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Simultaneous extracellular and intracellular quantification of EGFR using paired-agent imaging in an *in ovo* tumor model

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Abstract

Quantification of protein concentrations is often a static and tissue destructive technique. Paired-agent imaging (PAI) using matched targeted and untargeted agents has been established as a dynamic method for quantifying the extracellular domain of epidermal growth factor receptor (EGFR) *in vivo* in a variety of tumor lines. Here we extend the PAI model to simultaneously quantify the extracellular and intracellular regions of EGFR using novel cell membrane permeable fluorescent small molecules, TRITC-erlotinib (targeted) and BODIPY-N-erlotinib (non-binding control isoform) synthesized in house. An EGFR overexpressing squamous cell carcinoma cell xenograft tumor, A431, was implanted on the chorioallantoic membrane (CAM) of the embryonated chicken egg. In total six fluorescent molecules were administered and monitored over 1 h using multi-spectral imaging. EGFR concentrations were determined using both extracellular and intracellular PAI methods. The fluorescent molecules used for extracellular PAI were ABY-029, an anti-EGFR Affibody molecule conjugated to IRDye 800CW, and a Control Imaging Agent Affibody molecule conjugated to IRDye 680RD. The intracellular PAI (iPAI) fluorescent molecules were cell membrane penetrating TRITC-erlotinib, BODIPY-N-erlotinib, and BODIPY TR carboxylate, as well as cell membrane impermeant control agent, Alexa Fluor 647 carboxylate. Results from simultaneous imaging of both the extracellular and intracellular binding domains of EGFR indicate that concentrations of intracellular EGFR are higher than extracellular. This is anticipated as EGFR exists in two distinct populations in cells, cell membrane bound and internalized, activated protein. iPAI is a promising new method for quantifying intracellular proteins in a rapid tumor model on the chicken CAM.

Keywords

paired-agent imaging; intracellular paired agent imaging; multi-spectral imaging; chicken chorioallantoic membrane; epidermal growth factor receptor

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1. INTRODUCTION

Substantial efforts to characterize and categorize cellular response mechanisms are underway, where the most commonly recognized mechanisms are based on monitoring cell signaling pathways [1, 2]. Current methods to evaluate cell signaling are limited to techniques that destroy tissue samples (Western blot = lysis, immunohistochemistry = fixation and permeabilization, etc.) and so can only represent a “snapshot in time.” Additionally, analysis of cell lysates, involves homogenization where known tumor heterogeneity confounds interpretation. A dynamic method of monitoring intracellular protein concentrations is needed in living systems in order to capture changes associated with cell signaling and intracellular processes.

Our “paired-agent” imaging (PAI) approach has been developed over the past decade, where the biological and computational parameters have been rigorously tested [3–12]. PAI uses a second, spectrally distinct, paired control imaging-agent that is of similar size to the targeted agent, but does not bind to the target. The control agent is co-injected with any targeted imaging agent. Since the two agents are spectrally distinct, their respective dynamics in any tissue can be measured; the dynamics of the control agent can be used to account for all non-specific aspects of the targeted agent dynamics (Figure 1; ROI_{EC} , ROI_{ET}), and thus provides quantifiable binding information of targeted agents [13]. Intracellular PAI (iPAI) extends the cell-surface PAI method to include cell membrane permeable targeting agents in order to quantify intracellular protein expression (Figure 1; ROI_{EC} , ROI_{IC} , and ROI_{IT}). In the case of iPAI, a triple-agent modeling approach is incorporated where one control imaging agent assesses tissue extracellular concentration, a second control agent assesses the extracellular and intracellular concentration, and a targeted imaging agent assesses the concentration bound to the intracellular receptors. By solving the differential equations that govern the 1-, 2-, and 3-tissue compartment models associated with each of these three imaging agents, a simplified equation can be achieved relating the uptake of all three imaging agents and the binding potential, BP , a parameter proportional to the concentration of the targeted biomolecule. The iPAI model is mathematically complex, so is not easily represented, but for clarity is simply expressed as a function in Figure 1.

