Optical Metabolic Imaging Identifies Glycolytic Levels, Subtypes, and Early-Treatment Response in Breast Cancer

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Abstract
Abnormal cellular metabolism is a hallmark of cancer, yet there is an absence of quantitative methods to dynamically image this powerful cellular function. Optical metabolic imaging (OMI) is a noninvasive, high-resolution, quantitative tool for monitoring cellular metabolism. OMI probes the fluorescence intensities and lifetimes of the autofluorescent metabolic coenzymes reduced NADH and flavin adenine dinucleotide. We confirm that OMI correlates with cellular glycolytic levels across a panel of human breast cell lines using standard assays of cellular rates of glucose uptake and lactate secretion (P < 0.05, r = 0.89). In addition, OMI resolves differences in the basal metabolic activity of untransformed from malignant breast cells (P < 0.05) and between breast cancer subtypes (P < 0.05), defined by estrogen receptor and/or HER2 expression or absence. In vivo OMI is sensitive to metabolic changes induced by inhibition of HER2 with the antibody trastuzumab (herceptin) in HER2-overexpressing human breast cancer xenografts in mice. This response was confirmed with tumor growth curves and stains for Ki67 and cleaved caspase-3. OMI resolved trastuzumab-induced changes in cellular metabolism in vivo as early as 48 hours posttreatment (P < 0.05), whereas fluorodeoxyglucose-positron emission tomography did not resolve any changes with trastuzumab up to 12 days posttreatment (P > 0.05). In addition, OMI resolved cellular subpopulations of differing response in vivo that are critical for investigating drug resistance mechanisms. Importantly, OMI endpoints remained unchanged with trastuzumab treatment in trastuzumab-resistant xenografts (P > 0.05). OMI has significant implications for rapid cellular-level assessment of metabolic response to molecular expression and drug action, which would greatly accelerate drug development studies. Cancer Res; 73(20); 6164–74. ©2013 AACR.

Introduction
Cellular metabolism produces energy and macromolecules necessary for cell survival. Abnormal metabolism is involved in many of the diseases that cause the greatest burden of morbidity and mortality in the developed world. Many malignant cancer cells maintain high rates of glycolysis in the presence of oxygen (1) and oncogenic transformation is linked with changes in metabolic rates. For example, the HER2 receptor tyrosine kinase, which is amplified in about 20% of invasive breast cancer, potently activates the phosphatidylinositol-3 kinase (PI3K)/Akt/mTOR pathway, a master regulator of glucose metabolism (2, 3). Patients with HER2 gene-amplified breast cancers present with more aggressive disease and generally have a poor prognosis (4). HER2 inhibitors such as the antibody trastuzumab (herceptin) provide substantial clinical benefits. However, the action of HER2 inhibitors is limited because of innate and acquired drug resistance (5).

Clinically and in preclinical drug development, there is a need for high-resolution, noninvasive, functional imaging tools to monitor and predict drug efficacy versus lack of efficacy. In cancer research, the primary endpoint of drug efficacy is tumor regression. However, cellular and molecular changes precede changes in tumor size. If these molecular endpoints could be identified and measured, they would provide biomarkers predictive of drug response or drug resistance. Cellular metabolism is particularly sensitive to upstream molecular interventions and therefore may be a powerful biomarker of early-drug response. The HER2 inhibitor trastuzumab, for example, inhibits PI3K-mediated glucose metabolism (6–8). Current preclinical and clinical methodologies to assess metabolic state in tumors in situ include fluorodeoxyglucose-positron emission tomography (FDG-PET), immunohistochemical (IHC) assessment of levels of metabolic regulators, and metabolic flux analyses (7, 9–13). Yet each of these techniques fails to capture dynamic changes in metabolic state and poorly reflect sensitivity to drug efficacy (7, 9, 14–18).
Optical metabolic imaging (OMI) exploits the autofluorescent properties of reduced NADH and flavin adenine dinucleotide (FAD), two metabolic coenzymes. We use multiphoton fluorescence and time-correlated single photon counting to measure the optical redox ratio and fluorescence lifetimes of NADH and FAD in living cells and tissues. The optical redox ratio is the ratio of NADH fluorescence intensity divided by FAD fluorescence intensity (19) and provides a dynamic measure of cellular metabolism (8, 19–21). The fluorescence lifetime, the time a molecule remains in the excited state, is independent of inter- or intrainstrument variability, resolves free and bound protein configurations, and is influenced by preferred protein-binding of the molecules and proximity to quenchers (e.g., oxygen; ref. 22). NADH and FAD each have two-component fluorescence decays. For NADH, the short lifetime ($\tau_1$) corresponds to NADH free in solution, whereas the long lifetime ($\tau_2$) corresponds to protein-bound NADH (23). Conversely, protein-bound FAD corresponds to the short lifetime, whereas free FAD corresponds with the long lifetime (24). The shorter fluorescence lifetimes of both protein-bound FAD and free NADH are due to dynamic quenching by the adenine moiety (22, 25). The mean fluorescence lifetime ($\tau_m$) is the weighted average of the short and long lifetime components, $\tau_m = \alpha_1\tau_1 + \alpha_2\tau_2$, where $\alpha_1$ and $\alpha_2$ are the fractional contributions of the short and long lifetimes, respectively.

