**In vivo** mouse fluorescence imaging for folate-targeted delivery and release kinetics

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**Abstract:** Many cancer cells over-express folate receptors, and this provides an opportunity for both folate-targeted fluorescence imaging and the development of targeted anti-cancer drugs. We present an optical imaging modality that allows for the monitoring and evaluation of drug delivery and release through disulfide bond reduction inside a tumor in vivo for the first time. A near-infrared folate-targeting fluorophore pair was synthesized and used to image a xenograft tumor grown from KB cells in a live mouse. The in vivo results are shown to be in agreement with previous in vitro studies, confirming the validity and feasibility of our method as an effective tool for preclinical studies in drug development.

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**References and links**

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1. Introduction

Fluorescence has proven to be a useful medical imaging modality [1, 2], specifically as an optical source for guided surgery [3–5] and for studying targeted fluorescence kinetics [5]. Forty percent of human cancer cells (including ovarian, lung, breast, kidney, brain, and colon cancer) over-express folate receptors, and this provides an opportunity to identify tumor nodules using folate-targeted fluorescence imaging, as well as to develop targeted anti-cancer drugs [6–8]. Moreover, inappropriate activated macrophages may result in autoimmune and inflammatory diseases (including lupus, rheumatoid arthritis, and ulcerative colitis) and these macrophages also over-express folate receptors [7,9], allowing for folate-targeting. To maximize the potency of folate-targeted drugs, the drug delivery kinetics (where, when, and how much drug is delivered and released) must be well understood. An optimized dose of the drug should be administered to maximize delivery to unhealthy cells while minimizing negative effects on healthy cells.

To study kinetics, a fluorophore may be attached to the targeting agent (folate) and tracked in a live animal over time using an optical imaging method. Such optical imaging is hindered however by the high degree of scatter present in tissue, and popular methods, such as two-photon microscopy, are useful only within a few hundred micrometers of the tissue surface [10]. Therefore, these methods are insufficient for the study of anti-cancer drug kinetics, which requires knowledge of concentration in deep tissue where tumors occur, and can only be measured in vivo. To account for this high scatter in tissue, a diffusion model can be used to describe the photon transport. By treating light as particles and with sufficient scatter, optical diffusion tomography (ODT) allows for deep-tissue imaging by using a diffusion equation to describe the spatially dependent optical properties [11–13]. Here, we use ODT with fluorescence and a kinetics compartment model incorporated into the forward model to image a folate-targeted fluorophore pair in a mouse tumor in vivo. In terms of drug delivery kinetics, we determine the release rate due to disulfide bond reduction in the tumor, and show that it is consistent with prior in vitro measurements, thus demonstrating the feasibility of our method as a preclinical drug development tool.

The paper is organized as follows. Section 2 describes the folate-targeting kinetics, forward model, the kinetics compartment model, and image reconstruction. Details on the chemical synthesis, mouse preparation, and experimental setup are provided in Section 3. Sections 4 and 5 give the results and discussion, and Section 6 the conclusions.

2. Methods

2.1. Folate-targeting kinetics

Pharmacokinetics describes the change in the number of drug molecules inside the body due to absorption, distribution, and elimination. After an intravenous injection, a drug is partly distributed to the extracellular extravascular space, eliminated (mostly by the liver and kidneys), or remains in circulation. Here, we examine the pharmacokinetics of a folate-targeted anti-cancer drug. In folate targeting strategies [6–8,14–16], the ‘cargo’, which is a drug or imaging agent, is attached to folic acid via a linker and together this system is known as a folate conjugate. Figure 1 illustrates the process of receptor-mediated endocytosis (RME), by which folate bound to the folate receptor is internalized by a cancer cell. In a mouse, the folate conjugate is distrib-
Fig. 1. The folate-DA conjugate (folic acid + linker + DA), bound to the folate receptor, is internalized by the cell. An enzyme cleaves the disulfide (S-S) bond, releasing the acceptor [17].

uted throughout the entire blood supply within 30 seconds after injection. Within 5 minutes, the folate conjugate is internalized by tumor cells, and after 30 minutes, the remaining chemical is mostly cleared from the plasma. In our work, the ‘cargo’ attached to folic acid is a fluorophore donor-acceptor (DA) pair used in lieu of a drug, forming a folate-DA conjugate. The donor and acceptor are connected through a disulfide bond, and upon internalization through RME by the tumor cells, this disulfide bond is cleaved, resulting in free donors and acceptors inside the tumor, and an increase in donor fluorescence. The release process (disulfide bond cleavage) in the tumor cells has a half-time of approximately 6 h in vitro [14]. The unloading rate of the folate conjugate from the tumor into plasma is relatively slow compared to the internalization and release processes. The time at which the release process occurs is important for the design of anti-cancer drugs, as the drug should be released inside the tumor cells (after internalization but before being unloaded from the tumor into the plasma).

We show that the change in the fluorescence and concentration of the fluorophore (DA pair, free donor, and acceptor) molecules allows the delivery and release mechanism to be imaged non-invasively through optical imaging, giving information about the pharmacokinetics. When folate-fluorophores collect at the kidney, disulfide bond reduction does not occur in the kidney and thus there is no acceptor (drug) release nor change in fluorescence [15]. Importantly, the conjugation of molecules (either fluorophores or drug) to folic acid has been shown to not interfere with the high affinity of folate for its receptor nor with its endocytosis into the cell [16]. Therefore, one might expect that the in vivo kinetics measured with a folate-DA indicator is representative of the kinetics of a folate-drug. Additionally, the relevance of a DA pair as a reporter has been verified in vitro [14]. The goal of our work is to monitor, study, and evaluate the folate-targeting mechanism in vivo, allowing for the design of more potent anti-cancer drugs.

