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The effect of flow on BOLD (R_2^*) MRI of orthotopic lung tumors

Heling Zhou¹, Olivier Belzile², Zhang Zhang³, Jo Wagner¹, Chul Ahn⁴, James A. Richardson⁵, Debabrata Saha³, Rolf A. Brekken^{2,6}, and Ralph P. Mason¹

¹Department of Radiology, University of Texas Southwestern Medical Center, Dallas, TX, United States

²Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas, TX, United States

³Department of Radiation Oncology, University of Texas Southwestern Medical Center, Dallas, TX, United States

⁴Department of Clinical Science, University of Texas Southwestern Medical Center, Dallas, TX, United States

⁵Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX, United States

⁶Department of Surgery, University of Texas Southwestern Medical Center, Dallas, TX, United States

Abstract

Purpose: Blood oxygen level dependent (BOLD) MRI based on R_2^* measurements can provide insights into tumor vascular oxygenation. However, measurements are susceptible to blood flow, which may vary accompanying a hyperoxic gas challenge. We investigated flow sensitivity by comparing R_2^* measurements with and without flow suppression (fs) in two orthotopic lung xenograft tumor models.

Methods: H460 (n=20) and A549 (n=20) human lung tumor xenografts were induced by surgical implantation of cancer cells in the right lung of nude rats. MRI was performed at 4.7 T after tumors reached 5–8 mm diameter. A multi-echo gradient echo MRI sequence was acquired with and without spatial saturation bands on each side of the imaging plane to evaluate the effect of flow on R_2^* . fs and non-fs R_2^* MRI measurements were interleaved during an oxygen breathing challenge (from air to 100% O_2). T_2^* -weighted signal intensity changes (SI(%)) and R_2^* measurements were obtained for regions of interest and on a voxel-by-voxel basis and discrepancies quantified with Bland-Altman analysis.

Results: Flow suppression affected SI(%) and R_2^* measurements in each tumor model. Average discrepancy and limits of agreement from Bland-Altman analyses revealed greater flow-related bias in A549 than H460.

Conclusion: The effect of flow on R_2^* , and hence BOLD, was tumor model dependent with measurements being more sensitive in well-perfused A549 tumors.

Keywords

Flow-suppression; oxygen-sensitive MRI; hypoxia; blood oxygen level dependent (BOLD); lung cancer

Introduction:

Hypoxia is associated with tumor aggressiveness, metastatic spread and resistance to various treatments, particularly radiation therapy [1–3]. Several studies have demonstrated the connection of hypoxia with poor prognosis in lung cancer. Through examination of tumor samples from surgery, hypoxia-inducible factor (HIF)-1 α [4, 5] and HIF-2 α [4] have been identified as independent prognostic indicators in non-small-cell lung cancer (NSCLC). Eppendorf polarographic electrodes were used intra-operatively to measure human lung tumor oxygenation directly and relative hypoxia correlated with elevated levels of osteopontin, CAIX and poor prognosis in terms of local relapse and overall survival [6]. Imaging methods have also been explored including PET based on ^{18}F MISO or ^{60}Cu -ATSM [7, 8]. These studies provided evidence that hypoxia in lung cancer impacts treatment outcomes; however, the invasive nature of assessment techniques or the need for radioactive isotopes limited the feasibility of repeat measurements and general adoption of the techniques into clinic.

MR imaging methods have been developed to provide information regarding tumor oxygenation [9–11]. R_2^* is sensitive to the concentration of deoxyhemoglobin and changes in compartmentalized deoxyhemoglobin yield BOLD contrast [12–14]. Both semi quantitative T_2^* -weighted signal intensity changes and quantitative R_2^* measurements have been explored and gained popularity in cancer imaging due to the simplicity of the technique and clinical translatability [11, 15–18]. An intervention, such as hyperoxic gas challenge, is frequently used to perturb the biological system to obtain additional information on tumor oxygenation [12, 18–25]. However, BOLD signal is affected by a number of physiological factors in addition to oxygenation, notably blood flow, vascular volume, hematocrit and the Hill coefficient for oxygen binding to hemoglobin (itself sensitive to 2, 3-DPG, CO_2 and pH [26]). Indeed, early studies coined the concept “flow and oxygen level dependent” (FLOOD) MRI contrast [27, 28], and a breathing gas intervention, such as oxygen challenge, may induce changes in blood flow, potentially affecting the BOLD signal [29].