The images acquired by OMI provide sufficient resolution and contrast to distinguish the cellular and extracellular tissue compartments, as the collagen-enriched ECM generates a strong second harmonic signal that has a lifetime and spectral emission distinct from cellular NADH and FAD (26). Resolution is adequately high to isolate single cells, allowing identification of inflammatory infiltrates in the stroma and tumor epithelia. This single cell-level resolution may be useful for identifying resistant subpopulations of cells that preexist in the tumor and are responsible for cancer relapse.

Because OMI is inexpensive, fast, and directly measures dynamic changes in cellular metabolism that reflect glycolysis, oxidative phosphorylation, and metabolic enzyme microenvironment interactions, we investigated the potential of OMI as a tool for monitoring metabolic response to targeted therapies in human breast cancer cells and xenografts. OMI was validated by measuring metabolic inhibition by cyanide, and in comparison with standard assays of glycolytic metabolism. The sensitivity of OMI to breast cancer subtypes was confirmed. Finally, the OMI-measured response of mouse xenograft models treated with trastuzumab was compared with FDG-PET, IHC, and tumor size measurements. This work represents a significant advancement in the tools available to study cellular metabolism and tumor response to treatment in living systems.

Materials and Methods

Fluorescence lifetime instrumentation

A custom built, commercial multiphoton fluorescence microscope (Prairie Technologies) was used to acquire fluorescence images. A 40X water-immersion objective (1.15 NA) or a 40X oil-immersion objective (1.3 NA) coupled the excitation and emitted light through an inverted microscope (TiE, Nikon). A titanium:sapphire laser (Coherent Inc.) was tuned to 750 nm for excitation of NADH and 890 nm for FAD excitation. The average laser power was 7.5 to 7.8 mW for NADH and 8.4–to 8.6 mW for FAD. A pixel dwell time of 4.8 µs was used. A GaAsP PMT (H7422P-40, Hamamatsu) detected emitted photons. A 400 to 480 nm bandpass filter isolated NADH fluorescence. A 500 nm high pass dichroic mirror and a 500 to 600 nm bandpass filter isolated FAD fluorescence.

Fluorescence lifetime images were acquired using time correlated single photon counting (TCSPC) electronics (SPC-150, Becker and Hickl). TCSPC uses a fast detector PMT to measure the time between a laser pulse and fluorescence event. Each image of 256 × 256 pixels was acquired using an integration time of 60 seconds. No change in the photon count rate was observed, ensuring that photobleaching did not occur. The instrument response function (measured from the second harmonic generated signal of urea crystals excited at 900 nm) full width at half maximum was measured to be 260 ps. The single-component fluorescence lifetime of a fluorescent bead (Polysciences Inc.) was measured daily. The measured fluorescence lifetime of the bead was 2.1 ± 0.08 ns (n = 18), which is consistent with published studies (20, 27).

Cell culture

All cell lines were acquired from the American Type Culture Collection except the HR6 cell line (28), which was provided by the Arteaga Laboratory. The noncancerous mammary epithelium cell line, MCF10A, was cultured in MEBM (Lonza) supplemented with cholera toxin, penicillin: streptomycin, bovine pituitary extract, hydrocortisone, insulin, and human epidermal growth factor. All malignant cell lines were grown in DMEM (Invitrogen) with 10% FBS and 1% penicillin: streptomycin. The growth media for the HR6 cell line was further enhanced with 25 µg/mL trastuzumab (Vanderbilt Pharmacy). For fluorescence imaging, cells were plated at a density of 10^6 cells per 35 mm glass-bottom imaging dish (MatTek Corp.) 48 hours before imaging.

The MCF10A cell line was used as a daily fluorescence standard for the redox ratio and imaged each day measurements were acquired. All other cell lines were imaged on at least two different days. A total of 18 different locations were imaged for each cell line (58 for MCF10A cells) from six different dishes (three images were acquired from each dish, see Supplementary Table S1).

