Supplementary material to manuscript

Rinsing paired-agent model (RPAM) to quantify cell-surface receptor concentrations in topical staining applications of thick tissues

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I. Compartment modeling for imaging agent staining and rinsing of thick tissues

Compartment modeling is a standard method to derive mathematical expressions that approximate the characteristics of imaging agent distribution in molecular imaging studies (Innis *et al*., 2007). For 3D cell culture or thick tissue staining and rinsing, a three-compartment model can be used to model the distribution of a cell-surface receptor targeted imaging agent, with the three compartments including: 1) a "bound" compartment representing the concentration of imaging agent bound to the targeted receptor, C_b, 2) a "free" compartment representing the concentration of unbound agent in the medium, C_f, and 3) a "stain/rinse" compartment representing the concentration of the agent in the staining or rinsing solution, C_{sr} (**Fig. S1a**). A control (untargeted) imaging agent can be modeled similarly by two compartments, including only a "free" compartment, C_f', and a "stain/rinse" compartment, C_{sr}' (**Fig. S1b**).

In general, imaging agents in any compartment are free to transfer to an adjacent compartment. The rate of transfer between compartments is assumed to follow first-order kinetics where the rate of change of concentration in any one compartment is directly proportional to a weighted sum of the concentrations in that compartment and the adjacent compartments. As such, the three- and two-compartment models characterizing targeted and control imaging agent

distributions, respectively, during staining or rinsing phase can be described by the following set of differential equations:

$$\begin{aligned}
\frac{dC_f}{dt} &= k_{in}(t)C_{sr}(t) - k_{out}(t)C_f(t) - k_3C_f(t) + k_4C_b(t), \\
\frac{dC_b}{dt} &= k_3C_f(t) - k_4C_b(t), \\
\frac{dC_{sr}}{dt} &= -k_{in}(t)C_{sr}(t) + k_{out}(t)C_f(t) \quad ,
\end{aligned} \tag{S1}$$

$$\begin{aligned}
\frac{dC'_f}{dt} &= k_{in}(t)C'_{sr}(t) - k_{out}(t)C'_f(t) \quad , \\
\frac{dC'_{sr}}{dt} &= -k_{in}(t)C'_{sr}(t) + k_{out}(t)C'_f(t) \quad ,
\end{aligned}$$

where k_{in}, k_{out}, k_3, and k_4 are rate constants associated with the likelihood of diffusion of imaging agent from the rinsing/staining solution to the cell medium (k_{in}), diffusion from the cell medium to the rinsing/staining solution (k_{out}), binding to the targeted receptor (k_3), and disassociation from the targeted receptor (k_4), respectively. The system of differential equations in **Eq. (S1)** is directly adaptable for simulating the staining process, in addition to the rinsing process. For rinsing procedures, where t_r is the time the rinse is initiated, $C_{sr}(t_r)$ and $C_{sr}'(t_r) = 0$, indicating no imaging agent in the rinsing solution. For staining procedures, where t_s is the time the stain is initiated, $C_{sr}(t_s)$ and $C_{sr}'(t_s) = C_0$, where C_0 is the concentration of imaging agent in the staining solution. Imaging is typically done without the staining solution or rinsing solution in contact with the medium. In this case, k_{in} and k_{out} are both set to zero in **Eq. (S1)**, which is why these rate constants are represented as functions of time in the equations. Under these conditions, the signal measured from the targeted (S_T) and control (S_C) imaging agents as a function of time, t, can be expressed as:

$$\begin{aligned}
S_T(t) &= \eta_T\left[C_f(t) + C_b(t)\right], \\
S_C(t) &= \eta_C C'_f(t) \quad ,
\end{aligned} \tag{S2}$$

where η_T and η_C are constants relating the concentration to measured signal of targeted and control imaging agents, respectively, in the medium. The signals, S_T and S_C, are assumed to be in units proportional to fluorescence measured by the imaging system.