The extent to which BOLD (*viz* R_2^*) is sensitive to potential flow changes has not previously been quantified in lung cancer models. We expected it to be particularly relevant and challenging for orthotopic lung tumors giving the proximity to major blood vessels and the potential motion artifacts. We have now examined and quantified the effect of flow on BOLD measurement by comparing two sequences with different flow suppression (fs) settings applied to two orthotopic lung xenograft tumor models.

Methods:

Cell Preparation

Luciferase transfected human lung tumor cells were kindly provided by Professor John Minna. Briefly, the A549-luc and H460-luc human lung cancer cells (ATCC, Manassas, VA) were established using a lentivirus encoding the luciferase gene driven by a ubiquitin promoter. Stable clones were then isolated by G418 selection. The cells were incubated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 1% *L*-glutamine and 1% penicillin-streptomycin at 37 °C with 5% CO₂. Once 80% confluence was reached, the cells were harvested, and suspended in serum-free medium and 50% Matrigel (BD Biosciences, Mississauga, Canada).

Surgical Orthotopic Tumor model

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center. The surgical procedure was described previously [30]. Briefly, female nude rats (8–10 weeks old, 150–200 g; National Cancer Institute, Frederick, MD) were anesthetized and placed onto a Hallowell tilting workstation (Hallowell EMC, Pittsfield, MA). One to two drops of 2% lidocaine were applied to the back of the mouth to locally anesthetize the epiglottis. The rats were then intubated and connected to the ventilator. Anesthesia was maintained with 2% isoflurane in oxygen, and the ventilator was set to achieve a tidal volume of approximately 10 ml/kg and a respiratory rate of 79–90 breaths/min. A 1–2 cm incision was made in the shaved and cleaned skin over the 5th and 6th ribs on the right side of the chest and the muscle was bluntly dissected. The chest wall was opened with an incision through the 5th and 6th intercostal space. The bottom lobe of the right lung was carefully exteriorized using curved forceps, and a portion of the tissue was clamped with a microvascular clamp. A cell suspension (3×10^6 H460-luc or A549-luc human lung cancer cells in 15 μ l) was injected into the isolated lung using a 28-gauge insulin syringe with a total of 40 rats (20 with H460-luc and 20 with A549-luc).

Bioluminescence imaging (BLI)

BLI was performed weekly using an IVIS® Lumina or Spectrum Imaging System (Xenogen/Caliper Life Sciences, Alameda, CA). Rats were anesthetized by 2% isoflurane inhalation followed by SC injection of *D*-luciferin (150 mg/kg; Gold Biotechnology, St. Louis, MO) [30]. BLI was acquired 10 min after luciferin injection with typical images shown in Supporting Information Figure S1.

MRI

MRI was performed using a horizontal bore 4.7-T magnet (Agilent Technologies, Santa Clara, CA, USA) with a Doty rat body coil (Doty Scientific, Columbia, SC, USA). Animals were anesthetized with isoflurane (2%) in air (1 L/min) and kept warm with a circulating warm water blanket. Body temperature and respiration were monitored with a small animal physiological monitoring system (Small Animal Instruments Inc., Stony Brook, NY) throughout the experiment. Shimming was performed on the water resonance achieving a

half-maximum linewidth of 150 Hz or narrower on each occasion. T_1 -weighted images were acquired to provide anatomical information using a fast spin-echo (FSE) sequence: TR = 500 ms; effective TE = 10 ms; echo train length = 2; slice thickness = 2 mm; field of view 50×50 or 55×55 mm; and matrix size 128×128 . BOLD MRI (multi-echo gradient echo; TR = 150 ms, ten echo times from 2 to 29 ms, flip angle = 20°) was acquired with the intervention of an oxygen breathing challenge (from air to 100% O_2). Images were acquired in the sagittal plane. BOLD was acquired with ECG and respiratory triggering to reduce motion artifacts (Fig. 1A). Spatial saturation bands were placed on each side of the imaging plane (Fig. 1B) for flow suppression (fs). Five sets of maps interleaved with and without fs were acquired during air breathing and eight sets during oxygen breathing (Fig. 1C).