Cyanide experiment

NADH and FAD fluorescence lifetime images of three locations of three dishes were acquired. Media of two of the MCF10A dishes was removed and replaced with cyanide supplemented MCF10A growth media (4 mmol/L NaCN, Sigma). The cells were allowed 5 minutes for the cyanide to react and post-cyanide NADH and FAD fluorescence images were acquired from three unique locations from each dish.

Trastuzumab perturbation

The effect of HER2 inhibition by trastuzumab was tested in HER2-overexpressing cells. The cells were plated at a density of...
10^6 cells per imaging dish, 48 hours before imaging. At 24 hours before imaging, the growth media was exchanged for growth media containing 25 µg/mL trastuzumab. This dose of trastuzumab, 25 µg/mL, was chosen to mimic therapeutic drug dosage in patients (29).

**Mouse xenografts**

This study was approved by the Vanderbilt University Animal Care and Use Committee and meets the NIH guidelines for animal welfare. MDA-MB-361 cells (10^6), BT474 cells (10^6), or HR6 cells (10^6) in 100 µL Matrigel were injected into the inguinal mammary fat pads of female athymic nude mice (J:NU; Jackson Laboratories). Tumors were allowed to grow to approximately 150 mm^3. Tumor-bearing mice were treated with trastuzumab (Vanderbilt University Medical Center pharmacy) or control human immunoglobulin G (IgG) 10 mg/kg twice weekly for two weeks. This dose of trastuzumab was chosen to mimic therapeutic drug dosage in patients (30).

**OMI xenograft imaging**

Isoflurane-anesthetized mice were used for vital imaging, by removing the skin overlying the tumor, overlaying the tumor with a coverslip, and placing the mouse on the microscope stage. NADH and FAD fluorescence lifetime images of three different tumor locations were acquired each day. After imaging, mice were humanely euthanized while under anesthesia. Each OMI group contained 3 mice, each with 2 tumors for a total n of 6 tumors at each time point.

**FDG-PET imaging**

The FDG-PET protocol follows published methods (7, 31, 32). The mice were fasted overnight and allowed to acclimate to the PET facility for 1 hour on a warm water pad. A single retroorbital injection of approximately 200 µCi (100 µL) of [18F]FDG was administered. Following a 40-minute distribution period, 20-minute static PET scans were collected on a Concorde Microsystems microPET Focus 220 (Siemens), whereas mice were anesthetized with isoflurane. PET images were reconstructed using the ordered subsets expectation maximization algorithm (33). FDG-uptake values were obtained by isolating the uptake of each tumor volume and correcting for the injected dose. Each FDG-PET group contained 5 mice, each with 2 tumors for a total n of 10 tumors.

**Quantification of the optical redox ratio**

The optical redox ratio was computed from the NADH and FAD fluorescence lifetime data. The photons detected at each pixel in an image were integrated over time to compute the sum of photons per pixel. The total number of NADH photons was divided by the total number of FAD photons at each pixel to create a redox ratio image (MATLAB, MathWorks). The redox ratio image was thresholded to remove background and nuclear fluorescence and the average redox ratio for the remaining cell cytoplasmics was computed. This approach has been confirmed to be consistent with redox ratios obtained with steady-state detection (8, 21).

**Quantification of fluorescence lifetime components**

For each image, a threshold was selected to eliminate background and nuclear fluorescence (SPCImage, Becker and Hickl). A binning of nine surrounding pixels was used. Then, the fluorescence lifetime components were computed for each pixel by deconvolving the measured system response and fitting the resulting exponential decay to a two-component model, \( I(t) = a_1 \exp^{-t/t_1} + a_2 \exp^{-t/t_2} + C \), where \( R(t) \) is the fluorescence intensity at time \( t \) after the laser excitation pulse, \( a_1 \) and \( a_2 \) are the fractional contributions of the short and long lifetime components, respectively (i.e., \( a_1 + a_2 = 1 \)), \( t_1 \) and \( t_2 \) are the fluorescence lifetimes of the short and long lifetime components, and \( C \) accounts for background light. A two-component decay was used to represent the lifetimes of the free and bound configurations of NADH and FAD (20, 23, 24). The average lifetime component values and a mean fluorescence lifetime \( \langle t_m = a_1 t_1 + a_2 t_2 \rangle \) for each image were computed in MATLAB.