2.2. Forward model

In fluorescence optical diffusion tomography (FODT), the coupled diffusion model is given by [18–20]

\[
\nabla \cdot \left[ D_x(r) \nabla \phi_x(r,\omega) \right] - \left[ \mu_{ax}(r) + j\omega / \mu_n \right] \phi_x(r,\omega) = -S_x(r,\omega) \tag{1} \\
\nabla \cdot \left[ D_m(r) \nabla \phi_m(r,\omega) \right] - \left[ \mu_{am}(r) + j\omega / \mu_n \right] \phi_m(r,\omega) = -\phi_x(r,\omega) S_f(r,\omega), \tag{2}
\]

\[
\nabla \cdot \left[ D_x(r) \nabla \phi_x(r,\omega) \right] - \left[ \mu_{ax}(r) + j\omega / \mu_n \right] \phi_x(r,\omega) = -S_x(r,\omega) \tag{1} \\
\nabla \cdot \left[ D_m(r) \nabla \phi_m(r,\omega) \right] - \left[ \mu_{am}(r) + j\omega / \mu_n \right] \phi_m(r,\omega) = -\phi_x(r,\omega) S_f(r,\omega), \tag{2}
\]

\[
\nabla \cdot \left[ D_x(r) \nabla \phi_x(r,\omega) \right] - \left[ \mu_{ax}(r) + j\omega / \mu_n \right] \phi_x(r,\omega) = -S_x(r,\omega) \tag{1} \\
\nabla \cdot \left[ D_m(r) \nabla \phi_m(r,\omega) \right] - \left[ \mu_{am}(r) + j\omega / \mu_n \right] \phi_m(r,\omega) = -\phi_x(r,\omega) S_f(r,\omega), \tag{2}
\]
where \( \mathbf{r} \) denotes the position, \( \phi \, (\text{W/mm}^2) \) is the photon flux density, \( \omega \) is the angular modulation frequency, \( \mu_a \, (\text{mm}^{-1}) \) is the absorption coefficient, \( D \, (\text{mm}) \) is the diffusion coefficient, \( c \) is the speed of light in the medium, the subscripts \( \chi \) and \( m \), respectively, denote parameters at the excitation and emission wavelengths, \( \lambda_\chi \) and \( \lambda_m \), \( S_\chi \, (\text{W/mm}^3) \) is the excitation source term, and \( S_f = \eta \mu_a (1 + j\omega\tau)^{-1} \, (\text{mm}^{-1}) \) is the fluorescence source term. The fluorescence parameters are the lifetime \( \tau \, (\text{ns}) \) and the fluorescence yield \( \tilde{\eta} = \eta \mu_a \, (\text{mm}^{-1}) \), where \( \eta \) and \( \mu_a \) are the quantum yield and absorption of the fluorophore, respectively. The forward model solution to (1) and (2) was formed on an unstructured finite element method (FEM) mesh (based on the TOAST package [21]).

2.3. Kinetic model

With fluorescence-labeling, it is possible to obtain spatial and temporal information of a targeted drug’s delivery to cancer cells through fluorescence imaging, as we show here. To model the kinetics of this process, we incorporate a compartment model into FODT, specifically in the description of the fluorescence source [22]. A three-compartment model (depicted in Fig. 2) is used, where the number of folate conjugate molecules in each compartment is assumed to be uniform. As illustrated in Fig. 2, \( k_1 \, (\text{h}^{-1}) \) is the uptake rate (of the folate conjugate) from the plasma to the tumor, \( k_2 \, (\text{h}^{-1}) \) is the unloading rate from the tumor back to the plasma, \( k_3 \, (\text{h}^{-1}) \) is the elimination rate from the plasma (predominantly by the kidneys), and \( k_4 \, (\text{h}^{-1}) \) is the acceptor cleavage rate (drug release rate) inside the tumor due to disulfide bond reduction. These rates are considered constants, i.e., assumed to be time-invariant. The release rate \( (k_4) \) for folate-targeting is obtained \textit{in vivo} for the first time in this work. We denote \( X_{pDA} \) as the number of folate-DA molecules in the plasma compartment, and \( X_{tDA} \) and \( X_{tD} \) as the number of DA pair molecules and the number of free donor molecules in the tumor compartment, respectively. The steps used to relate \( X_{pDA}, X_{tDA}, \) and \( X_{tD} \) to the fluorescence source \( S_f \) in (2), which can be determined through FODT with optical measurements (fluorescence data), are as follows.

1. We express the change of \( X_{pDA}, X_{tDA}, \) and \( X_{tD} \) with respect to time as functions of
rates $k_1$-$k_4$, resulting in a set of differential equations.

2. We solve the differential equations formed in Step 1 for $X_{pDA}$, $X_{tDA}$, and $X_{tD}$.

3. We define the number of DA pairs in a voxel ($X_{DA}$) and the number of free donors in a voxel ($X_D$) based on the number of fluorophores in each compartment ($X_{pDA}$, $X_{tDA}$, and $X_{tD}$) and the volume fraction of each compartment in a voxel ($v_p$ for plasma and $v_t$ for tumor).

4. We convert $X_{DA}$ and $X_D$ into fluorescence yield $\tilde{\eta}_{DA}$ and $\tilde{\eta}_D$, respectively. The fluorescence source is then given by $S_f = \tilde{\eta}_{DA}(1 + j\omega \tau_{DA})^{-1} + \tilde{\eta}_D(1 + j\omega \tau_D)^{-1}$, with $\tau_{DA}$ the donor lifetime in the presence of the acceptor and $\tau_D$ the free donor lifetime.

5. We perform reconstructions using FODT to retrieve the kinetic information. The fluorescence source $S_f$ in Step 4 follows a multi-exponential model and the exponential parameters (including magnitude and decay constants, shown later as $\gamma_1$-$\gamma_6$) are reconstructed directly through our inversion algorithm, giving the fluorescence source $S_f$ as a secondary (indirect) reconstructed result.