Patient derived (PDX) models are useful for developing personalized therapy [14–19]. However, murine PDX models are often not effective for therapeutic planning due to the length of time it takes to grow and expand tumors in mice. A more rapid method is required for optimal personalized therapy [14, 18, 19]. Rapid and cost-efficient PDX models, such as the chicken chorioallantoic membrane (CAM) and *in vitro*, 3D-tumor pieces (‘tumoroids’) are being investigated. The CAM is a highly angiogenic tissue bed that lines the eggshell of a chicken embryo during its 20-21 day gestation period [20]. The CAM has been used extensively for studying vascular growth and remodeling, [21–23] (anti-)angiogenic factors [24], biosensors and biomaterials [25, 26], photodynamic therapy [27–29], and as a rapid vehicle for tumorigenesis [30–33], including PDXs [34, 35], Table 1 summarizes the benefits and drawbacks of PDXs in the CAM and tumoroid models, in comparison to the mouse model [14–16, 36, 37]. Advantages of the CAM model as a rapid *in ovo* avatar screen are: 1) implanted xenograft tumors are re-vascularized with CAM vessels within three-days of implantation (Figure 2) [31, 32]; 2) rapid implantation-to-imaging time (<1 week) maintains

xenograft integrity; 3) direct, visible, repeatable tumor access without invasive surgical intervention (*i.e.*, mouse skin chamber window) for quantitative imaging [30]; and 4) cost efficiency compared to rodent PDX models. The CAM offers an exciting intermediate between the mouse and tumoroid models for growing PDXs and for facilitating a rapid, *in vivo* assay.

2. MATERIALS AND METHODS

2.1 Embryonated chicken eggs

Premium Specific Pathogen-Free (SPF) embryonated chicken eggs were purchased from Charles River (Norwich, CT). Eggs were received and either immediately placed in a 37 °C incubator at 60% humidity to initiate gestation, or placed at 10 °C, for up to 14 days, to halt gestation and delay incubation. Eggs were rotated daily until Day 4 of incubation, when the eggshell was “windowed.” The windows were sealed with cellulose tape and were returned to the incubator to continue gestation. On Day 9 of incubation, the cellulose tape was removed and up to 5 1-mm³ A431 xenograft pieces were implanted on the CAM by creating a small abrasion with a 25-gauge needle and placing the tumor piece over the top. Small wounds in the CAM surface can aid in vascularization of the tumor piece. The window was again sealed with cellulose tape and returned to the incubator. On Day 13 of incubation, 100 µL of rocuronium bromide was dropped onto the CAM away from the xenografts at least 20 minutes prior to imaging. Rocuronium bromide is an agent that provides skeletal muscle relaxation and minimizes movement of the embryo for imaging.

2.2 Cell culture and tumor production

A431 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% v/v fetal bovine serum (FBS) and 1% penicillin:streptomycin. Subcutaneous A431 tumors were grown in athymic nude mice by implanting 1x10⁶ cells in 50 µL in the subcutaneous space on the right hind quarter. Tumors were allowed to grow until they reached a volume of ~1,000 mm³, measured from calipers. The mice were sacrificed using cervical dislocation under isoflurane anesthesia (1 L/min O₂; 2% isoflurane) and the tumor was excised. The tumors were diced into ~1 mm³ sections in complete cell culture media and either immediately implanted on the surface of the chicken CAM or frozen at 80 °C in 10% dimethylsulfoxide, 40% FBS and 50% complete cell culture medium for long term storage.

2.3 Imaging agents

Six fluorescent agents were used in this study, with the following functions: 1) ABY-029, which is IRDye 800CW (LI-COR Biosciences, Inc., Lincoln, NE) conjugated to anti-EGFR Affibody molecule (Affibody AB, Sweden) was used as the PAI targeted agent; 2) IRDye 680RD (LI-COR Biosciences, Inc.) conjugated to Imaging Agent Control Affibody molecule (Affibody AB) was used as the PAI untargeted control agent; 3) TRITC-erlotinib was used as the cell membrane penetrating iPAI targeted agent; 4) BODIPY-N-erlotinib is a non-functional isoform of erlotinib that was used as the cell membrane penetration iPAI untargeted control agent; 5) Alexa Fluor 647 carboxylate was used as the iPAI cell membrane impermeant untargeted control agent; and 6) BODIPY TR carboxylate was administered as an additional cell penetrating fluorophore for demonstration of spectral

unmixing but was not used to determine PAI or iPAI binding potential. All agents were administered at 0.1 nmole per egg, via intravenous injection using a 30-gauge needle.

2.4 Multispectral paired-agent imaging

The Nuance EX (Perkin Elmer, Waltham, MA) multispectral camera (450-950 nm) was used in conjunction with an Olympus BX51 microscope and a PhotoFluor LM-75 light source (89 North, Burlington, VT). Basis spectra for the chicken CAM autofluorescence were collected from a naïve egg and basis spectra from each individual fluorophore were collected in the chicken CAM. The collected basis spectra were used in conjunction with the Nuance EX software to spectrally separate the signal from individual fluorophores from the CAM and tumor autofluorescence at each time point.