**Statistical analysis**

A rank sum test of means was used to test for significant differences due to cyanide. A Bonferroni correction for multiple-comparisons was used on rank sum tests of means of the metabolic values from the panel of cell lines. A rank sum test of means was used to identify significant differences when cell lines were treated with trastuzumab and to find differences in the in vivo xenograft experiments. A Student \( t \) test of means was assumed to be two-way. Spearman rank correlation coefficient was used to identify correlations. Both a correlation coefficient \( (r) \) and a \( P \) value were computed. An \( \alpha \) level of less than 0.05 was used and the test was assumed to be two-way.

**Computation of intra- and intercellular variation**

Intercellular variation was visualized by histogram representation of the mean metabolic measure (optical redox ratio, NADH \( t_{mr} \) or FAD \( t_{mr} \)) for all cells. The histogram was fit to one, two, and three component Gaussian curves to determine the number of modes within the data. The fit with the lowest Akaike information criterion was selected to represent the probability density function of the histogram (34). Intracellular variation was computed as the average coefficient of variation (SD divided by mean) for each cell and averaged over all cells.

**Percentage of mitotic cells**

The percentage of proliferating cells was measured by flow cytometry. Cells were plated at a density of 10^4 cells per 35 mm dish. After 48 hours, the cells were labeled with Phospho-Histone H3 (Ser10) antibody (Cell Signaling Technology) and a secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) enabled detection of labeled cells by flow cytometry.
**Glycolytic index**

Media glucose and lactate concentrations were measured using standard assay kits [Amplex Red Glucose/Glucose Oxidase Assay Kit (Invitrogen) and r-Lactate Assay Kit (Eton Bioscience Inc.)]. Concentrations of glucose and lactate in the cell growth media were determined at the time of plating (0 hour) and at the time of imaging (48 hour). The "glycolytic index" was computed as the moles of glucose consumed within 48 hours divided by the moles of lactate produced in 48 hours.

**Histologic analysis**

Tumors were collected and placed in buffered formalin, paraffin embedded, sliced, and stained with hematoxylin and eosin stain. Additional slides were stained for Ki-67 and cleaved caspase-3. Staining protocols were verified in positive control samples. The percentage of positively stained cells was quantified from five fields of view from three tumors in each group.

**Results**

Figure 1A shows the relationship between HER2 and the fluorescent molecules NADH and FAD. HER2 activation drives an increase in glycolysis, which generates NADH. The pyruvate produced in glycolysis can enter the mitochondria as a reactant in oxidative phosphorylation. Oxidative phosphorylation consumes NADH and produces FAD. A net gain of NADH relative to FAD is observed with HER2 activation due to a relative increase in glycolysis.

**Validation**

Chemical inhibition of oxidative phosphorylation affects the relative fluorescence intensities of NADH and FAD in a cell (35), so this perturbation was used to validate the optical imaging approach (Fig. 1B–D, Supplementary Fig. S1). Cyanide (which disrupts the electron transport chain) induced the predicted trends 23, 27): an increase in the optical redox ratio and a decrease in NADH $\tau_m$. Unreported until now, FAD $\tau_m$ increased with cyanide treatment.

**Metabolic profiling of breast cancer cells**

High-resolution images (Fig. 2) allowed visualization of cellular morphology and cell-to-cell variability of the optical redox ratio and NADH and FAD fluorescence lifetimes for human breast cell lines (Supplementary Table S1). Cellular fluorescence localized to the cytoplasm of cells. ER$^+$/HER2$^+$ cells displayed increased redox ratios over that seen in triple-negative cells ($P < 0.001$; Fig. 3A), and the greatest redox ratios were measured in HER2 gene-amplified cells ($P < 0.001$). To account for any differences in cellular proliferation rates among the cell lines and show that the redox ratio is not a reporter of cellular proliferation (Supplementary Fig. S2A), we normalized the redox ratio to the percentage mitotic cells (Supplementary Fig. S2B) and found similar trends, increased redox ratio/percentage mitotic cells in ER$^+$ cells and the greatest redox ratio/percentage mitotic cells in HER2$^+$ cells.

**Figure 1.** A, graphical representation of the relationship between HER2, NADH, and FAD. HER2 activation drives an increase in glycolysis, which generates NADH. The pyruvate generated in glycolysis can enter the mitochondria as a reactant in oxidative phosphorylation. Oxidative phosphorylation consumes NADH and produces FAD. A net gain of NADH relative to FAD is observed with HER2 activation due to a relative increase in glycolysis. Inhibition of HER2 by trastuzumab reduces cellular glycolysis rates, resulting in a decrease of cellular NADH relative to FAD. NADH and FAD are highlighted as the fluorescent molecules in this diagram and molecules in bold indicate the net direction of the reaction. Optical redox ratio (B), NADH $\tau_m$ (C), and FAD $\tau_m$ (D) of MCF10A (nonmalignant) cells before ($n = 9$) and after ($n = 6$) treatment with 4 mmol/L NaCN. $\tau_m$ is the mean lifetime ($\tau_m = \tau_{m1}/\alpha_1 + \tau_{m2}/\alpha_2$)$^{-1}$, $P < 0.05$.