II. Rinsing paired-agent model (RPAM) estimate of binding potential (BP_{RPAM})

In standard fluorescence imaging systems, it is hard to monitor the concentration of the imaging agent in the staining or rinsing solution as a function of time, as would be necessary to solve the system in **Eqs. (S1)** and **(S2)**. As such, the staining/rinsing compartments C_{sr} and C_{sr}' were represented in terms of general functions, $k_r(t)$ and $k_r'(t)$, as follows:

$$\begin{aligned} k_r(t) &= k_{out} - k_{in}\frac{C_{sr}(t)}{C_f(t)} \quad &, for\ staining/rinsing, \\ k_r(t) &= 0 \quad &, for\ "imaging", \end{aligned} \tag{S3}$$

$$\begin{aligned} k_r'(t) &= k_{out} - k_{in}\frac{C_{sr}'(t)}{C_f'(t)} \quad &, for\ staining/rinsing, \\ k_r'(t) &= 0 \quad &, for\ "imaging", \end{aligned}$$

where "imaging" refers to the time window of imaging when the medium is not in contact with either the staining or rinsing solutions. In general, a rinsing solution is chosen so that $C_{sr} << C_f$ for all time points, and a staining solution is chosen so that $C_{sr} = C_{sr}' >> C_f, C_f'$. Under these conditions, it can be assumed that $k_r(t)$ is equivalent to $k_r'(t)$. Based on these assumptions, the system in **Eq. (S1)** can be simplified to:

$$\begin{aligned} \frac{dC_f}{dt} &= -k_r(t)C_f(t) - k_3C_f(t) + k_4C_b(t), \\ \frac{dC_b}{dt} &= \quad k_3C_f(t) - k_4C_b(t), \end{aligned} \tag{S4}$$

$$\frac{dC_f'}{dt} = -k_r(t)C_f'(t) \quad .$$

The compartment models of targeted and control agents for RPAM are represented in **Figs. S1c** and **d**, respectively. Combination of the first two expressions in **Eq. (S4)** results in:

$$\begin{aligned} \frac{d(C_f + C_b)}{dt} &= -k_r(t)C_f(t), \\ \frac{dC_f'}{dt} &= -k_r(t)C_f'(t). \end{aligned} \tag{S5}$$

If the free and bound compartments are assumed to be in rapid equilibrium (adiabatic approximation) (Lammertsma and Hume, 1996), **Eq. S5** can be further simplified to:

$$C_f(t) \cong \frac{C_f(t) + C_b(t)}{1 + \frac{k_3}{k_4}}, \tag{S6}$$

where k_3/k_4 is an important ratio in kinetic modeling of targeting imaging agents, referred to as the "binding potential" (*BP*) (Mintun *et al*., 1984). *BP* can be shown to be equivalent to the product of the targeted receptor concentration and the affinity of the imaging agent for the receptor (Innis *et al*., 2007). Since an imaging agent's affinity is generally assumed to be constant, *BP* is often considered a direct estimate of the receptor concentration, which is the parameter of interest in most molecular imaging studies (Innis *et al*., 2007).

Combining **Eqs (S2)**, **(S5)**, and **(S6)**, the system can be further simplified to:

$$\begin{aligned} \frac{dS_T(t)}{dt} &= -\frac{1}{1 + BP_{RPAM}}k_r(t)S_T(t), \\ \frac{dS_C(t)}{dt} &= -k_r(t)S_C(t) \quad . \end{aligned} \tag{S7}$$

Eq. (S7) can be tricky to solve, since $k_r(t)$ is a function of S_T and S_C; however, under rinsing conditions, when the C_{sr}/C_f terms can be considered small compared to the k_{out} term in **Eq. (S3)**, it is possible to approximate $k_r(t)$ strictly as a function of t (demonstrated in simulations in this study). Solving these differential equations separately leads to:

$$\begin{aligned} S_T(t) &= S_T(t_i)\, e^{-\frac{1}{1+BP_{RPAM}}\int_0^t k_r(u)\,du}, \\ S_C(t) &= S_C(t_i)\, e^{-\int_0^t k_r(u)\,du}, \end{aligned} \tag{S8}$$

which upon combination and further simplification can be expressed as:

$$\frac{S_T(t)}{S_T(t_i)} = \left(\frac{S_C(t)}{S_C(t_i)}\right)^{\frac{1}{1+BP_{RPAM}}}, \tag{S9}$$

where a nonlinear least-squares fitting algorithm can be used to fit for BP, the estimate of which is referred to as the staining paired-agent model estimate of BP, BP_{RPAM}, in this article. Alternatively, **Eq. (S9)** can be linearized as:

$$\ln\left[\frac{S_C(t)}{S_C(t_i)}\right] = \left(BP_{RPAM}+1\right)\ln\left[\frac{S_T(t)}{S_T(t_i)}\right], \tag{S10}$$

where the plotting of $\ln(S_C(t)/S_C(t_i))$ vs. $\ln(S_T(t)/S_T(0))$ would have a linear relationship with a slope equal to $BP_{RPAM}+1$.