We describe these steps now in detail. The parameters discussed are summarized in Table 1. Donor emission is considered and thus only free donors and DA pairs are included in the model (the acceptor is not considered). Following Step 1, we assume that the change in $X_{pDA}$ follows a single exponential model, giving

$$\frac{dX_{pDA}(t)}{dt} = -(k_1 + k_3) X_{pDA}(t).$$

The solution to (3) is

$$X_{pDA}(t) = X_0 e^{-(k_1+k_3)t},$$

where $X_0$ is the initial number of folate-DA molecules (injected dose) in the plasma. By conservation of mass, the number of molecules in each compartment are related by

$$\frac{dX_{tDA}(t)}{dt} = k_1 X_{pDA}(t) - (k_2 + k_4) X_{tDA}(t),$$

$$\frac{dX_{tD}(t)}{dt} = k_4 X_{tDA}(t) - k_2 X_{tD}(t).$$

The slow unloading rate ($k_2$), and the fast removal of the chemical from the plasma by elimination ($k_3$) and the tumor uptake ($k_1$), allow for the approximation of $X_{pDA}$ with a single exponential model with a fast decay rate $k_1 + k_3$, as seen in (4) [22–25]. The folate-DA conjugate

| Parameter  | Description                                      |
|------------|--------------------------------------------------|
| $k_1$      | Chemical uptake rate from plasma to tumor (h$^{-1}$) |
| $k_2$      | Chemical unloading rate from tumor to plasma (h$^{-1}$) |
| $k_3$      | Chemical clearing rate from plasma (h$^{-1}$) |
| $k_4$      | Acceptor cleavage rate (h$^{-1}$) |
| $X_{pDA}$  | Number of folate-DA molecules in the plasma compartment |
| $X_{tDA}$  | Number of folate-DA molecules in the tumor compartment |
| $X_{tD}$   | Number of free donor molecules in the tumor compartment |
| $X_0$      | Initial number of folate-DA molecules in the plasma compartment |
| $v_p$      | Volume fraction of a voxel in the plasma |
| $v_t$      | Volume fraction of a voxel in the tumor |
| $\alpha_{DA}$ | Fluorescence of a folate-DA molecule (mm$^{-1}$) |
| $\alpha_D$ | Fluorescence of a free donor molecule (mm$^{-1}$) |

Table 1. Parameter definitions used in the kinetics model.
is internalized and cleaved inside the tumor cell and the resulting free donor is unloaded from the tumor into the plasma. Thus, with small unloading rate \( k_2 \) and large elimination rate \( k_3 \), the number of free donor molecules in the plasma is negligible [16]. The specifics of the folate uptake and release process (described in Section 2.1) support these assumptions.

In Step 2, we solve differential equations (5) and (6) using (4), giving

\[
X_{\text{DA}}(t) = \left[ \frac{X_0}{k_1} \right] e^{-(k_1+k_3)t} - e^{-(k_2+k_4)t}, \tag{7}
\]

\[
X_{\text{D}}(t) = \left[ \frac{X_0}{k_1} \right] \frac{k_4}{k_2} e^{-(k_1+k_3)t} + e^{-(k_2+k_4)t}, \tag{8}
\]

With a known injection dose \( X_0 \), determining \( k_1\)-\( k_4 \) yields the number of fluorophore molecules in each compartment as a function of time through (4), (7), and (8). In addition, with known compartment volumes (\( V_p \) and \( V_t \)), we can determine the chemical concentration (the number of molecules per unit volume) in each compartment. The plasma volume (\( V_p \)) can be estimated from the mouse weight [26], and the tumor volume (\( V_t \)) can be determined from the reconstructed absorption and fluorescence images.

To perform numerical calculations and form images, the image subject is discretized into \( N \) voxels (as mentioned in Step 3), with \( s \) the voxel index \( (s = 1, \ldots, N) \) and \( V_{\text{vox}} \) the voxel volume. The number of fluorophore molecules in a voxel can be expressed as a weighted sum of the number of molecules in each compartment. Thus, in voxel \( s \), the number of folate-DA molecules has contributions from both the plasma compartment and the tumor compartment, giving

\[
X_{\text{DA}}(s,t) = v_p(s) X_{\text{DA}}(t) + v_t(s) X_{\text{DA}}(t), \tag{9}
\]

where the volume fraction \( v_p(s) = \) (plasma volume in voxel \( s \))/\( V_p \), and \( v_t(s) = \) (tumor volume in voxel \( s \))/\( V_t \). Notice that \( \sum_{s=1}^{N} v_p(s) = 1 \) and \( \sum_{s=1}^{N} v_t(s) = 1 \), given that the discretization covers the entire plasma compartment and tumor compartment, respectively. Similarly, the number of free donor molecules in voxel \( s \) is given by

\[
X_{\text{D}}(s,t) = v_t(s) X_{\text{D}}(t). \tag{10}
\]

To incorporate kinetics into FODT, as described in Step 4, the number of fluorophore molecules needs to be converted to fluorescence, \( \tilde{\eta} \) (mm\(^{-1}\)). To do so, we introduce constants \( \alpha_{\text{DA}} \) (mm\(^{-1}\)) and \( \alpha_{\text{D}} \) (mm\(^{-1}\)), which are related to the quantum yield and \( V_{\text{vox}} \), as the fluorescence of one folate-DA molecule and one free donor molecule, respectively. The total fluorescence in voxel \( s \) due to DA pairs and free donors is then given by

\[
\tilde{\eta}_{\text{DA}}(s,t) = \alpha_{\text{DA}} X_{\text{DA}}(s,t) \tag{11}
\]

\[
= \alpha_{\text{DA}} v_p(s) X_{\text{DA}}(t) + \alpha_{\text{DA}} v_t(s) X_{\text{DA}}(t) \tag{12}
\]

\[
= w_{\text{DA}}(s) X_{\text{DA}}(t) + w_{\text{DA}}(s) X_{\text{DA}}(t) \tag{13}
\]

\[
\tilde{\eta}_{\text{D}}(s,t) = \alpha_{\text{D}} X_{\text{D}}(s,t) \tag{14}
\]

\[
= \alpha_{\text{D}} v_t(s) X_{\text{D}}(s,t) \tag{15}
\]

\[
= w_{\text{D}}(s) X_{\text{D}}(t), \tag{16}
\]

where weights \( w_{\text{DA}}(s) = \alpha_{\text{DA}} v_p(s) \), \( w_{\text{DA}}(s) = \alpha_{\text{DA}} v_t(s) \), and \( w_{\text{D}}(s) = \alpha_{\text{D}} v_t(s) \). The fluorescence source term in (2) then becomes
\[ S_f = (w_{PD} X_{PD} + w_{TD} X_{TD}) \frac{1}{1 + j\omega \tau_{DA}} + w_{TD} X_{TD} \frac{1}{1 + j\omega \tau_D}. \]  

(17)

