2 (min^{-1})	0.10	0.100 ± 0.002	0.10 ± 0.03
BP	3.0	3.00 ± 0.12	4.8 ± 14.0

Table 2

Physiological parameters from the dual-probe fitting routine in various tissue types

	Leg	Pancreas	Pancreas Tumor
K₂ (min⁻¹)	0.28	0.31	0.64
BP	0.11	8.57	25.64

Values are presented as mean $\pm$ SD.* $p < 0.05$ compared to leg value.# $p < 0.05$ compared to pancreas tumor.