III. Estimation of k_{in} and k_{out}

The parameters, k_{in} and k_{out}, were tested through a series of staining and rinsing procedures on cell-free 3D matrix. Twelve wells (in 2, 6-well plates) containing 1-mL of 0.3% agarose gel were prepared using the same protocol as the "blank" well in the cell culture experiment. Background fluorescence images were taken for all wells.

Staining was carried out in 6 wells for durations of 1, 2, 3, 5, 10, and 20 min, followed by extraction of the staining solution and fluorescence imaging. Typical k_{in} and k_{out} values were then determined by fitting the following equations, through least squares optimization, to the measured fluorescence time-curves:

$$\begin{aligned} s_{Stain}(t) &= \frac{k_{in}}{k_{in}+k_{out}} S_{w0} - \frac{k_{in}}{k_{in}+k_{out}} S_{w0} e^{-(k_{in}+k_{out})t}, \\ s_{Rinse}(t) &= \frac{k_{in}}{k_{in}+k_{out}} S_{f0} + \frac{k_{out}}{k_{in}+k_{out}} S_{f0} e^{-(k_{in}+k_{out})t}, \end{aligned} \tag{S11}$$

where the staining curve, $S_{Stain}(t)$, rinsing curve, $S_{Rinse}(t)$, initial dye signal, S_{w0}, and "zero-rinse" signal, S_{f0}, were imaged and measured for the targeted and control imaging agent, individually.

Six wells were stained with a 1 mL solution of 44-nM IRDye® 800CW EGF and 4-nM IRDye® 700DX NHS Ester solution (LICOR Biosciences) for a duration of 45 min. Staining solutions were extracted and pre-rinse fluorescence images were acquired. Each well was "rinsed" by adding 1 mL of Phosphate Buffered Saline solution for 1, 2, 3, 5, 10, and 20 min, at which time the rinsing solution was extracted, followed by final fluorescence imaging.

IV. RPAM Evaluation Simulation

The full system of differential equations that characterizes staining and rinsing of topically applied imaging agents on thick tissue is expressed in **Eq. (S1)**. The staining time was set to be 45 minutes (time to reach equilibrium given experimental conditions). The effect of BP, rinsing time

and staining time (for dye removal and imaging procedures) on RPAM accuracy were evaluated separately. Time between repeated rinses was also evaluated; however, no effect was observed, so the results were left out the manuscript. Further analysis over a range of *BP* and rinsing time were evaluated. Given input values of k_{in}, k_{out}, k_3, and k_4, targeted and control imaging agent curves were simulated using numerical methods in the form of function ode45() in MATLAB (Mathworks, Natick, MA). The parameters k_{in} and k_{out} were estimated for 3D cell culture using the aforementioned experiments. Since the difference between estimated control and targeted k_{in} and k_{out} were close to each other (within the standard deviation), these numbers were kept the same in the simulation procedure. The parameter k_4 was kept as a constant 0.1 min^{-1} obtained from past studies that explored the affinity between EGF and EGFR (Zhou *et al*., 1993), and *BP* was set to change from 0.1 to 10 to evaluate a 2 orders-of-magnitude range typical of *in vivo* cell conditions. k_3 were then calculated as:

$$k_3 = BP \times k_4. \tag{S12}$$

0.1% Gaussian noise was applied to both curves (outputs from the numerical solution to **Eqs. S1**) separately to obtain fluorescence signal, and binding potential was evaluated using the Ratiometric, DPM-NS, and RPAM methods (BP_{Ratio}, $BP_{DPM\text{-}NS}$ and BP_{RPAM}). This level of noise was similar to conservative estimates from experimental data and calculated as the ratio of the "energy" of random Gaussian noise to the "energy" of the signal – where "energy" of a signal refers to the area underneath the square of the signal over a set time interval.