When there is no lifetime change (\(\tau_{DA} = \tau_D\)), as would be the case for molecular beacons or in some fluorescence resonance energy transfer (FRET) work [27], (17) becomes

\[ S_f = \tilde{\eta} (1 + j\omega \tau_D)^{-1}, \]

where \(\tilde{\eta}\) is given by

\[ \tilde{\eta}(s, t) = w_{PD} s X_{PD}(s) + w_{TD}(s) X_{TD}(s) + w_{TD}(s) X_{TD}(s), \]

(18)

with \(X_{PD}, X_{TD}\), and \(X_{TD}\) given by (4), (7), and (8), respectively. If there is change in lifetime after cleavage (\(\tau_{DA} \neq \tau_D\)), fluorescence data at multiple modulated frequencies can be used (thus a larger data set) for reconstruction through the frequency-dependent fluorescence source (17), and more accurate reconstructions can potentially be obtained due to the additional information in the large data set. For the results presented in this work, we considered (18) because there was no significant change in fluorescence lifetime after acceptor release. Notice that \(\tilde{\eta}\) in (18) has the form of a multi-exponential model and can be expressed by

\[ \tilde{\eta}(s, t) = \gamma_1(s) e^{-\gamma_2 t} - \gamma_3(s) e^{-\gamma_4 t} + \gamma_5(s) e^{-\gamma_6 t}, \]

(19)

where

\[ \gamma_1(s) = X_0 \left[ w_{PD}(s) - \frac{w_{TD}(s) k_1}{(k_1 + k_3) - (k_2 + k_4)} + \frac{w_{TD}(s) k_1 k_4}{(k_1 + k_3) - (k_2 + k_4)} \right], \]

(20)

\[ \gamma_2 = k_1 + k_3, \]

(21)

\[ \gamma_3(s) = X_0 \left[ \frac{k_1 w_{TD}(s) - k_1 w_{TD}(s)}{(k_1 + k_3) - (k_2 + k_4)} \right], \]

(22)

\[ \gamma_4 = k_2 + k_4, \]

(23)

\[ \gamma_5(s) = X_0 \left[ \frac{w_{TD}(s) k_1}{(k_1 + k_3) - k_2} \right], \]

(24)

\[ \gamma_6 = k_2. \]

(25)

Reconstructions of \(\gamma_1 - \gamma_6\) through FODT (as mentioned in Step 5) allow for the retrieval of information on delivery and release kinetics. The spatially-dependent variables (images) \(\gamma_1\) and \(\gamma_6\) give information on the spatial distribution of the tumor compartment, as both \(w_{TD}\) and \(w_{TD}\) depend on \(v_j\). In other words, we can expect that the reconstructed \(\gamma_1\) and \(\gamma_6\) images show the tumor depth, size, and location. With reconstructed images \(\gamma_1, \gamma_3,\) and \(\gamma_6\), we can determine the plasma compartment distribution through \(\gamma_1 - \gamma_3 + \gamma_5 = X_0 w_{PD}\) at \(t = 0\) in (19) (as \(v_j\) in \(w_{PD}\) gives the spatial map of the plasma). As shown in (7) and (8), the absolute (as opposed to relative) number of folate-DA molecules and the absolute number of free donor molecules in the tumor can be determined if \(k_1 - k_4\) are known. From the reconstructed \(\gamma_2, \gamma_4,\) and \(\gamma_6\), the rates \(k_1 + k_3, k_2 + k_4,\) and \(k_2\) can be determined. The drug release rate (\(k_4\)) is obtained by forming \(\gamma_4 - \gamma_6\). The uptake rate (\(k_1\)) needs to be extracted to determine (7) and (8) quantitatively. With \(X_0\) known, if \(\alpha_D\) and \(\alpha_{DA}\) can be measured and the discretization covers the entire tumor compartment \((\sum_{i=1}^N v_i(s) = 1)\), \(k_1\) can be deduced from reconstructed \(\gamma_2, \gamma_5,\) and \(\gamma_6\), shown by

\[ \sum_{i=1}^N \gamma_i(s)(\gamma_2 - \gamma_6) = X_0 k_1 \alpha_D \sum_{i=1}^N v_i(s) = X_0 k_1 \alpha_D, \]

(26)

where the caret (\(\tilde{\cdot}\)) represents the reconstructed result. Similarly, if \(\alpha_D\) and \(\alpha_{DA}\) are known, \(k_1\) can be extracted from reconstructed \(\gamma_2, \gamma_5,\) and \(\gamma_6\), shown by

\[ \sum_{i=1}^N \gamma_i(s)(\gamma_2 - \gamma_6) = X_0 k_1 \alpha_D \sum_{i=1}^N v_i(s) = X_0 k_1 \alpha_D, \]
The elimination rate ($k_3$) can be extracted by determining $\gamma_2 - k_1$, with reconstructed quantities. With $k_1$-$k_4$ known, (7) and (8) can be evaluated, giving information on the acceptor (in lieu of the drug) release process.

### 2.4. Simplified kinetics model

To reduce the degrees of freedom (DOF) in the reconstruction, we relate $\gamma_3$ and $\gamma_5$ by a constant as

$$R_{\gamma_3\gamma_5} = \frac{\gamma_5(s)}{\gamma_3(s)} = \left[ \frac{(k_1 + k_3) - (k_2 + k_4)}{(k_1 + k_3) - k_2} \right] \frac{\alpha_D}{\alpha_D - \alpha_{DA}}.$$  

(28)

Therefore, we reconstruct the image $\gamma_3$ and the constant $R_{\gamma_3\gamma_5}$ (giving $N + 1$ DOF) instead of two images, $\gamma_3$ and $\gamma_5$ (giving $2N$ DOF). The fluorescence is then given by

$$\tilde{\eta}(s,t) = \eta_0(s) e^{-\gamma_5 t} + \gamma_3(s) (R_{\gamma_3\gamma_5} e^{-\gamma_3 t} - e^{-\gamma_5 t}).$$  

(29)

The unknowns in (29) to be solved through inversion are the images $\gamma_1$ and $\gamma_3$, and the constants $\gamma_2$, $\gamma_4$, $\gamma_5$, and $R_{\gamma_3\gamma_5}$.