2.5 Kinetic modeling for paired-agent imaging

The kinetic model describing the quantification of extracellular PAI has been well described previously [6, 10–12, 38]. The iPAI model employed in this work assumed that transport of the intracellular imaging agents (both targeted and control) between the extra- and intracellular spaces was relatively slow compared to extravascularization (i.e., $k_5, k_6 < K_1, k_2$; see Fig. 1). Under these conditions a single control agent is not sufficient to account for nonspecific effects and it becomes necessary to have two different control agents: one (the extracellular control) that exhibits similar K_1/k_2 to the targeted agent but has $k_5 = 0$, and another (the intracellular control) that exhibits similar K_1/k_2 and k_5/k_6 to the targeted agent, with $k_7 = 0$. A system of first order differential equations for all three agents that are co-administered (intracellular targeted, 3-tissue compartment model; intracellular control, 2-tissue model; and extracellular control, 1-tissue model) is solved simultaneously with inputs of the intracellular control and extracellular control kinetics as inputs. Unknowns parameters, k_2 and k_7/k_8 (the binding potential, BP) are then estimated by fitting these equations to the kinetics of the targeted intracellular agents via Levenberg-Marquardt non-linear least squares fitting.

3. RESULTS AND DISCUSSION

The embryonated chicken CAM has long been used as a model for the growth of tumors and testing both drugs and biomaterials. Here, A431 xenograft tumor pieces were successfully implanted onto the chicken CAM and demonstrated typical radiating vascular pattern indicative of revascularization (Figure 2). The chicken embryo was successfully immobilized using rocuronium bromide as a paralytic agent. Typically, paralytic agents in animal models requires the animal be intubated to maintain regular breathing. However, because the chicken embryo does not utilize lungs for breath, but rather uses the chicken CAM for gas exchange through the shell, paralytic agents can be used without detrimental effects to the embryo itself. Using this method for immobilization also allows repeated imaging sessions, although only a single imaging session was used here.

The Nuance EX multispectral camera was used to collect multispectral images of the A431 tumor implanted in the CAM (Figure 3). Autofluorescence images were collected prior to injection of the fluorescent agents (Figure 3). As expected, the measured autofluorescence in

the A431 tumor (grey dashed line) closely matched the autofluorescence basis spectrum (black line) collected on previous samples. The mixture of six fluorescent agents (ABY-029, IRDye 680RD Affibody Control Imaging Agent, TRITC-erlotinib, BODIPY-N-erlotinib, Alexa Fluor 647, and BODIPY TR carboxylate) were injected into a large CAM feeder vessel and multispectral images were collected at approximately 2 min intervals for an hour. In the 6-color fluorescence image, the grey dashed line in the corresponding spectrum represents the measured fluorescence (Figure 3). Each multispectral image was saved as an “image cube” in the Nuance EX software and each fluorophore contribution was determined by spectrally unmixing the image cube at each time point using the basis spectra collected in naïve eggs and represented in corresponding colors in the autofluorescence and 6-color fluorescence images. A composite fluorescence image was created after spectrally unmixing that removed the autofluorescence contribution and represents the distribution of each individual fluorophore. The deconvolved fluorescence intensity of each imaging agent in the tumor region is also demonstrated (Figure 3).

The kinetics of each individual fluorophore averaged within the tumor over time showed the fluorescence signal of both the extracellular and intracellular targeted agents (ABY-029 and TRITC-erlotinib, respectively) were the highest and retained the longest within the tumor (Figure 4). The untargeted agents had lower normalized signal contribution and were cleared from the tumor faster than the targeted agents. In addition, the intracellular agents (Figure 4, *left*) had a slower uptake within the tumor as compared to the extracellular agents (Figure 4, *right*) that displayed a more rapid uptake into the tumor space. BODIPY-N-erlotinib and BODIPY TR were both non-binding intracellular agents and displayed similar uptake trajectories. BODIPY-N-erlotinib emission was evident in the 500-550 nm region but was difficult to spectrally separate due to the strong autofluorescence contribution in that region and the relatively low quantum yield of the imaging agent. In future studies, the concentration of BODIPY-N-erlotinib could be increased or alternative fluorophores could be investigated to overcome this limitation. It can also be noted that IRDye 680RD-Affibody Control Imaging Agent and Alexa Fluor 647 were both non-binding extracellular imaging agents and demonstrate nearly identical trajectories.

Binding potential maps of extracellular and iPAI for the A431 tumor region were generated from the corresponding fluorescent agents that were used to determine the extracellular PAI binding potential (IRDye 680RD Affibody Control Imaging Agent and ABY-029) and the iPAI binding potential (BODIPY-N-erlotinib, Alexa Fluor 647, and TRITC-erlotinib, Figure 5). Notably, the extracellular and intracellular PAI binding potential maps showed similar distribution. However, the intracellular PAI binding potential showed higher EGFR concentration as expected. This was likely due to the internalization of EGFR during its signaling cascade, creating higher intracellular concentrations of EGFR because TRITC-erlotinib could bind to both membrane bound EGFR and internalized EGFR, representing two distinct populations. Only the membrane bound EGFR was exposed to the extracellular fluorescent agents, and therefore will have a lower contribution to the EGFR concentration observed in the extracellular PAI.

