Delivery Rate Affects Uptake of a Fluorescent Glucose Analog in Murine Metastatic Breast Cancer

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Abstract

We demonstrate an optical strategy using intravital microscopy of dorsal skin flap window chamber models to image glucose uptake and vascular oxygenation in vivo. Glucose uptake was imaged using a fluorescent glucose analog, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-y1)amino]-2-deoxyglucose (2-NBDG). SO2 was imaged using the differential absorption properties of oxygenated [HbO2] and deoxygenated hemoglobin [dHb]. This study was carried out on two sibling murine mammary adenocarcinoma lines, 4T1 and 4T07. 2-NBDG uptake in the 4T1 tumors was lowest when rates of delivery and clearance were lowest, indicating perfusion-limited uptake in poorly oxygenated tumor regions. For increasing rates of delivery that were still lower than the glucose consumption rate (as measured in vitro), both 2-NBDG uptake and the clearance rate from the tumor increased. When the rate of delivery of 2-NBDG exceeded the glucose consumption rate, 2-NBDG uptake decreased with any further increase in rate of delivery, but the clearance rate continued to increase. This inflection point was not observed in the 4T07 tumors due to an absence of low delivery rates close to the glucose consumption rate. In the 4T07 tumors, 2-NBDG uptake increased with increasing rates of delivery at low rates of clearance. Our results demonstrate that 2-NBDG uptake in tumors is influenced by the rates of delivery and clearance of the tracer. The rates of delivery and clearance are, in turn, dependent on vascular oxygenation of the tumors. Knowledge of the kinetics of tracer uptake as well as vascular oxygenation is essential to make an informed assessment of glucose demand of a tumor.

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Introduction

In spite of heterogeneities in causal molecular events and signaling pathways, almost all cancers exhibit enhanced glucose uptake relative to normal cells. This enhanced glucose uptake has been utilized clinically for identifying cancers using Positron Emission Tomography (PET) of Fluorodeoxy-glucose (FDG), a radiolabeled analog of glucose. FDG-PET has found widespread use in identifying and staging cancers, and in predicting response to therapy [1–5]. PET images are interpreted based on the Standardized Uptake Value (SUV) of FDG. Although computationally simple, this method only provides a snapshot of the kinetics of glucose uptake and clearance, measuring FDG uptake at a single time point approximately 60 minutes after injection [6]. Knowledge of the initial kinetics of tracer uptake is important because blood flow rates and hence the delivery of FDG can influence tracer uptake [7]. FDG does not provide information regarding tumor blood flow or oxygenation and this typically requires the injection of additional tracers. Knowledge of tracer kinetics assumes added importance in the case of predicting response to therapy because therapies such as radiation can cause changes to tumor vasculature, potentially affecting blood flow in addition to expected changes in tumor glycolytic demand.

Here, we demonstrate an optical strategy using intravital microscopy of dorsal skin flap window chamber tumor models to image glucose uptake and vascular oxygenation (SO2) in vivo, and examine the effects of delivery and decay kinetics on glucose uptake. Intravital microscopy of window chamber models provides the requisite spatial resolution to differentiate vascular from tissue/tumor compartments. Glucose uptake was imaged using a fluorescent analog of FDG, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-y1)amino]-2-deoxyglucose (2-NBDG). 2-NBDG has been validated in multiple model systems by our group and others as a viable marker of glucose uptake [8–14]. Optical imaging is amenable to repeated measures to capture the delivery and clearance kinetics of glucose uptake via 2-NBDG fluorescence. SO2 was imaged using the differential absorption properties of oxygenated and deoxygenated hemoglobin. SO2 in blood vessels is strongly associated with tissue pO2 [15]. Our group has previously established the ability to image SO2 of hemoglobin in blood vessels using label-free trans-illumination microscopy in window chamber models [16]. This study was carried out on two sibling murine mammary adenocarcinoma lines, 4T1 and 4T07, derived from the same parental line. These cell lines were selected due to differences in their metastatic potential, glycolytic phenotypes and oxygen consumption properties [17–20]. First, the glucose uptake rate of
both cell lines was characterized in vitro. Second, in vivo tumors were established for each cell line. Their baseline vascular and metabolic characteristics were imaged using intravital microscopy. Third, a hypoxia/reoxygenation protocol was used to perturb delivery of the tracer to the tumor. The purpose of this protocol was to perform continuous imaging of SO$_2$ and 2-NBDG in response to a reoxygenation-associated increase in blood flow.

Two endpoints were obtained from trans-illumination microscopy: concentrations of oxy-hemoglobin ([HbO$_2$]) and deoxy-hemoglobin ([Hb]), from which SO$_2$ ([HbO$_2$]/[Hb]+[HbO$_2$]) was derived. Three endpoints were obtained from the 2-NBDG fluorescence kinetic profile: rate of delivery (ratio of the maximum 2-NBDG intensity and the time to maximum), rate of clearance (rate of decay of 2-NBDG intensity from its maximal value to that at 60 minutes), and finally, uptake of 2-NBDG by the tumor after wash-in and wash-out through the vasculature at approximately 60 minutes.