Histology

Following MRI some animals were sacrificed and tumors resected for histology. Tissues were placed in liquid nitrogen and formalin. Slides for hematoxylin and eosin (H&E) were fixed in 4% PFA, rinsed and then subjected to a progressive staining line containing Leica Selectech reagents (Hematoxylin 560 and Alcoholic Eosin-Y 515) according to well established standard protocols [21, 31]. At the time of imaging, multiple 1600×1200 pixel subfields were acquired to cover the entire tumor and surrounding tissue at 10x objective magnification on a Nikon E600 compound photomicroscope (Melville, NY) equipped with epi-fluorescence and bright-field illumination, an Applied Scientific Instruments x-y-z motorized stage (Eugene, OR) and Nikon DS-Fi2 CCD camera. Stage registry, illumination and camera were controlled by Nikon Imaging Solution Elements v4.20.00 software.

Data Analysis

Data were processed on a voxel by voxel basis using Matlab and statistical analysis performed on both voxel by voxel and regional basis. Regions of interest were identified and outlined using respiratory- and ECG-triggered T_1 -weighted images.

R_2^* was generated by fitting the signal intensity of the multi-echo gradient echo images to TE as a monoexponential function on a voxel-by-voxel basis:

$$SI = S_0 \times e^{-TE \times R_2^*} + k \quad [\text{Eq. 1}],$$

where SI is the signal intensity at each TE, S_0 represents the net magnetization. A constant k was included as a precaution for possible long T_2 component in the voxel, but was ultimately found to be close to zero most of the time or at maximum about 2% of S_0 . Starting values of S_0 and R_2^* for curve fitting were calculated by first solving Eq. 1 based on SI at the first two TE values [20, 32]. $R^2 > 0.8$ was set as a threshold to ensure curve fitting reliability.

The change of R_2^* (R_2^*) due to 100% O_2 challenge was then calculated as:

$$\Delta R_2^* = R_2^*(O_2) - R_2^*(air) \quad [\text{Eq. 2}]$$

Semi-quantitative percentage signal intensity changes ($\Delta SI(\%)$) were calculated using the images acquired at TE = 8 ms. $\Delta SI(\%)$ was calculated as:

$$\Delta SI(\%) = \left(\overline{SI_{O_2}} - \overline{SI_{air}} \right) / \overline{SI_{air}} \times 100\% \quad [\text{Eq. 3}],$$

where $\overline{SI_{air}}$ is the mean signal intensity during air breathing and $\overline{SI_{O_2}}$ is the mean signal intensity of the last five time points during 100% O_2 breathing. The TE = 8 ms was chosen to match the typical R_2^* of about 120 s^{-1} .

Discrepancy of measurements in all four parameters ($R_2^*_{air}$, $R_2^*_{O_2}$, R_2^* and $\Delta SI(\%)$) with fs and non-fs settings was quantified with Bland-Altman analysis to obtain the average and standard deviation of discrepancy. Differences of two sequences were taken as non-fs minus fs. Student's t-tests were used to investigate if there were significant differences in $\Delta SI(\%)$, $R_2^*_{air}$, $R_2^*_{O_2}$ and R_2^* between H460 and A549 tumors. Paired t-tests were conducted to examine if there were significant differences in $R_2^*_{air}$, $R_2^*_{O_2}$, R_2^* and $\Delta SI(\%)$ between fs and non-fs.

Results:

Characteristics of BOLD in the two lung tumor models

Interleaved BOLD sequences with and without fs were successfully acquired in 38 of 40 orthotopic lung tumors. Two A549 tumors were so small as to provide very few voxels in the MRI plane and obvious through plane partial volume effects and these were omitted from analysis. The effectiveness of flow suppression was emphasized by comparison of signal intensity (S_0) maps (Supporting Information Figure S2). Dynamic R_2^* measurements with and without fs responded similarly to the oxygen breathing challenge over time, as shown for size matched H460 and A549 tumors (Fig. 2). A range of responses was observed (Supporting Information Figure S3), however most tumors showed a positive change in the semi-quantitative signal intensity $\Delta SI(\%)$ and negative quantitative R_2^* indicating improved oxygenation in response to oxygen breathing challenge. Comparing the two tumor types, more A549 tumors were responsive (83%) than H460 (55%).

H460 tumors had significantly lower mean R_2^* than A549 while rats breathed air or oxygen, as assessed with or without fs (Table 1). Meanwhile, A549 tumors showed a significant response to oxygen breathing challenge, which was significantly greater than for H460 when fs was applied. Paired t-tests showed that quantitative R_2^* measurements were significantly different with and without fs for A549 tumors, but only for air breathing in H460.