To simplify the problem, we make the assumption that the first exponential in (18) is negligible for $t \gg 0$ because $(k_1 + k_3) \gg (k_2 + k_4)$, i.e., $\gamma_2 \gg \gamma_4$, and thus

$$\tilde{\eta}(s,t) \approx \gamma_3(s) (R_{\gamma_3\gamma_5} e^{-\gamma_3 t} - e^{-\gamma_5 t}).$$  

(30)

This assumption is supported by prior studies that showed $(k_1 + k_3) \gg (k_2 + k_4)$ experimentally (see Section 4.1). Moreover, this also indicates that there is a negligible number of folate-DA molecules remaining in the plasma after some time, i.e., $X_{DA} = \sum_{t=1}^{N} X_{tDA} = X_0 e^{-\left(k_1 + k_3\right) t} \approx 0$ at time $t \gg 0$ due to large $k_1 + k_3$. This is substantiated by a prior study that showed very little chemical remaining in the plasma 4 h after injection [16]. In this work, we started collecting fluorescence data around 4 h after chemical injection. This assumption should not be applied to data collected right after the injection. Thus, the forward diffusion model, (1) and (2), with the incorporated fluorescence, (30), was used.

### 2.5. Image reconstruction

We are interested in the pharmacokinetics, including rates $k_1$-$k_4$, which offer information on when and how much drug is delivered to the tumor and released, and the spatial map $\eta_i$, which shows where the drug is delivered. These parameters can be obtained by directly reconstructing $\gamma_1$-$\gamma_4$, $R_{\gamma_3\gamma_5}$, and $\gamma_6$; together they characterize the fluorescence source. It can be expected that spatial distributions of $\gamma_1$ and $\gamma_5$ show the tumor shape and location through $\eta_i$. Knowledge of release rate ($k_4$) is important for the design of targeted anti-cancer drugs and it has never been measured in vivo. Moreover, with known $k_1$-$k_4$, the number of folate-DA molecules and the number of free donor molecules in each compartment can be determined as a function of time, i.e., $X_{DA}$, $X_{tDA}$, and $X_{t_0}$, become known through (4), (7), and (8), respectively. In this section, we describe the inversion algorithm that allows for the extraction of the kinetic information.

First, we give a brief description of our image reconstruction (inversion) algorithm, which is based on a Bayesian nonlinear optimization framework [12, 20, 28]. The method facilitates the image reconstruction by using maximum a posteriori (MAP) estimation. The spatial correlation between image voxels is modeled by a generalized Gaussian Markov random field (GGMRF). The problem becomes the minimization of a cost function and it is solved by the iterative coordinate descent (ICD) algorithm [12], described by
\[
\hat{x}_i = \arg \min_{\hat{x}_i} [||y-f(\hat{x}_i)||^2_\Lambda + \frac{1}{\rho \sigma^p} \sum_{j \in \mathcal{N}_i} b_{ij} |\hat{x}_i - x_j|^p],
\]

where \( \mathbf{x} \) is the image to be reconstructed, subscript \( i \) represents the voxel being updated, \( \hat{x}_i \) is the updated (reconstructed) value, \( \mathbf{y} \) is a vector of length \( P \) representing the measurements (calibrated experimental data), \( f(\mathbf{x}) \) is the solution to the forward model, (1) and (2), for assumed \( \mathbf{x} \), and for an arbitrary vector \( \mathbf{w} \), \( ||\mathbf{w}||^2_\Lambda = \mathbf{w}^H \mathbf{\Lambda} \mathbf{w} \), where \( H \) denotes Hermitian transpose with \( \mathbf{\Lambda}^{-1} = \text{diag}[|y_1|, \ldots, |y_P|] \). The prior model, the GGMRF, is characterized by \( \sigma \) and \( \rho \), which are constants representing scale and shape parameters for the distribution, respectively, and \( b_{ij} \), which provides a local 26-neighborhood \( \mathcal{N}_i \) weight. For this work, we choose \( \rho = 2 \), which gives a Gaussian prior model. The iterative method is terminated when a measure of convergence is reached which, in our case, is five consecutive iterations in which the change in cost is less than 2% of the mean of the five largest cost reductions. We allow for a maximum of 50 iterations. This stopping criterion does not need to be specified \( \textit{a priori} \) (e.g., using a predefined threshold).

The image reconstruction method relies on solving an ill-posed inversion problem. The challenge resides in finding the global minimum of the cost functional. The step-wise linear approximation using the Fréchet derivative reduces the problem to one of finding the minimum of a quadratic function. The validity of the linear approximation and the regularization parameter dominates the accuracy of the inversion problem solution. The regularization parameter (related to the \( \sigma \) in the prior model) not only provides the weight of the prior model but also determines the validity of the linear approximation. For example, with a small prior model weight, we trust the forward model and experimental data more by enforcing less smoothness, allowing for a large variation in the image. This may render the linear approximation that assumes a perturbational change in the image inappropriate. Therefore, the regularization parameter needs to be within a reasonable range to obtain a meaningful solution (reconstructed image). The method for choosing a proper regularization parameter has been discussed previously [29].

The inversion algorithm described by (31) is applied to the kinetic problem for the reconstruction of the absorption and kinetic parameters. Specifically, at \( \lambda_1 \), the forward model (1) is solved and the absorption \( (\mu_\text{at}) \) image is reconstructed. At \( \lambda_m \), using the reconstructed absorption (assuming \( \mu_\text{at} = \mu_\text{at,0} \)), the coupled diffusion equations (1)-(2) are solved and \( \gamma_1-\gamma_4 \), \( R_{\phi_\gamma} \), and \( \gamma_6 \), which characterize the fluorescence source through (29), are reconstructed directly. In other words, \( \mathbf{x} \) in (31) represents \( \gamma_1-\gamma_4 \), \( R_{\phi_\gamma} \), and \( \gamma_6 \), and is updated in each iteration. Positivity constraints can be easily imposed on (31) when needed. Images \( \gamma_1 \) and \( \gamma_6 \) are both greater than zero because \( \alpha_D > \alpha_{DA} \) and \( (k_1 + k_3) > (k_2 + k_4) \) (due to slow unloading rate, see the description in Section 2.1 and in detail in Section 4.1). The rates \( \gamma_2 \), \( \gamma_3 \), and \( \gamma_5 \) are greater than zero. For the simplified model, (30) in Section 2.4, \( \gamma_1 \) and \( \gamma_2 \) are not considered.