NADH $\tau_m$ of HER2$^+$ and ER$^+$ cells was increased over that measured in nonmalignant cells ($P < 0.001$; Fig. 3B), but were statistically similar to each other ($P = 0.5$). Triple-negative cells exhibited the shortest NADH $\tau_m$. Reduced-free and protein-bound NADH lifetimes ($\tau_1$ and $\tau_2$) were observed in the triple-negative and ER$^+$ cells (Supplementary Fig. S3A and S3B). The portion of free NADH ($\alpha_1$) was decreased in the HER2$^+$ cells compared with the nonmalignant cells (Supplementary Fig. S3C). Compared with nonmalignant cells, FAD $\tau_m$ was increased in all malignant cells ($P < 0.001$), with the longest FAD $\tau_m$ observed in ER$^+$/HER2$^+$ cells ($P < 0.001$; Fig. 3C). Increased FAD $\tau_1$ and $\tau_2$ values, as well as a reduced $\alpha_1$ (contribution of bound FAD) contributed to the increased FAD $\tau_m$ observed in the malignant cells (Supplementary Fig. S3D–S3F). A scatterplot of NADH $\tau_m$ versus redox ratio/percentage mitotic cells (Fig. 3D) allowed accurate clustering of data-points of nonmalignant, triple-negative, ER$^+$, and HER2$^+$ cells. Scatterplots of FAD $\tau_m$ versus redox ratio and...
NADH $\tau_m$ versus FAD $\tau_m$ allow separation of triple-negative and nonmalignant cells (Supplementary Fig. S4).

We compared OMI with cellular rates of glucose uptake and lactate secretion or the "glycolytic index." A Spearman rank correlation coefficient ($r$) of 0.89 ($P < 0.05$) defined a positive correlation between the optical redox ratio/percentage mitotic cells and the glycolytic index (Fig. 3E). Neither NADH $\tau_m$ nor FAD $\tau_m$ correlated with the glycolytic index or the redox ratio (Supplementary Table S2).

**Trastuzumab response in vitro**

The effect of trastuzumab on cellular metabolism was investigated in three HER2-overexpressing cell lines: BT474 cells that are responsive to trastuzumab, MDA-MB-361...
cells that partially respond to trastuzumab, and HR6 cells, which were derived from a BT-474 xenograft that acquired resistance to trastuzumab in vivo (28). The redox ratio ($P < 0.05$), NADH $\tau_m$ ($P < 0.05$), and FAD $\tau_m$ ($P < 0.001$) of BT-474 cells decreased upon trastuzumab treatment for 24 hours (Fig. 4A–C). Lifetime component analysis showed significant increases in the portions of free NADH ($\alpha_1$) and bound FAD ($\alpha_2$) of BT-474 cells treated with trastuzumab (Supplementary Fig. S5). Similarly, the redox ratio of the MDA-MB-361 cells decreased ($P < 0.05$) with trastuzumab treatment (Fig. 4A). The high-resolution OMI enabled analysis of cellular heterogeneity in response to trastuzumab (Fig. 4D, Supplementary Fig. S6). Distribution modeling of cellular redox ratios of MDA-MB-361 cells revealed two subpopulations characterized by differing redox ratios (Fig. 4D). The mean redox ratio of the first population, representing 70% of cells, showed no change (1.34 to 1.31) upon trastuzumab treatment ($P = 0.07$), suggesting that trastuzumab did not affect metabolic processes in the majority of MDA-MB-361 cells. However, 30% of the cells responded to trastuzumab with a mean redox ratio that decreased from 1.89 to 1.65 ($P < 0.001$).

HR6 cells are a trastuzumab-resistant BT-474-derived subline that retains HER2 overexpression. Previous studies have shown maintenance of HER2 overexpression and PI3K/Akt signaling in trastuzumab-treated HR6 cells (28). Consistent with this observation, the redox ratio remained unchanged upon treatment with trastuzumab, as was FAD $\tau_m$. NADH $\tau_m$ decreased in HR6 cells upon treatment with the antibody ($P < 0.05$; Fig. 4A–C). An increased portion of free NADH ($\alpha_1$) contributed to the decreased NADH $\tau_m$ observed in the HR6 cells (Supplementary Fig. S5).