Our results initially revealed a simple relationship between SO$_2$ and 2-NBDG uptake. The 4T07 tumors were better oxygenated than the 4T1 tumors and mean 2-NBDG uptake was significantly higher in the 4T1 tumors. Breathing hypoxic gas significantly increased SO$_2$ and blood flow in the 4T1 tumors and decreased mean 2-NBDG uptake in the 4T1 tumors to the level of the 4T07 tumors. Detailed analysis revealed that both 4T1 and 4T07 tumors demonstrated distinct patterns of 2-NBDG uptake that depended on the rates of uptake and clearance of 2-NBDG that were, in turn, dependent on tumor SO$_2$. The results presented in this manuscript establish the importance of tracer kinetics and SO$_2$ in order to accurately interpret glucose uptake data from tumors in vivo.

**Methods**

**Ethics Statement**

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Duke University Institutional Animal Care and Use Committee (Protocol Number: A170-12-06). All experiments were performed under isoflurane gas anesthesia, and all efforts were made to minimize suffering.

**In vitro Cell Culture**

A 4T1 murine mammary carcinoma line was transduced by retroviral siRNA to constitutively express the red fluorescent protein (RFP) DsRed, allowing easy demarcation and growth tracking of tumor cells both in vitro and in vivo [21]. 4T1-RFP and 4T07 cell lines were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics and plated for 24 hours for in vitro experiments. After 24 hours, 3 cell plates of each cell line were incubated with 2-NBDG for increasing durations ranging from 1–75 minutes (Incubation time periods were 1, 2, 3, 4, 5, 10, 20, 30, 40, 50 and 75 minutes). For each incubation period, cells were washed once with PBS and incubated with 3 ml of 100 µM 2-NBDG dissolved in glucose-free and serum free-media. At the end of incubation, cells were washed with PBS and imaged immediately using a two photon microscope. 2-NBDG used in these experiments was synthesized and characterized at the Duke University Small Molecule Facility.

**Two Photon Imaging of Cells**

2-NBDG fluorescence in the cells was excited at 960 nm and imaged over the wavelengths 495–540 nm. 960 nm was selected to reduce contribution from fluorescence of flavin adenine dinucleotide (FAD). The image size was 512 x 512 pixels and this corresponded to a field of view of 510 x 510 µm. Dwell time for each pixel was 8 µs and total image acquisition time was 8.31 s. From each image, the 10 brightest cells were selected to compute the mean fluorescence intensity. Fluorescence images were calibrated using a rhodamine solution (90.8 µM) using the same microscope settings.

**In vivo Studies**

8 to 10 weeks old mice weighing between 20 and 25 g were used for these studies. Titanium window chambers were surgically implanted on the back of female athymic nude mice (nu/nu, NCI, Frederick, Maryland) under anesthesia (i.p., administration of ketamine (100 mg/kg) and xylazine (10 mg/kg)). A 20 µL suspension (20,000 cells) of 4T1-RFP or 4T07 cells was injected into the dorsal skin fold and a glass coverslip (dia = 12 mm, No. 2, Erle Scientific, Portsmouth, New Hampshire) was placed over the exposed tissue. All animals were housed in an on-site housing facility with ad libitum access to food and water and standard 12-hour light/dark cycles. A flowchart depicting the experiment protocol is presented in Figure 1. For baseline measurements, the animals were kept in a chamber filled with 21% oxygen for 6 hours. For hypoxia, the animals were exposed to alternating 1-hour cycles of 21% oxygen and 10% oxygen for 6 hours as described below. During this 6-hour period, the animals were only provided water.

**Hypoxia Chamber**

The hypoxia chamber used for the in vivo experiments was developed in our lab. The chamber was designed using a 37” x 37” x 25” Glove Bag (Glas-Col, Terre Haute, IN). The bag included an outlet opening to which a flexible tubing piece 1 cm in diameter with a 6 mm bore was attached. An inlet hole was also made with a #10 scalpel blade to accommodate another piece of identical tubing. The area around each hole was sealed with Blenderm (3 M, St. Paul, MN). The inlet tubing was used to connect to either a 10% oxygen tank (AirGas National Welders, Raleigh, NC) or to house air. This inlet tube could also be attached to a vacuum for gas removal. The outlet tube was fed into an Erlenmeyer flask filled with water in the fume hood, and the tip of the tube was placed under 1-cm of water in the flask. This tube acted as an overflow valve for the system and also allowed the user to visually confirm that the bag was receiving adequate gas flow by assessing bubbling in the water. The large opening of the bag was sealed by wrapping the open end around a piece of rigid tubing and sealing with binder clips. Control mice breathed room air and were kept in the same part of the room; therefore, they experienced similar lighting and noise environments to the treatment mice.

**Imaging Platform**

We used a Zeiss Axioskop 2 microscope for recording all images. The imaging platform used in our experiments has been described in detail in a previous publication [22]. Briefly, a 100 W halogen lamp was used for trans-illumination imaging and a 100 W mercury lamp was employed for epi-illumination fluorescence imaging of 2-NBDG and RFP expression. Hyperspectral imaging was accomplished using a liquid crystal tunable filter (LCTF). Trans-illumination images were acquired from 520 to 620 nm in 10 nm increments. Epi-Illumination 2-NBDG fluorescence images were acquired at 525 nm with a 470 nm bandpass excitation filter (40 nm bandwidth) and a 510 nm longpass dichroic beamsplitter. Image acquisition time was 300 ms. RFP
fluorescence was recorded from 610 to 690 nm using a 560 nm bandpass excitation filter (30 nm bandwidth) and a 600 nm longpass dichroic beamsplitter. The image acquisition time was 100 ms. All images were collected with a 2.56 objective (NA = 0.075) and a DVC 1412 CCD camera (DVC Company). Lamp throughput images at each wavelength were collected by placing a neutral density filter (to avoid saturating the camera) in the optical path. A rhodamine solution (90.8 μM) was used to calibrate images and compare to 2-photon microscope images.