Flow effects on average values of tumor ROIs

Bland-Altman analyses of mean tumor values of H460 (n=20) and A549 (n=18) tumors indicated that the sensitivity of BOLD measurement to flow is tumor type dependent (Fig. 3). Specifically, the average discrepancy (dotted orange line) indicated greater differences between the measurements of fs and non-fs BOLD in A549 than H460. The limits of

agreement (solid blue line; defined as 95% confidence interval) were also greater in A549 than H460, suggesting a higher sensitivity to flow effects in A549. The limits of agreement for the semi quantitative SI(%) were much wider compared with the quantitative measurements ($R_2^*(\text{air})$, $R_2^*(\text{O}_2)$ and R_2^*).

Flow effects on spatial patterns

Distinct intratumoral heterogeneity was observed in all parametric maps in both tumor models (Figs. 4 and 5). Voxel-by-voxel comparison of fs and non-fs BOLD parametric maps appeared similar, but Bland-Altman analysis revealed larger average discrepancy and wider limits of agreement in spatial patterns of A549 compared to H460 tumors (Fig. 6).

Histological differences revealed by H&E

Histology revealed extensive avascular areas in H460 (Fig. 7 A&B), while A549 was well vascularized (Fig. 7 C&D) and each tumor type showed only small areas of necrosis.

Discussion:

Oxygen sensitive MRI was successful in orthotopic lung tumors growing in rats with respect to oxygen breathing challenge. Respiratory- and ECG-triggering facilitated effective acquisition of quantitative R_2^* maps with or without flow suppression. The general trends in responses were similar with or without flow suppression, but Bland Altman analysis revealed significant bias in the measurements for the better vascularized A549 tumor in the absence of flow suppression.

Many studies have considered flow effects on BOLD signal response [33, 34], but most have focused on vasodilation rather than flow itself. The pioneering work of Griffiths *et al.* examined hyperoxic and hypercapnic gases and reported very different behavior in various tumors [35] which could be related to the extent of vascularization [27, 36]. Notably, well perfused G3H tumors showed strong effects, whereas poorly perfused RIF-1 showed much less [35] and these differences were related to radiation response [37]. Historically carbogen (95% O_2 plus 5% CO_2) was a favored intervention in radiation oncology, but increasingly oxygen is being evaluated, as applied here, to avoid the respiratory distress.

In early studies, we applied a pulse burst saturation sequence prior to echo planar imaging (EPI) to detect semi quantitative BOLD responses in Dunning prostate AT1 tumors, which effectively removed flow effects [14, 38]. EPI is particularly effective at revealing changes in susceptibility associated with hemoglobin saturation, but is also subject to image distortions, whereas gradient recalled echo (GRE) sequences provide much better image definition. Saturation bands are widely used to reduce motion, flow and vessel/CSF pulsation artifacts [39, 40]. It is also used routinely in time-of-flight (TOF) MR angiography [41–43]. However, those applications are mostly focused on the flow effect of large blood vessels with rapid blood flow. The vascular network in tumors is often chaotic, tortuous and with irregular diameters and flow rate.

The effect of flow is well-known with respect to BOLD measurements. Howe *et al.* demonstrated the flow effect on traditional BOLD measurements and suggested the term

FLOOD (Flow and Oxygen level Dependent contrast) to emphasize the importance of flow in tumor studies [27, 28]. They suggested additional measurements to account for flow when interpreting BOLD results. The FLOOD studies particularly focused on blood flow modifiers (carbogen or carbogen + nicotinamide). Oxygen breathing is known to affect blood pressure and may cause vascular contraction and therefore a change blood flow, but this is mostly observed in normal vasculature [44]. Due to the lack of systemic regulation and smooth muscle lining in tumor vasculature, oxygen challenges are often assumed to have less effect on the blood flow in tumors, especially in subcutaneous models with associated ectopic vasculature.