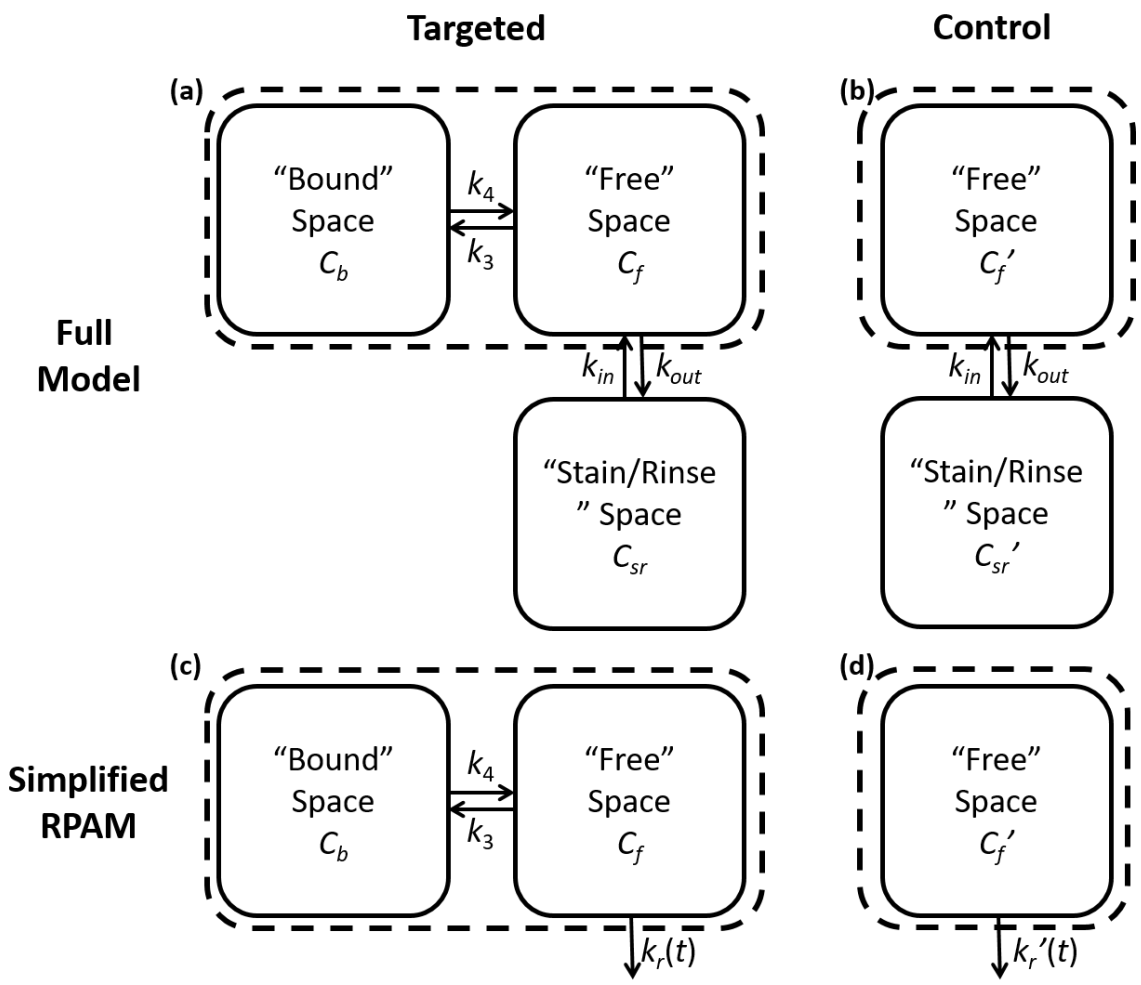

Figure S1. Illustration of the compartment models forming the foundation of the rinsing paired-agent model, RPAM. (a) A three-compartment model representing the distribution of the targeted imaging agent in "thick tissue," between the stain/rinse compartment (staining or rinsing solution), C_{sr}, the "free" space (unbound agent in the tissue/medium), C_f, and the "bound" space (concentration of imaging agent bound to the targeted receptor), C_b. The rate constants k_{in}, k_{out}, k_3, and k_4, govern the rates of transport of the imaging agent from stain/rinse space to free space, free space to stain/rinse space, free space to bound space, and bound space to free space, respectively. (b) A two-compartment model representing the distribution of the control imaging agent in "thick tissue." (c) A simplified two-compartment model that represents the RPAM for the targeted imaging agent. Here $k_r(t)$ is a function replacing the mathematical contributions from k_{in}, k_{out}, and the stain/rinse compartment. (d) A simplified one-compartment that represents the rinsing paired-agent model (RPAM) for the control imaging agent.

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