Hyperspectral Imaging of Vascular Oxygenation and Glucose Uptake

After 6 hours of breathing room air or hypoxic gas, mice were anesthetized using isofluorane mixed with 21% oxygen (1.5% v/v) administered through a nose cone. Once breathing was stable, mice were placed on a heating pad for the duration of the experiment. Trans-illumination images, RFP fluorescence (for 4T1-RFP cells) and a background image at 525 nm corresponding to pre-NBDG injection were recorded. Any background fluorescence at this wavelength was due to FAD. 100 μl of 2-NBDG (2.5 mg/ml) dissolved in sterile saline was injected through the tail vein. The 2-NBDG fluorescence was recorded for 75 minutes as follows: continuously for the first 8 minutes, every 30 seconds for the next 30 minutes and every 3 minutes for the final 35 minutes of imaging.

High resolution images of 2-NBDG uptake and constitutive tumor RFP fluorescence were recorded using a Zeiss upright confocal microscope. 2-NBDG fluorescence was excited at 488 nm and imaged from 510–550 nm. RFP fluorescence was recorded from 580–620 nm. All images were collected using a 10x objective (NA = 0.45). Field of view for the first set of images (baseline –40 minutes) was 850×850 μm. After 40 minutes, field of view was 297×297 μm.

Calculation of Vascular and Metabolic Parameters

A modified form of the Beer-Lambert law that describes absorption of chromophores in thin slices is fit to the trans-illumination image cube (x,y,l) to obtain the concentration of the primary absorbers – [HbO2] and [dHb] at each pixel [16]. This is possible due to knowledge of the extinction coefficients of both absorbers. Based on this information, we can calculate total hemoglobin content, [THb] ([HbO2]+[dHb]) and SO2 ([HbO2]/[THb]) at each pixel. Because [THb] is negligible in tissue space (hemoglobin exists in blood vessels only), [THb] was used to segment the blood vessels and create a map of SO2.

2-NBDG fluorescence images were acquired continuously for a period of 75 minutes to construct a (x,y,l) data cube. At each (x,y) pixel location, a time course of 2-NBDG uptake was obtained. Based on the time course, three metabolic parameters were calculated at each pixel (Fig. 1): the initial rate of delivery (RD), the rate of clearance (RC), and glucose uptake (2-NBDG60).
rate of clearance (RC), and glucose uptake (2-NBDG$_{60}$). RC was calculated from the falling part of the curve as $\frac{(I_{\text{max}}-I_0)}{T_{60}-T_{\text{max}}}$, where subscript 0 corresponds to a pre-2-NBDG baseline image. RC was calculated from the falling part of the curve as $\frac{(I_{\text{max}}-I_{60})}{(T_{60}-T_{\text{max}})}$. 2-NBDG$_{60}$ is defined as the glucose uptake by the tumor after delivery and clearance from the extracellular space.

**Statistical Analysis**

*In vitro* glucose consumption rates were compared using an unpaired Student’s t-test. Statistical analyses of the effects of hypoxia/reoxygenation were performed using a paired Student’s t-test. Correlations between parameters were evaluated by computing Spearman’s rank correlation coefficient ($\rho$). Survival curves or cumulative probability distributions were compared using repeated measures ANOVA. All statistical analyses were performed using the Statistics Toolbox in MATLAB.

**Results**

4T1 and 4T07 Cells have Similar Glucose Uptake Rates *in vitro*

Figure 2A presents the dynamics of 2-NBDG-uptake in 4T1 and 4T07 cells *in vitro*. The data show a monotonic increase in 2-NBDG-fluorescence with incubation time. *In vivo*, this is analogous to 2-NBDG being taken up by cancer cells from the interstitial space. The initial rate of glucose uptake was calculated using the 1-minute time point after incubation (Fig. 2B). The rate of glucose uptake is similar in both cell lines (2.33 ± 0.09 s$^{-1}$ for 4T1 and 2.69 ± 0.45 s$^{-1}$ for 4T07 cells). Statistical analysis confirmed that the initial rates were not significantly different from each other ($p = 0.67$).