**In vivo xenografts** To verify the OMI response observed in cultured cells, we conducted OMI on HER2-overexpressing xenografts and compared these findings with tumor size, IHC, and FDG-PET measurements. Established BT-474 xenografts treated with control IgG continued to grow throughout the course of the experiment while the trastuzumab-treated tumors decreased in size (Fig. 5A). IHC staining confirmed lower rates of proliferation and higher rates of apoptosis in the trastuzumab-treated group (Fig. 5B and C). A representative FDG-PET image displays the location of the tumors in the mammary fat near the hind limbs and shows increased FDG-uptake in the tumors compared with the surrounding tissue (Fig. 5D). FDG-uptake increased in the control mice between days 2 and 12 post-treatment; however, no difference was observed between control and trastuzumab-treated tumors at any time point (day 2, 5, or 12 post-treatment; Fig. 5E). OMI imaging of an identical cohort of tumors allowed cellular-level visualization of metabolism (Fig. 5F) and resolved metabolic differences in the redox ratio (Fig. 5G). NADH $\tau_m$ (Fig. 5H, Supplementary Fig. S7A–S7C), and FAD $\tau_m$ (Fig. 5I, Supplementary Fig. S7D) between control and trastuzumab-responding tumors as early as 2 days after the first dose of trastuzumab.

Tumor size measurements of the HR6 xenografts showed similar growth of both control and trastuzumab-treated HR6
tumors (Fig. 6A). IHC confirmed that the trastuzumab-treated HR6 tumors retain proliferative capabilities and express similar rates of apoptosis compared with control-treated tumors (Fig. 6B and C). A representative FDG-PET image of the HR6 tumors shows increased FDG-uptake by the tumor compared with the surrounding tissue (Fig. 6D). No change in FDG-PET

Figure 5. A, BT474 tumors treated with trastuzumab (10 mg/kg, 2× weekly) decrease in size (mean ± SE) compared with control IgG-treated tumors. Trastuzumab versus control at each time point. B, Ki67 staining shows reduced proliferation in trastuzumab-treated tumors. C, cleaved caspase-3 staining shows increased apoptosis in trastuzumab-treated tumors at day 5. D, representative FDG-PET image (T, tumor). E, FDG-uptake increases in control tumors at day 12 compared with control tumors at day 2. No significant difference in FDG-uptake between trastuzumab-treated and control tumors is observed. n = 10. F, representative OMI images. Scale bar, 50 μm. G, decreased redox ratio, NADH (H), and FAD (I) are observed in trastuzumab-treated tumors at 2 days after trastuzumab-treatment. *, P < 0.05; **, P < 0.01; ***, P < 0.001; n = 6 tumors.

Figure 6. A, HR6 tumor size (mean ± SE) treated with trastuzumab (10 mg/kg, 2× weekly) compared with control IgG-treated tumors. B, Ki67 staining of HR6 control IgG and trastuzumab-treated tumors. C, cleaved caspase-3 staining of HR6 control IgG and trastuzumab-treated tumors. D, representative FDG-PET image (T, tumor). E, FDG-uptake increases in control tumors at day 12 compared with control tumors at day 2, **, P < 0.05. No significant difference in FDG-uptake between trastuzumab-treated and control tumors is observed at any time. n = 10. F, representative OMI images; scale bar, 50 μm. G, redox ratio, NADH (H), and FAD (I) of control and trastuzumab-treated tumors. *, P < 0.05; n = 6 tumors.
was observed between control and trastuzumab-treated tumors at any time point; however, the FDG-uptake was increased in the control mice at 12 days compared with 2 days (Fig. 6E). No difference was observed in the redox ratio between control and treated HR6 tumors at any time point (Fig. 6F and G). NADH \( t_m \) decreased in the HR6-treated tumors relative to controls at 2 days posttreatment, but was similar to controls at days 5 and 14 (Fig. 6H, Supplementary Fig. S8). No change in FAD \( t_m \) was observed at any time point (Fig. 6I, Supplementary Fig. S8).

The redox ratio \( (P < 0.05) \), NADH \( t_m (P < 0.05) \), and FAD \( t_m (P < 0.05) \) decreased in trastuzumab-treated MDA-MB-361 xenografts as compared with IgG-treated controls (Fig. 7A–D; Supplementary Fig. S9) 2 days after treatment. Modeling of the optical redox ratio and FAD \( t_m \) values on a cell-by-cell basis (intercell variability) identified two subpopulations of tumor cells in trastuzumab-treated animals, whereas an unimodal cellular distribution was identified for NADH \( t_m \) (Fig. 7E–G). High-resolution analysis revealed that intracellular variability in redox ratios and FAD \( t_m \) increased \( (P < 0.05) \) in trastuzumab-treated samples (Fig. 7H). These results fully characterize OMI and show its potential for monitoring early-drug response in cell culture and mouse xenografts on a single-cell level.