3. Experiment

3.1. Chemical preparation

For our targeting fluorophore pair (Folate-Dylight680B-S,S-Promofluor750), we used Asp-Lys-Cys as the spacer and folic acid as the targeting ligand, where cysteine (Cys) is linked via a releasable disulfide bond to Promofluor750 (PromoKine) and lysine (Lys) is linked to Dy-light680B (Thermo Fisher Scientific) via a non-releasable amide bond, as illustrated in Fig. 3. The development of a folate-DA conjugate expressing fluorescence at near-infrared (NIR) wavelengths is advantageous because of the low tissue absorption in the NIR region [1, 30]. As a result, lower laser powers can be used to excite fluorescence in deep tissue. Moreover, the folate-DA is pH-insensitive, making it suitable for targeting and imaging tumor cells.

The synthesis procedure is as follows. The folate cysteine linker was prepared by following standard Fmoc chemistry [31] on an acid-sensitive chlorotrityl resin loaded with Fmoc-L-
Cys(Trt)-OH. The crude folate cysteine linker was purified using preparative RP-HPLC at \( \lambda = 285 \) nm (1%B to 30%B for 30 min; A = 0.1% TFA, pH = 2; B = ACN; column: Waters, xTerra C18 10 m; 19 x 250 mm, flow rate = 10 mL/min). HPLC-purified (pH = 2) was reacted in the presence of excess N,N-diisopropylethylamine (DIPEA) with dithiopyridyl activated Promofluor750 in DMSO to afford folate-cysteine-S,S-promofluor750. The reaction was monitored by analytical HPLC (C18 reverse phase, mobile phase 1.0 mM sodium phosphate, pH 7.0 and acetonitrile). The product (folate-cysteine-S,S-promofluor750) was isolated by preparative RP-HPLC (1%B to 30%B for 30 min; A = 10 mM ammonium acetate, pH = 7.0; B = ACN; pH 7.0) and the fractions were lyophilized for purity. The final conjugation was performed by mixing excess DIPEA with folate-cysteine-S,S-promofluor750 in DMSO, followed by the addition of Dylight680B NHS ester predissolved in DMSO. After the reaction reached completion by LC-MS, the compound (Folate-Lys-NHCO-Dylight680B-Cysteine-S,S-promofluor750) was isolated by preparative RP-HPLC using the same condition, and pure fractions were lyophilized to give the NIR folate-DA chemical.

Due to efficient coupling of the donor and acceptor, the donor fluorescence is quenched in the presence of the acceptor. After the folate-DA is injected into the body, the disulfide bond is reduced over time due to RME (illustrated in Fig. 1), resulting in a cleaved acceptor and an increase in the donor fluorescence. In this work, time-domain gated pulse measurements revealed that our folate-DA conjugate behaves as a molecular beacon [27], expressing a change in fluorescence intensity with acceptor release, but not a significant change in fluorescence lifetime. We tested the release process in vitro using confocal microscopy, as shown in Fig. 4. The chemical was injected at various concentrations into KB Cells over-expressing folate-receptors [32] for 1 h and 8 h incubation cycles. KB cells belong to a subline of the ubiquitous keratin-forming tumor cell line HeLa and are derived from human cervical tissue. They belong to a cell type of epidermoid carcinoma. The cells are positive for keratin by immunoperoxidase staining. Here, we show images at 100 nM, the concentration resulting in the highest intensity contrast. The strong increase in donor fluorescence after 8 h indicated efficient donor-acceptor coupling before acceptor release. This is as expected from the 6 h half-time of the disulfide bond reduction process measured previously [14]. Our goal is to measure this release rate in tumor cells in vivo.
3.2. **Mouse preparation**

Female nu/nu mice purchased from NCI Charles River Laboratories were maintained on folate deficient rodent chow for 3 weeks prior to experimental study and kept on a standard 12 h light-dark cycle. Normal rodent diets containing excessive amounts of folic acid were not used because they elevate serum folate levels significantly above normal physiological concentrations. The animal procedure was carried out with the approval of the Purdue Animal Care and Use Committee in accordance with NIH guidelines.

A six-week-old female nu/nu mouse (~22 g) was inoculated subcutaneously in the right hind limb with KB cells (1.0 × 10⁶ cells/mouse in RPMI medium) over-expressing folate-receptors using a 25-gauge needle. Growth of the xenograft tumor was measured in two perpendicular directions every 2 days using a caliper. The tumor volume was calculated as 0.5 × L × W² (L was the measurement of the longest axis and W was the measurement of the axis perpendicular to L in mm). The imaging experiment was performed 3 weeks after tumor cell implantation, when the tumor reached approximately 100 mm³. The experiment was conducted before the interior of the necrotic tumor began to die from lack of nutrients, to ensure all the tumor cells’ ability to internalize the folate conjugate. Three hours before the imaging experiment, the tumor bearing mouse was injected intravenously into the tail vein with 10 nmol of the NIR folate-DA conjugate (described in Section 3.1) dissolved in 100 μL of saline, giving \( X_0 = 10 \times 10^{-9} \times \text{Avogadro’s constant} \). The chemical attached itself to the folate-receptors present in the tumor, as shown in previous studies [7], allowing for fluorescence imaging. Once the chemical was absorbed into the tumor, the acceptor that acted as a quencher was cleaved from the donor, resulting in an increase in donor emission, as mentioned previously.

The mouse was kept alive during the experiment using a table-top anesthesia machine (Parkland Scientific Inc., FL). It was anesthetized (3 h post injection) using a table top anesthesia machine with isoflurane as anesthetizing agent. Anesthesia was maintained by a constant flow rate of 2% isoflurane in oxygen with the vaporizer. The anesthetized mouse was then imaged to measure the change in donor fluorescence emission as the acceptor (linked via a releasable disulfide bond) was cleaved inside the tumor. The mouse was euthanized through CO₂ asphyxiation after the experiment.