2-NBDG-fluorescence at 60 Minutes after Delivery (2-NBDG$_{60}$) is Indicative of Glucose Uptake within the Tumor *in vivo*

To image 2-NBDG *in vivo*, it is necessary to account for the kinetics of 2-NBDG through the vasculature and extracellular space. Thus, a time point for measurement after injection was identified that would be free of the effects of delivery and clearance of 2-NBDG. Figure 3A shows confocal fluorescence images of a dorsal window chamber model of a 4T1 tumor. Constitutive RFP expression allows for visualization of the tumor cells at high resolution. There is no significant background fluorescence prior to the injection of 2-NBDG. At 15 minutes after injection, 2-NBDG is present in the entire field of view but is not yet confined to tumor cells. By 30 minutes, 2-NBDG preferentially localizes within the tumor. A population of cells in the lower left corner helps visualize this phenomenon. The fifth and sixth columns present higher resolution images from this region of interest. After 60 minutes, 2-NBDG fluorescence is largely confined to RFP-positive areas. Therefore, 2-NBDG-fluorescence at 60 minutes—2-NBDG$_{60}$—was used to reflect steady state glucose uptake within the tumor *in vivo*.

Figure 3B presents 2-NBDG fluorescence images from a tumor acquired using the hyperspectral imaging microscope over a larger field of view. These images are similar to the confocal fluorescence images that exhibit maximum 2-NBDG fluorescence at 5 minutes and a steady decline over the course of 60 minutes.

**Blood Flow and SO$_2$ of Tumors Increase after Breathing Hypoxic Gas**

Figure 4 shows representative images of SO$_2$ from the 4T1 and 4T07 tumors at baseline and after breathing hypoxic gas. Because both tumor lines exhibited a wide range of SO$_2$ values at baseline, representative images from animals with high and low SO$_2$ are...
shown. The 4T1 and 4T07 tumors with low SO2 possess a hypoxic core and a negative gradient in SO2 towards the center of the tumor. The tumors with high SO2 do not exhibit a distinct gradient in SO2. After breathing hypoxic gas, the differences between the low and high SO2 images at baseline are no longer apparent in either tumor line. Breathing hypoxic gas significantly increased SO2 of the 4T1 tumors (Fig. 4B; p = 0.03). Although SO2 in 4T07 tumors increased after hypoxia, this was not statistically significant (p = 0.06). In the 4T1 tumors, the significant increase in SO2 with hypoxic gas breathing was a transient effect with SO2 going back to baseline levels 24 hours after the perturbation (Fig. 4C; p = 0.04). The increase in SO2 was strongly, but negatively associated with baseline SO2 in both cell lines (Fig. 4D; r = 0.74, p = 0.01). Further, the increase in SO2 was driven by an increase in [THb] (data not shown; r = 0.72, p = 0.02) and specifically [HbO2] (Fig. 4E; r = 0.87, p = 0.001), reflecting increased blood flow in both cell lines after breathing hypoxic gas.

2-NBDG60 is Dependent on SO2 and Hence Perfusion of 4T1 and 4T07 Tumors

Figure 5 presents representative 2-NBDG60 images of the low and high SO2 tumors shown in Fig. 4. At baseline, 2-NBDG60 is higher in the high-SO2 4T1 tumor compared to the low-SO2 tumor. However, in the 4T07 tumors, 2-NBDG60 is lower in the high-SO2 tumor. At baseline, mean 2-NBDG60 of the 4T1 tumors is significantly higher than the 4T07 tumors (p<0.05). After breathing hypoxic gas, mean 2-NBDG60 decreases in the 4T1 tumors and increases in the 4T07 tumors (Fig. 5B). However, this is not statistically significant. Mean 2-NBDG60 is not significantly different between the two cell lines after hypoxia. There is no crosstalk between SO2 and 2-NBDG60 fluorescence with these measurements because SO2 images are acquired prior to 2-NBDG injection. To determine the relationship between SO2 and 2-NBDG60, survival curves (cumulative distributions) of 2-NBDG60 for all animals over the entire tumor were plotted as a function of SO2 range (Fig. 5C). 2-NBDG60 in the 4T1 tumors is lowest for the lowest oxygenation level (SO2<10%), demonstrating perfusion-limited delivery of 2-NBDG. There are no significant differences in the survival curves of 2-NBDG60 at higher SO2 levels. Post-hypoxia survival curves show no significant differences in 2-NBDG60 as a function of SO2. In addition, the lowest SO2 range no longer exists after hypoxia due to increased SO2. The overall 2-NBDG60 is lower post-hypoxia in the 4T1 tumors compared to that at baseline. In the 4T07 tumors, 2-NBDG60 is lowest when SO2 is highest (SO2<80%, p = 0.02). There is no significant difference between the other SO2 ranges. After breathing hypoxic gas, the same CDF profile is retained in the 4T07 tumors. It is interesting to note that 2-NBDG60 is lowest in the 4T1 tumors at lowest SO2 and in the 4T07 tumors at highest SO2. To understand whether these results were due to increased blood flow, the kinetic profiles of 2-NBDG uptake and specifically, the effect of increasing SO2 on R0 was evaluated.

Increased SO2 Leads to Increased Rate of Delivery (R0) and Clearance (Rc) of 2-NBDG

Figure 6A shows the kinetic profiles of 2-NBDG-uptake calculated from the 4T1 and 4T07 tumor regions of interest shown in Fig. 3. 2-NBDG-fluorescence in the tumor’s extracellular space reaches a maximum and begins to decay at approximately 7–10 minutes after injection. The rising slope of the curve (indicated by a blue solid line) represents the rate of delivery of 2-NBDG (R0) to the tumor. The rate of clearance of 2-NBDG (Rc) is calculated from the falling slope of the curve. Recovery from hypoxia clearly increases R0 (reducing time to maximum) and this is especially apparent in the low-SO2 tumor. In both tumors, the falling part of the curve or Rc is greater after hypoxia, indicating a slightly faster decay. This leads to lower 2-NBDG60 in both low- and high-SO2 tumors. Similarly, the R0 and Rc of the high-SO2 4T07 tumor are higher than the low-SO2 tumor at baseline. Breathing hypoxic gas caused a significant increase in R0 of 4T1 tumors (Fig. 6B; p = 0.002) and Rc of the 4T1 (p = 2×10−4) and 4T07 tumors (p = 0.03).