4. CONCLUSIONS

PAI for the quantification of protein concentrations *in vivo* is a promising technique. Extracellular EGFR is readily quantified using PAI, while the intracellular binding domain can be quantified by using cell membrane penetrating targeted and non-targeted agents. Further validation will be required to correlate absolute extracellular and intracellular EGFR concentrations to the PAI and iPAI determined concentrations, respectively. In addition, alternative fluorophores are being investigated in order to increase the observed signal and reduce the contribution from autofluorescence during spectral unmixing.

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Figure 1. Illustrated kinetic models for paired agent imaging (PAI) and intracellular paired-agent imaging (iPAI).

Established PAI requires two imaging agents, a targeted agent (ROI_{ET}) and an extracellular control (ROI_{EC}) to quantify extracellular receptor concentrations. The novel iPAI model employs 3 agents, ROI_{EC} , an intracellular control (ROI_{IC}), and an intracellular targeted (ROI_{IT}) to quantify intracellular protein expression. K_1 and k_2 are the rates of extravascular leakage and tissue efflux, k_3 and k_4 represent the “on” and “off” rates of extracellular target binding, k_5 and k_6 are the rate constants of transport across the cell membrane, and k_7 and k_8 represent the “on” and “off” rates of intracellular protein binding. **PAI Equation:** Is the Lammertsma equation. **iPAI Equation:** A complex solution involving analytical solutions to the 1-, 2-, and 3-compartment models associated with ROI_{EC} , ROI_{IC} , and ROI_{ET} . **Binding Potential (BP) Equation:** A key parameter proportional to B_{avail} , the concentration of available binding sites, and k_A , the receptor-target binding affinity.

Figure 2.

Embryonated chicken eggs (*left*) develop a chorioallantoic membrane (CAM) between Days 4-13 of gestation. These blood vessels are undergoing angiogenic growth and are able to support the revascularization and growth of tumor explants. A xenograft A431 tumor was implanted onto the chicken CAM on Day 9 of gestation (*right*). The white arrows indicate blood vessels that have revascularized the tumor explant and are visible in a radial pattern around the tumor.

Figure 3. Imaging an in ovo avatar using 6-spectrally distinct fluorophores with the Nuance EX multispectral camera (Perkin Elmer).

Top row: *In ovo* raw images of multispectral autofluorescence (510-850 nm), multispectral fluorescence post-injection of 6 fluorophores (0.1 nmol/100 μ L, each), and RGB. *Middle row:* Basis spectra for each fluorophore (colors correspond to last row), average autofluorescence (black), and signal from middle of tumor (grey dashed). The spectrally unmixed composite fluorescence of each fluorophore without autofluorescence contribution (*right*). *Bottom row:* Individual spectrally unmixed images of each fluorescent agent.

Figure 4. Uptake of fluorescent agents within the A431 tumor implanted in the chicken CAM. The cell-membrane-permeable “intracellular agents” are displayed on the *left* and the cell-membrane-impermeable “extracellular agents” are displayed on the *right*. The time course of the average fluorescence intensity within the tumor was determined by individual unmixing the multi-spectral images and averaging the fluorescence intensity within the tumor region-of-interest.

Figure 5.

Validation of iPAI compared to Paired-Agent Imaging (PAI). *Bottom:* iPAI of EGFR intracellular tyrosine kinase domain was performed using a cell-permeable control BODIPY-N-Erlotinib, an extracellular control Alexa Fluor 647 (AF647) and a cell-membrane-permeable targeted TRITC-Erlotinib to produce a spatial map of iPAI binding potential (BP; i.e., receptor concentration). *Top:* An *in ovo* avatar white-light image and the PAI agents – extracellular control agent (IR680-Affibody control) and anti-EGFR-IRDye800 agent (ABY-029) – and the corresponding PAI BP map.

Table 1.

Comparison of patient-derived tumor models.

TUMOR CHARACTERISTIC	PATIENT DERIVED MODEL		
	MOUSE	CAM	TUMOROID
Biological System	<i>In vivo</i>	<i>In ovo</i>	<i>In vitro</i>
Multiple tumor regions in one sample	✗	✓	✓
Immune System Involvement	✓	✓	✗
Vascular Involvement	✓	✓	✗
Extracellular matrix from host	✓	✓	✗
Ability to repeatedly image exposed tumor	✗*	✓	✓
Cost	\$\$\$\$	\$\$	\$
Time to Analysis	⌚⌚⌚⌚	⌚	⌚