3.3. **Measurement**

The mouse laid comfortably on a stage and constantly breathed anesthetic through a tube during the experiment, as shown in Fig. 5(a). A 3-D topography laser line scanner was used to obtain the 3-D profile of the mouse on which an unstructured FEM mesh was formed [33]. We used a 633 nm pulsed laser (Horiba Jobin Yvon, pulse duration 230 ps) as our source to excite the
donor fluorophore and a cooled gated image-intensified CCD camera (Roper PIMAX, 512×512 pixels) for detection, as shown in Fig 5(b). Reflection data was collected. Fluorescence (donor emission) data was collected through a 710 nm bandpass filter (Andover Corporation, bandwidth 10 ± 2 nm). The laser was operated in a quasi-CW mode with a repetition rate of 10 kHz (giving an average power of 0.12 μW) for ODT data (at λx) and 1 MHz (giving an average power of 12 μW) for fluorescence data, to achieve adequate signal levels for imaging. The camera exposure time was 60 ms.

For data at the excitation wavelength, calibration was done through the optimization-based inversion [28]

\[ \hat{\Gamma} = \arg \min_{\Gamma} \| y_{\text{raw}} - f(x) \|_A^2, \]

where \( y_{\text{raw}} \) is the raw data obtained experimentally from the CCD camera, \( f(x) \) is the numerical forward model solution, given the initial (homogeneous) absorption image, and \( \Gamma \) is the calibration constant. The calibrated measurement used in (31) for reconstruction was then \( y = y_{\text{raw}} \hat{\Gamma} \).

The fluorescence data was calibrated using \( \hat{\Gamma} P_{\lambda m} / P_{\lambda x} \), where \( P_{\lambda x} \) and \( P_{\lambda m} \) were the measured powers at \( \lambda_x \) and \( \lambda_m \), respectively.

4. Results

4.1. Parameter estimation

Previously, the residual folate conjugate in various tumor cell lines, including KB cells, was evaluated by sacrificing multiple mice at different times [16]. These results allowed us to estimate the unloading rate \( (k_2) \) to be around 0.01 h\(^{-1}\) by applying an exponential fit to the data. The disulfide cleavage rate \( (k_4) \) of a folate-DA conjugate was evaluated in a cell culture [14], giving an approximate half-time of 6 h and thus a release rate of \( \ln(2)/6 = 0.12 \text{ h}^{-1} \). This gave us a reference to assess the accuracy of our reconstructed \( k_4 \) (see Section 4.2). The binding of the folate conjugate to the tumor cells was completed within roughly 30 minutes of injection. The number of folate conjugate molecules in the tumor as a function of time was described by the compartment model, given by

\[ X_{\text{folate}}(t) = \frac{X_0 k_1}{(k_1 + k_3)} - \frac{k_2}{(k_1 + k_3)} \left[ e^{-k_2 t} - e^{-(k_1 + k_3) t} \right]. \]
which has a similar form to $X_{\text{DA}}$ in (7), except with no $k_4$, because there was no disulfide bond cleavage. With $k_2 = 0.01 \text{ h}^{-1}$, we then estimated $k_1 + k_3 = 14.3 \text{ h}^{-1}$ by assuming the maximum of (33) occurred at 30 minutes. This shows that $k_1 + k_3$ is larger than $k_2$ and $k_4$ by at least two orders of magnitude, supporting the approximation in (30).

We measured experimentally (data not shown here) that, at laser repetition rates of 100 kHz and 1 MHz, free donors have 15-20 times more fluorescence than DA pairs, giving $\alpha_D/(\alpha_D - \alpha_{\text{DA}}) \approx 1.1$. Thus, with (28) and the fact that $k_1 + k_3$ is much larger than $k_2$ and $k_4$, we have $R_{\gamma_5} \leq 1.1$, allowing us to define a reasonable range for $R_{\gamma_5}$ in reconstruction.

### 4.2. In vivo mouse imaging

We show the imaging of a live, tumor-bearing mouse injected with the folate-DA conjugate (shown in Fig. 3). Figure 6(a) shows a photo of the live mouse during the experiment and its corresponding 3-D surface profile obtained using a laser line scan [33]. The mouse with a tumor in its right hind leg is shown in Fig. 6(b). The forward model, (1) and (2), was solved with 20541 FEM nodes (discretizing the mouse). For reconstruction, images were formed on a Cartesian grid with voxel size $(0.5 \text{ mm})^3$, giving $V_{\text{vox}} = 0.125 \text{ mm}^3$. The general location of the 10 sources and 775 detectors used for the reconstructions are shown by the black dotted circle in Fig. 6(a). For each source, only data at detectors that were more than one transport mean free path away from the source were used in order to avoid singularities in the diffusion approximation (forward model). This gave $P = 7284$ for the number of measurements. We assumed that the absorption and diffusion images are the same at $\lambda_x$ and $\lambda_m$ and that the diffusion image is independent of absorption [34] and known. The initial images (backgrounds) were $\mu_a = 0.0524 \text{ mm}^{-1}$ and $D = 0.2 \text{ mm}$ (reduced scattering coefficient $\mu_s' \approx 1.6 \text{ mm}^{-1}$) [35].

We reconstructed the absorption image with $\sigma = 0.015$ (the prior model parameter in (31)). Figures 7(a)-(c) show the isosurface and cross-section of the reconstructed absorption, respectively. The reconstructed absorption ($\mu_a$) image gives the shape and location of the tumor, as expected. The reconstructed tumor has a dimension of around 5 mm in the $x$-direction and 4 mm in both the $y$- and $z$-directions (as shown in Figs. 7(a)–7(c)), giving a volume of 80 mm$^3$, which is close to the 100 mm$^3$ described in Section 3.2.

For the kinetic FODT reconstruction, we used (30) with $k_2 = 0.01 \text{ h}^{-1}$ and $R_{\gamma_5} = 1.1$ as the fluorescence source and reconstructed $\gamma_3$ and $\gamma_4$ (i.e., $x$ in (31) represents $\gamma_3$ and $\gamma_4$). The choice of $k_2$ and $R_{\gamma_5}$ was explained in Section 4.1. In this work, regularization (with a GGMRF prior model) was used for the reconstruction of $\gamma_4$. The initial images were $\gamma_3 = 0$ and $\gamma_4 = 0.01 \text{ h}^{-1}$.