Survival curves of R0 as a function of SO2 reveal the relationship between the two parameters (Fig. 6C). At baseline, R0 of the 4T1 tumors is lowest for the lowest range of SO2 (SO2<10%) and increases with SO2 however, there are no significant differences in R0 at higher SO2 ranges. Post-hypoxia, there is a large increase in R0 as evidenced by the right-shift in the curves (p<0.05). There is also an SO2-dependent increase in R0 post hypoxia (p<0.05). In the 4T07 tumors, R0 of 2-NBDG is greatest at the highest SO2 level. Post-hypoxia, no significant differences in R0 were observed over the different SO2 ranges below the highest SO2 value. It is interesting to note that when R0 is lowest, the SO2 and 2-NBDG60 of the 4T1 tumors are also at their lowest. On the other hand, 2-NBDG60 of the 4T07 tumors is the lowest when R0 and SO2 levels are highest (Fig. 4B). These data imply that the R0 of 2-NBDG influences uptake in both the
4T1 and 4T07 tumors. However, there does not appear to be a simple relationship between the two parameters. Therefore, the relationship between RD, RC and 2-NBDG60 for the 4T1 and 4T07 tumors was analyzed next. The values of RD and RC are summarized in Table 1 for the representative pair of animals in the 4T1 and 4T07 groups.

**RD in Excess of Glucose Consumption Rate Leads to Lower 2-NBDG60**

Figure 7A illustrates how RD, RC and 2-NBDG60 are derived from the uptake curve of 2-NBDG. Figure 7B presents the relationship between RD, RC and 2-NBDG60. RD and RC represent the x- and y-axes, respectively, while 2-NBDG60 represents the z-axis projecting out of the x-y plane. The data shown here are from all tumors within a group. For the 4T1 tumors at baseline, the RD range is 0.5–3 s⁻¹. 2-NBDG60 is lowest when RD and RC are low (lower left quadrant) and corresponds to poorly oxygenated tumor regions, indicating perfusion-limited uptake. When RD increases, there is a corresponding increase in 2-NBDG60 and RC, indicating higher uptake due to improved delivery. When RD exceeds approximately 2.5–3 s⁻¹, 2-NBDG60 plateaus and declines with any further increase in RD. This effect is observed clearly at high values of RC (>0.2 s⁻¹). The inflection point in the 4T1 tumors at baseline of 2.5–3 s⁻¹ is approximately equal to the glucose consumption rates calculated in vitro, demonstrating that RD in excess of the glucose consumption rate leads to a decrease in 2-NBDG60. After hypoxia, the RD range extends to 6 s⁻¹. The 4T1 tumors exhibit a similar decline in 2-NBDG60 beyond the glucose consumption rate; however, there is a slight shift in this inflection point to 4 s⁻¹, suggesting an increase in the glucose consumption rate after breathing hypoxic gas. Due to higher rates of delivery in the 4T07 tumors, regions with RD values, 3.5 s⁻¹ are mostly absent and the inflection point is not observed. Similar to the 4T1 tumors after hypoxia, a 2-NBDG60 maximum exists at high RD (>6 s⁻¹) and low RC (<0.15 s⁻¹). This is also observed in the 4T07 tumors after hypoxia.

Table 2 summarizes the interplay seen in the contour plots between the three metabolic endpoints and their relationship to SO₂. Regions of low 2-NBDG60 do not necessarily mean anoxic tissue and delivery limitations; they can also indicate regions of high SO₂ with delivery rate exceeding the glycolytic rate. Similarly, a high level of 2-NBDG60 may not mean hypoxic tissue.

**Figure 4. Effect of breathing hypoxic gas on vascular oxygenation of 4T1 and 4T07 tumors.** A. Representative intravital images of vascular oxygenation (SO₂) from 4T1 and 4T07 tumors at baseline and after breathing hypoxic gas (10% O₂, rest N₂). The effect of hypoxia is compared on tumors that showed low and high SO₂ values at baseline. White dotted line in each image represents the tumor. B. After breathing hypoxic gas, there was a statistically significant increase in SO₂ of the 4T1 tumors (n = 5; p = 0.03). There was no statistically significant increase in the 4T07 group (n = 5; p = 0.03). C. The change in SO₂ of 4T1 and 4T07 tumors after breathing hypoxic gas is inversely correlated with SO₂ value at baseline (r = −0.74; p = 0.01). D. The change in SO₂ of 4T1 and 4T07 tumors after hypoxia is strongly correlated with a change in oxy-hemoglobin concentration (r = 0.87; p = 0.001). E. 24 hours after breathing hypoxic gas, there was a significant decrease in SO₂ of the 4T1 tumors (n = 4; p = 0.04), which had returned to a mean value close to pre-hypoxia baseline. 1 animal had to be censored before the 24 hours post-hypoxia measurement. Statistical significance in B and D were assessed using Student’s paired t-test. doi:10.1371/journal.pone.0076524.g004
with great demand; it could potentially indicate tumors demonstrating aerobic glycolysis.