Discussion

OMI is advantageous for live cell and animal imaging due to several features, including direct and dynamic assessment of cellular metabolism \textit{in vivo}, cellular and subcellular resolution imaging capabilities, rapid acquisition, low-cost, use of intrinsic signals (no contrast agents, no radioactivity), and high sensitivity to metabolic changes. Our results have significant implications for rapid assessment of drug action in preclinical models, which would greatly accelerate drug development studies.

These results are the first to correlate OMI with a standard assay of metabolic behavior. Although the optical redox ratio is inferred from theory as the relative rates of glycolysis and oxidative phosphorylation within cells, we confirm a strong...
positive correlation ($r = 0.89, P = 0.03$) with a standard measure of cellular glycolytic levels (Fig. 3). In contrast, NADH $\tau_{nm}$ and FAD $\tau_{nm}$ are not correlated with the glycolytic index (Supplementary Table S2). The proportion of free NADH ($\alpha_1$) has been interpreted as an analog to the optical redox ratio (27); however, we did not find a correlation between the optical redox ratio and NADH $\alpha_1$ (Supplementary Table S2). Given the physical nature of these fluorescence lifetime measurements, which are sensitive to protein-binding, relative fractions of free and bound components, and proximity to quenchers, it is not surprising that they are sensitive to more cellular processes than just glycolysis. For example, changes in the distribution of NADH and FAD enzyme–binding sites associated with preferred metabolic pathways in breast cancer subtypes may be responsible for the changes in protein-bound lifetimes between cell lines (36). The changes in the lifetimes of the free components of NADH and FAD may reflect changes in dynamic quenching (22). Taken together, the data (Fig. 3, Supplementary Table S2) indicate that the redox ratio, NADH, and FAD lifetimes provide independent measures of cellular metabolism and the molecular microenvironment. The varied dynamics of these endpoints due to HER2 inhibition suggest that all three OMI endpoints provide added value when measured together.

We used these tools to differentiate breast cancer cells by subtype, defined by ER, and/or HER2 expression or absence (Fig. 3). OMI is sensitive to metabolic behaviors caused by ER and HER2 (Fig. 3), known oncogenic drivers of glycolytic metabolism in breast cancer cells (3, 8, 37, 38). ER regulates gene expression of glucose transporter proteins and proteins involved in oxidative phosphorylation and the citric acid cycle such as isocitrate dehydrogenase, which actively reduces NAD$^+$ to NADH (37, 39–41). HER2 mediates metabolism through signaling of the PI3K/Akt/mTOR pathway, which directs transcription of glycolytic enzymes (2, 3). When combined, the redox ratio and NADH lifetime fully separate the distinct subtypes of breast cancer (Fig. 3D), indicating that the complementary measures of the redox ratio and fluorescence lifetime allow robust characterization of cellular metabolism and molecular microenvironments associated with breast cancer subtypes.

We further show that the OMI endpoints optical redox ratio, NADH $\tau_{nm}$ and FAD $\tau_{nm}$ reflect impaired metabolic activity in trastuzumab-responsive BT474 cells (Fig. 4) in vitro, suggesting a large metabolic response of HER2-amplified cells to trastuzumab. Only one of the OMI endpoints, NADH $\tau_{nm}$ decreased in the trastuzumab-resistant HR6 cells (Fig. 4). Although HR6 cells maintain HER2-overexpression and PTEN and P-Akt protein levels in the presence of trastuzumab, blockage of HER2 with trastuzumab may affect internal signaling (28). Because of its highly sensitive nature, NADH $\tau_{nm}$ may be reporting such minute metabolism differences.

A subset of variables also resolved metabolic changes induced by trastuzumab in poorly responsive HER2-amplified cells (MDA-MB-361) in vitro (Fig. 4). Behavioral heterogeneity inherent to the MDA-MB-361 cell line suggests that 25% of the overall population would be growth inhibited by trastuzumab in vitro (42). Our data detected a 30% subpopulation within MDA-MB-361 cells that responded to trastuzumab through decreased redox ratios (Fig. 4D), suggesting correlative evidence that high-resolution optical imaging is capable of detecting responders and nonresponders at the single-cell level in the context of a heterogeneous tumor cell population with a mixed response. The complementary metabolic information gained from the OMI endpoints allowed identification of a large metabolic response to trastuzumab (BT474) but also resolved negative (HR6) and partial (MDA-MB-361) responses, showing high sensitivity and resolution of OMI.