We first defined a tumor region using the reconstructed absorption image and the reconstructed fluorescence magnitude (time-invariant) image, and subsequently, within this region, recon-
Fig. 7. (a)-(b) Isosurfaces of the reconstructed absorption ($\mu_a$) at 0.05 mm$^{-1}$ (showing the semi-transparent contour of the mouse) and 0.07 mm$^{-1}$ (showing the reconstructed tumor), with different viewing angles in (a) and (b). (c) Cross-section of the reconstructed absorption.

Structured both $\gamma_3$ and $\gamma_4$ in each ICD iteration. The prior model $\sigma$ was 0.001 for $\gamma_3$, and 0.2 for $\gamma_4$. Figures 8(a)-(c) show the isosurface and cross-section of the reconstructed fluorescence magnitude ($\gamma_3$), respectively, and Figs. 8(d)–8(f) show the reconstructed rate ($\gamma_4$). From (23) and (25), we calculated $k_4 = \gamma_4 - \gamma_2$ using the mean of $\gamma_4$ (average in the $\gamma_4 > 0.01$ region) and $k_2$, giving $k_4 = 0.178 - 0.010 = 0.168$ h$^{-1}$. The in vitro and in vivo release mechanisms are similar with comparable rates [14, 16], but there may be perturbational variations due to differences in the in vivo local environment. Here, we show that our reconstructed release rate ($k_4 = 0.168$) is similar to that from the cell culture study (see Section 4.1), suggesting that our reconstruction was reasonable and successful.

With the reconstructed $k_4$, we show the change in fluorescence (30) over time in Fig. 9(a) for the center region of the tumor (defined by the location with maximum fluorescence, and its neighboring 26 voxels). As an assessment of this result, we also observed the donor emission intensity (2-D CCD image intensity, not the 3-D reconstruction) relative to the excitation intensity at a constant position on the tumor hourly. Figure 9(b) shows the comparison of the 3-D reconstruction result (Fig. 9(a)) with the 2-D hourly measurements, both showing an increasing trend that indicates the acceptor release. Note that the 3-D reconstruction offers more information, including the depth and size of the tumor, and the possibility to determine the exact number of fluorophore indicator molecules (or released drug), allowing for quantitative molecular imaging.

Using a 3.47 GHz Intel X5690 with 96 GB RAM (not fully utilized), the $\mu_a$ reconstruction converged after 22 iterations in 5.7 h. The kinetics reconstruction (of $\gamma_3$ and $\gamma_4$) converged in 5 iterations after 0.5 h.

5. Discussion

We have demonstrated an optical imaging modality to determine the in vivo folate-targeting kinetics in tumor cells in a small animal. We used a folate-DA conjugate, a surrogate for the folate-drug conjugate, as previous studies have shown that attaching either fluorophores or anti-cancer drugs to folic acid does not interfere with the uptake of folic into cancer cells. To image in deep tissue where tumors occur, we used the diffusion equation to model photon transport in the heavily scattering regime [33,36,37]. This method provides the foundation for a unique and practical tool that can be used to monitor and evaluate pharmacokinetics in preclinical studies.

This work has broader impact related to in vivo molecular imaging. FRET [27, 38] is an energy transfer process between donor and acceptor molecules spatially separated by a distance less than 10 nm that can provide invaluable information for nanomedicine. With FODT, the FRET information is accessible in deep tissue [36, 37, 39]. The FRET distance (DA distance)
offers a quantitative measure of molecular activity as the donor fluorescence intensity and lifetime are dependent on the FRET distance. The FRET model that describes this relationship can be incorporated into the kinetics forward model (described in Section 2.2) to determine the kinetics of the FRET distance.

Our method can be improved by imaging all the kinetic parameters ($\gamma_1$-$\gamma_4$, $R_{\gamma_3\gamma_5}$, and $\gamma_6$), allowing for the retrieval of the number of folate-DA and free donor molecules in space and time. Experimentally, the setup can be improved by using a higher power pulsed laser to capture
pulsed time-domain data that can be Fourier-transformed to give data at multiple modulation frequencies. This would provide a larger data set, which would improve the reconstruction. Moreover, fluorescence data at the acceptor emission can be collected for additional information. In the reconstruction, a modified diffusion approximation or higher order approximations can be used to more accurately model the photon transport near the surface and sources. Computation efficiency can be improved through multigrid inversion [33, 40, 41] and parallel computing, allowing for finer discretization and higher resolution in a limited computation time.

In terms of the chemicals, different donor and acceptor fluorophores can be used that express FRET, allowing for another measurement metric through the difference in lifetime (between DA pairs and free donors). If the coupling between the donor and acceptor can be improved, a higher fluorescence contrast over time could be obtained as the acceptors are cleaved. By introducing steric crowding, the rate of cleavage of the disulfide bond can be modified in the carbon chain of disulfide linkage, allowing the accuracy of the imaging method to be tested. Finally, the injected dose can be increased to improve the fluorescence contrast between the tumor cells and healthy cells. This work serves as a pilot study for imaging in vivo kinetics and thus, in the future, a large number of mice may be imaged to establish the statistical significance.

6. Conclusion

We have shown successful imaging of optical and kinetic parameters in a live mouse using FODT incorporating a compartment model, which fundamentally allows imaging of changing fluorescence in heavy scatter. For this study, we developed a NIR folate-DA conjugate, which showed an increase in donor fluorescence intensity upon acceptor cleavage through disulfide bond reduction. The folate-drug kinetics can be determined by measuring folate-DA kinetics because conjugation of either fluorophores or anti-cancer drugs to folic acid has been shown to not interfere with the uptake of folate into cancer cells. Thus, optical methods can be used to determine the cleavage rate in vivo from the change in fluorescence due to acceptor release, which corresponds to the drug release rate inside cancer cells. Knowledge of this drug release rate is important for the design of anti-cancer drugs, as the drug should be released after the folate-conjugate has been internalized by the tumor, but before it is unloaded. The reconstructed release rate is close to in vitro confocal studies, confirming the validity of our imaging method. The method can be applied to determine the kinetics of other processes where both the compartment model and ODT are suitable. The method can also be extended to determine all the kinetic parameters, allowing the determination of the number of fluorophore molecules in each compartment and their variations over time. Our work offers a unique method for determining pharmacokinetics in preclinical studies and has the potential to become a powerful tool for the design and development of targeted anti-cancer drugs.

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