Figure 8 presents the ratio of 2-NBDG<sub>60</sub> to RD for combined pre- and post-hypoxia 4T1 and 4T07 tumors. The ratio was significantly higher (n = 13; p = 0.01) in the 4T1 tumors indicating a higher 2-NBDG<sub>60</sub> and lower RD relative to the 4T07 tumors.

**Discussion**

As much as it is important to measure glucose uptake in tumors, it stands to reason that the delivery of contrast agents such as [18F]-FDG will be affected by blood flow to the tumor. Therefore, it is important to understand the kinetics of tracer uptake by a tumor in order to understand the underlying implications of the scan. In this paper, we carried out a set of experiments that changed the delivery rate of a glucose tracer - 2-NBDG – to the tumor. Four parameters were established based on optical imaging of the tumor vasculature and uptake of 2-NBDG – vascular oxygenation (SO<sub>2</sub>), rate of delivery of 2-NBDG (RD), rate of clearance of 2-NBDG (RC), and glucose uptake (2-NBDG<sub>60</sub>). The 60-minute time point was based on overlap between 2-NBDG fluorescence and RFP-positive tumor areas seen in the window chamber and is similar to the time point used in FDG-PET scans.
imaging to calculate SUV. We then examined all four endpoints at baseline and after recovery from hypoxia in two tumor lines. Alternating 1-hour cycles of hypoxia and reoxygenation created a large increase in SO₂ in the 4T1 tumors that was dependent on baseline SO₂. The hypoxic or low SO₂ regions in the 4T1 tumors were absent after breathing hypoxic gas. An increase in blood flow following ischemia is a natural response to a transient oxygen deficit in normal tissue (reactive hyperemia). In this study, the increase in SO₂ in the 4T1 tumors can be attributed to increased \([\text{HbO}_2]\) and \([\text{THb}]\). This response was not observed in the well-oxygenated 4T07 tumors, presumably due to their high SO₂ at baseline. Further, there were no changes in vessel diameter or vascular length density in both cell lines following hypoxia. It has been shown previously that the CO₂ content of gases used in such animal studies could cause changes in tumor blood flow [23,24]. The gas mixture used in this study was 10% O₂ - 90% N₂ and did not contain any CO₂.

At baseline, the 4T1 and 4T07 tumors revealed contrasting relationships between 2-NBDG₆₀ and SO₂. 4T1 tumors had lowest 2-NBDG₆₀ when SO₂ was lowest. This phenomenon can be explained by perfusion-limited delivery, and hence uptake of 2-NBDG by the tumor. Pre-clinical and clinical studies have shown that lower rates of delivery lead to lower FDG uptake in tumors [7,25]. In the 4T07 tumors, 2-NBDG₆₀ was lowest when SO₂ was highest. In the absence of any kinetic data, this result would likely have been attributed to a Pasteur-like effect of lower glucose uptake in regions of low hypoxia. However, the kinetic profile of 2-NBDG uptake in the 4T07 tumors demonstrated that there was an SO₂-dependent increase in RD and that RD was highest in the region where 2-NBDG was lowest. A similar phenomenon has been noticed in untreated primary breast cancers, where Zasadny et al. observed a strong correlation between tumor SUV and blood flow but a drop-off in tumor SUV values at high blood flow rates [7]. The authors hypothesized that this was potentially due to the delivery rate exceeding the rate of phosphorylation by hexokinase. The basis for a reduction in 2-NBDG uptake at high flow rates was further established by subjecting the tumors to hypoxia. 2-NBDG₆₀ in the 4T1 tumors declined after hypoxia. Again, these results could have been attributed to a Pasteur-like effect where better oxygenation and perfusion leads to a lesser need for 2-NBDG. However, the large increase in flow in the 4T1 tumors led to a SO₂-dependent increase in R₉ that was similar to the 4T07 tumors at baseline. In addition, there was no SO₂-dependent trend in 2-NBDG₆₀ in the 4T1 tumors, suggesting that the changes in 2-NBDG₆₀ were not a direct result of changes in SO₂ but rather a result of changes in flow due to better oxygenation.

The contour plots presented in Fig. 7 allow simultaneous visualization of R₉, R₇, and 2-NBDG₆₀ and reveal an interesting interplay between the three parameters. At low rates of R₉ that do not exceed the glucose consumption rate, both 2-NBDG₆₀ and R₇.