Finally, we measured the in vivo metabolic response to trastuzumab in HER2-overexpressing mouse xenografts. Trastuzumab-induced metabolic repression in BT474 and MDA-MB-361 tumors was detected by 48 hours posttreatment (Figs. 5 and 7). The redox ratio and FAD $\tau_{nm}$ did not change in trastuzumab-resistant HR6 tumors treated with the antibody (Fig. 6). However, NADH $\tau_{nm}$ decreased in trastuzumab-treated HR6 tumors compared with controls at 48 hours (Fig. 6), which was consistent with the in vitro results (Fig. 4). Modeling of the intercellular variation of MDA-MB-361 xenograft tumors identified two subpopulations in response to trastuzumab for the optical redox ratio and FAD $\tau_{nm}$ (Fig. 7), suggesting in vivo cell-to-cell heterogeneity. This heterogeneous in vivo response is consistent with the in vitro results (in Fig. 4). We attempted to include only tumor cells in our image analysis, by evaluation of the cell morphology with respect to histology, but acknowledge that nontumor cell populations could have been included in this analysis. We speculate the greater intracellular variation of both the redox ratio and FAD $\tau_{nm}$ observed in the MDA-MB-361 tumors treated with trastuzumab (Fig. 7H) is due to heterogeneous responses of individual mitochondria (43). Elucidation of this possibility will require additional research.

The tumor size measurements and IHC analysis of cellular proliferation and apoptosis were consistent with an antitumor effect of trastuzumab against BT474 tumors and a lack of an effect against HR6 tumors (Figs. 5 and 6). Tumor size measurements first identified a difference in control versus trastuzumab-treated BT474 tumor size at 8 days after treatment was initiated (Fig. 5A), 6 days later than the first significant change in OMI endpoints between control and trastuzumab-treated tumors (day 2, Fig. 5G). Although Ki67 staining of BT474 tumors identified reduced proliferation in BT474 tumors treated with trastuzumab at 2 and 5 days posttreatment, OMI is advantageous over IHC as a measure of tumor response because OMI provides a dynamic measure of cellular metabolism and can be conducted in vivo and over time within the same animal. Although in vivo OMI data are presented here, OMI endpoints remain robust measures of metabolism in freshly excised tissues (21) allowing implementation of this metabolic imaging technique in situations when in vivo measurements are not feasible.

FDG-PET has also been explored as a potential tool for assessing response to therapeutic agents. Consistent with a prior study (7), in this study, FDG-PET failed to identify a metabolic difference between control and trastuzumab-treated BT474 tumors (Fig. 5). FDG-uptake did increase over time as the control tumors continued to grow. Unlike OMI, PET cannot resolve cellular subpopulations of differing response
that are critical for investigating drug resistance mechanisms (7, 13–17, 44). Furthermore, PET requires large upfront and continued costs due to radiolabeled tracers, whereas OMI can be implemented in low-cost, wide-field, or confocal microscopes without the need for external dyes (45–47). In addition, OMI allows for fast, accurate, dynamic in vivo monitoring of early-therapeutic response, potentially reducing time and animals required for drug development. Our studies have focused on breast cancer and HER2 inhibition, but the methods developed are also applicable to the array of diseases that are treated with metabolism-modulating drugs (48–50).

The results of this study validate OMI as a powerful tool for quantifying cellular metabolism, which is an active area of investigation across multiple diseases. As we show using human breast cancer cells and xenografts, optical metabolic measurements are sensitive to distinct tumor cell subtypes. In addition, we present significant findings suggesting that OMI can be used as an early indicator of metabolic response to treatment with a targeted therapy, both in cell culture and in vivo. These results represent the culmination of multiple imaging technologies, analysis tools, and their validation with standard assays and FDG-PET. The results in breast cancer cells and xenografts provide the first direct measurements relating cellular metabolism, HER2 expression, HER2 inhibition, and resistance to HER2 inhibitors in live cells and tissues. In addition, these methodologies are potentially broadly applicable outside the cancer and imaging communities, including those in drug development and metabolism research across multiple diseases. Although we see OMI as an immediately powerful tool in preclinical models, it also may directly impact patient care as an adjunct to current practice, either on freshly excised tissue (21) or through in vivo endoscopes adapted for fluorescence imaging.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.C. Skala
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Optical Metabolic Imaging Identifies Glycolytic Levels, Subtypes, and Early-Treatment Response in Breast Cancer

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