Table 1. Summary of R₉ and R₇ values for the representative curves shown in Figure 7A.

| Cell line | SO₂ | Low SO₂ | High SO₂ | Low SO₂ | High SO₂ |
|-----------|-----|---------|----------|---------|----------|
| 4T1       | Baseline | 0.89 | 4.37 | 0.17 | 0.27 |
|           | Post-hypoxia | 4.14 | 7.12 | 0.21 | 0.3 |
| 4T07      | Baseline | 2.14 | 6.58 | 0.11 | 0.22 |
|           | Post-hypoxia | 2.35 | 4.94 | 0.19 | 0.23 |

Figure 6. Effect of breathing hypoxic gas on 2-NBDG kinetics. A. Kinetic profiles of 2-NBDG uptake in 4T1 and 4T07 tumors for high and low SO₂. Blue line corresponds to the initial rate of delivery (R₀). Rate of clearance (R₇) is calculated from the falling part of the curve. Solid lines indicate baseline data and dashed lines indicate post-hypoxia data. B. Breathing hypoxic gas caused a significant increase in R₀ of 4T1 tumors (p = 0.002). There was significant increase in R₇ of 4T1 and 4T07 tumors after breathing hypoxic gas. Each set of bars in 6B represents data from one animal at baseline and after hypoxia (5 animals per cell line). C. Survival curves illustrating the relationship between R₉ and SO₂. In the 4T1 tumors, R₉ is lowest when SO₂ < 10%, illustrating that low SO₂ leads to poor perfusion. After hypoxia, the region of lowest SO₂ is no longer present in the 4T1 tumors, indicating an increase in SO₂. There is a small, but significant increase in R₉ with an increase in SO₂. In the 4T7 tumors, R₉ is highest for the highest level of SO₂ (>60%). Post hypoxia, there are no significant changes in R₉ as a function of SO₂. Statistical tests to compare 2-NBDG₆₀ survival curves were conducted using repeated measures ANOVA. For each SO₂ range, the number of animals is indicated in parentheses. Error bars represent standard error of the mean. *indicates statistical significance at a = 0.05.

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increase with \( R_D \). This is feasible because improved delivery is leading to improved uptake by the tumor. At the same time, not all of the delivered 2-NBDG is being taken up, leading to increased uptake.

**Figure 7. Relationship between \( SO_2 \), \( R_D \), \( R_C \) and 2-NBDG\textsubscript{60} for 4T1 and 4T07 tumors.**

A. Illustration of 2-NBDG uptake curve indicating how \( R_C \), \( R_D \) and 2-NBDG\textsubscript{60} are calculated.

B. Contour plots showing the relationship between delivery, clearance and uptake of 2-NBDG. \( R_D \) and \( R_C \) represent the x- and y-axes, respectively, while 2-NBDG\textsubscript{60} represents the z-axis projecting out of the x-y plane. For the 4T1 tumors at baseline, 2-NBDG\textsubscript{60} increases with \( R_D \) at low values of \( R_C \). At higher values of \( R_C \), 2-NBDG\textsubscript{60} reaches a maximum at \( R_D = 2.5 \text{ s}^{-1} \), levels off and then declines gradually for increasing \( R_D \). After hypoxia, a secondary maximum is seen at very high values of \( R_D \) (\( R_D > 6 \text{ s}^{-1} \)) and low \( R_C \). In the 4T07 tumors, 2-NBDG\textsubscript{60} increases with \( R_D \) and reaches a maximum at approximately 6 \text{ s}^{-1}. The same feature is also present after hypoxia. At higher values of \( R_C \), 2-NBDG\textsubscript{60} was nearly negligible for increasing values of \( R_C \).

**Table 2.** A summary of possible relationships between NBDG\textsubscript{60}, \( R_D \), \( R_C \), and \( SO_2 \) and final outcome corresponding to each combination.

| \( SO_2 \) | \( R_D \) (s\(^{-1}\)) | \( R_C \) (s\(^{-1}\)) | 2-NBDG\textsubscript{60} | Conclusion/tissue state |
|-----------|----------------|----------------|---------------|------------------------|
| Low       | Low            | Low            | Low           | Delivery-limited; anoxia |
| High      | Low            | High           | High          | High demand; hypoxic   |
| High      | High           | High           | Low           | Delivery rate->glycolytic rate |
| High      | Low            | High           | Aerobic glycolysis |

**Figure 8. Ratio of 2-NBDG uptake to delivery is higher in 4T1 tumors.** The ratio of 2-NBDG\textsubscript{60} and \( R_D \), which are indicative of glucose uptake and flow, respectively, is significantly higher in the 4T1 group (\( p = 0.01 \)) compared to the 4T07 group.

\[ \text{p} = 0.01 \]

\[ 2-\text{NBDG}_{60}/R_D \]

\[ x \times 10^4 \]

\[ 4T1 (13) \]

\[ 4T07 (13) \]
clearance. When \( R_D \) exceeds the glucose consumption rate, \( 2\text{-NBDG}_{60} \) decreases whereas \( R_C \) continues to increase. Because more \( 2\text{-NBDG} \) is being cleared or washed out, \( 2\text{-NBDG}_{60} \) decreases. Studies of glucose utilization in rat models of ischemic myocardium have revealed a strong dependence on coronary flow and glucose delivery rates. Specifically, glucose extraction as measured by F-FDG increased at lower flow rates and decreased with an increase in flow [26]. After hypoxia, the 4T1 tumors revealed a slight shift in the \( 2\text{-NBDG}_{60} \) inflection point from 2.5 s\(^{-1} \) to 4 s\(^{-1} \), suggesting a possible increase in glucose consumption rate after hypoxia. However, both mean \( 2\text{-NBDG}_{60} \) values as well as the survival curves do not suggest an increase in \( 2\text{-NBDG}_{60} \) after hypoxia. Recovery from hypoxia extended the range of \( R_F \) in the 4T1 tumors to reveal a secondary maximum in \( 2\text{-NBDG}_{60} \) at high \( R_F \) (\( >6 \text{ s}^{-1} \)) and low \( R_C \) (\(<0.15 \text{ s}^{-1} \)). It is interesting to note that the same crest in \( 2\text{-NBDG}_{60} \) is also observed in the 4T07 tumors at baseline and after hypoxia. Further, the tumor regions corresponding to this \( 2\text{-NBDG}_{60} \) zone (\( R_D=6 \) and \( R_C=0.15 \text{ s}^{-1} \)) were reasonably oxygenated (mean \( SO_2=35\% \)), indicating the presence of a potential aerobic glycolysis phenotype common to both cell lines.

These findings suggest that simply measuring glucose tracer uptake at a specific time-point is inadequate; knowledge of tracer kinetics and \( SO_2 \) as well is important to assess the tumor microenvironment. In a clinical study on primary breast tumors, Semple et al. showed that uptake of FDG was affected by blood flow and hence delivery of the glucose tracer [27]. One of their conclusions was that dynamic measurements of FDG uptake that account for the vascular characteristics of the tumor would provide an accurate measure of glucose uptake in tumors. Knowledge of \( SO_2 \) is important because \( R_D \), which is influenced by the vasculature, does not provide information about \( SO_2 \). Regions of high rates of delivery can be found at different \( SO_2 \) levels, leading to potentially different conclusions (Table 2). In addition, accounting for tracer delivery in addition to glucose metabolism has been shown to be of prognostic value in predicting response to therapy. Specifically, the ratio of glucose uptake to blood flow (which affects delivery of tracers) has been proposed as a biomarker of tumor aggression and response to therapy. A low ratio of metabolic rate of FDG uptake to blood flow measured pre-therapy was predictive of better disease-free survival in locally advanced breast cancers treated with neoadjuvant chemotherapy [5,28]. A high ratio of \([^{18}\text{F}]\text{-FDG}\) uptake to blood flow (measured using \([^{18}\text{O}]\text{H}_2\text{O}\)) has been correlated with tumor aggressiveness and resistance to therapy in pancreatic cancers [29] and poor local control in head and neck cancers [30]. Although our study does not provide direct measures of blood flow, we have shown that our hyperemia protocol increased oxy-hemoglobin and hence \( R_D \), indicating increased blood flow in the tumor. We used \( R_D \) and \( 2\text{-NBDG}_{60} \) to derive a similar ratio of metabolism to blood flow. The ratio of \( 2\text{-NBDG}_{60} \) to \( R_D \) of pre- and post-hypoxia tumors combined demonstrated a significantly higher ratio in the 4T1 tumors (Figure 8). It is interesting that a ratio that was found to be higher in therapy-resistant aggressive tumors in clinical studies was similarly higher in the metastatic 4T1 tumors in our study. On the other hand, the 4T07 tumors demonstrated a ratio similar to that of normal tissue in addition to being well oxygenated. A natural follow-up to this study would be to correlate longitudinal measures of endpoints derived in this study with metastatic progression and tumor recurrence in pre-clinical models of breast cancer. Such studies could potentially provide biomarkers to predict long-term outcome in breast tumors at the time of detection.

We are currently developing and validating the endpoints derived in this study using optical spectroscopy, a noninvasive optical fiber-based method. Optical spectroscopy affords the ability to make repeated and noninvasive \( \text{in vivo} \) measurements of tumor morphology and function over a long period of time. The results of this study also hold true for other imaging modalities such as PET in clinical studies and whole animal fluorescence molecular tomography (FMT) that may be used in pre-clinical studies to measure tumor glucose demand in response to therapeutic strategies such as targeted molecular agents or radiation. Targeted therapies such as bevacizumab [31] and PI3K inhibitors [32] have been shown to normalize the vasculature, which can potentially improve tracer delivery to the tumor and thus modulate glucose demand. We have previously demonstrated that radiation can upregulate HIF-1 and hence VEGF, which can stabilize vascular integrity and promote angiogenesis [33,34]. We have also shown separately that radiation promotes aerobic glycolysis [35]. Thus, therapy can lead to dynamic changes to the tumor vasculature which can influence glucose delivery as well as intrinsic glycolytic demand of tumor cells. Therefore, it is important to separate these effects to accurately determine changes in tumor metabolic demand.

In summary, we have presented a set of optical endpoints that can report on tumor glycolytic demand. Our findings demonstrate that the uptake of glucose tracers such as \( 2\text{-NBDG} \) is dependent on the rate of delivery to the tumor. Because this delivery rate is influenced by blood flow, it is essential to determine the kinetics of tracer uptake as well as \( SO_2 \) to make informed decisions based on changes in metabolic demand of a tumor. This is especially critical when analyzing tumors before and after events such as chemotherapy and radiation therapy, which are known to induce changes to the tumor microvasculature.

Author Contributions
Conceived and designed the experiments: NR MWD NR. Performed the experiments: NR AEF JZ. Analyzed the data: NR AEF ANF NR. Contributed reagents/materials/analysis tools: NR ANF KH MWD NR. Wrote the paper: NR MWD NR.

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