In this study, we implemented a simple spatial saturation technique to remove the flow component and compared the measurements with the standard non-fs BOLD measurements. Susceptibility artefacts from tissue/air interfaces are a major challenge for gradient echo imaging of tumors in the lung. Images were inspected to avoid inclusion of areas of severe partial volume at the edge of small tumors and a threshold $R^2 > 0.8$ was applied for acceptable R_2^* curve fitting on each voxel. We quantified the flow effect on four BOLD measurements (SI(%), $R_2^*(\text{air})$, $R_2^*(\text{O}_2)$ and R_2^*) using two different orthotopic lung cancer rat models. We found that flow affected all four BOLD measurements in each tumor type, however, the sensitivity to the flow effect was different depending on the type of measurement and tumor model. Bias in measurements from flow was much smaller in the poorly vascularized H460 tumors, as compared with A549 tumors based on the Bland Altman analysis (Figs. 3–6). Considering either regions of interest defining the tumors (Fig. 3) or assessing individual voxels (Fig. 6), the average discrepancy observed with or without fs was much smaller in H460 than A549 tumors.

Histology revealed that the two tumor types have very different vascular patterns when growing in the orthotopic location in rats. H460 were sparsely vascularized, whereas the A549 had extensive capillaries (Fig. 7). The architecture of A549 tumors was also different in the orthotopic setting compared to the subcutaneous site, where a multinodular structure was observed with very sparse vasculature generally limited to the stromal bed around the nodules [32]. This is also distinct from the vasculature reported for A549 growing subcutaneously in nude mice [45]. Others have reported substantially different patterns of vasculature and hypoxia in lung tumors developing spontaneously in transgenic mice or implanted subcutaneously (SC) or orthotopically [46, 47]. The significantly higher R_2^* in A549 tumors is consistent with extensive vasculature. We previously reported a typical BOLD signal response of 2 to 3 % for subcutaneous A549 compared with the 8 to 10% observed in the orthotopic setting using the same pulse sequence, though with the different TE required to match the observed T_2^* . A more rigorous comparison is R_2^* , which indicated $\sim 120 \text{ s}^{-1}$ for orthotopic A549 tumors here versus 58 s^{-1} for subcutaneous tumors [32]. We note that some values are greater than typically reported for tumors previously, but previous studies of SC tumors reported a mean value of 101.4 s^{-1} was reported for G3H prolactinomas (with a range $75\text{--}140 \text{ s}^{-1}$) [37] and values as high as 110 s^{-1} for DU-145 tumors and 130 s^{-1} for PC3 tumors at 4.7 T [48]. We are unaware of other studies of orthotopic lung tumors using oxygen sensitive MRI. Although, other studies have examined human lung tumor xenografts growing subcutaneously in rats [32] and mice [49].

We note relatively few previous reports of oxygen sensitive MRI in lung tumors. Ohno *et al.* did incidentally report on a lung tumor and stated that there was minimal BOLD response to oxygen breathing challenge compared to the surrounding lung [50]. Small studies of oxygen sensitive MRI have been reported for human lung tumors, which indicated detectable BOLD changes [51] and recently R_1 changes [52] with respect to an oxygen gas breathing challenge. Most studies of human lung have focused on COPD and asthma, thereby examining the lung itself [53–55]. Indeed, oxygen enhanced MRI of the lung is becoming an increasingly popular [56, 57].

Conclusions:

Flow suppression affects BOLD measurements including semi quantitative $SI(\%)$ and quantitative R_2^* . The range of discrepancy was smaller in quantitative measurements than the semi-quantitative SI . High similarity was found in spatial patterns when comparing maps with and without flow-suppression. ROI and spatial pattern analysis showed higher sensitivity to flow in A549 than H460 tumors, which was in agreement with histological results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. BOLD experiment design to assess flow sensitivity.

A. ECG and respiration trigger setting. B. Placement of spatial saturation bands (orange boxes) on both sides of imaging plane (blue box). Green arrow indicates a single nodular tumor in the lung. C. The scan scheme shows the interleaved acquisition of BOLD with and without flow suppression (fs).

H460

A549

Figure 2. R_2^* response to oxygen breathing challenge in size-matched H460 and A549 orthotopic lung tumor xenografts growing in right lung of nude rats.

Left: sagittal T_1 -weighted images showing central tumor cross section. Orange arrows indicate tumors in the lung. Right: dynamic variation in mean tumor R_2^* with respect to oxygen breathing challenge using flow suppressed or non-flow suppressed acquisition.

Figure 3. Higher flow-related discrepancy in mean ROI values was observed in A549 than H460 tumors (H460: n=20; A549: n=18).

In each Bland-Altman plot, y-axis is the difference of measurements with and without fs and x-axis is the average of the two measurements. Solid blue lines show the limits of agreement, defined as 95% confidence interval ($1.96 \times$ standard deviation), and the dotted orange lines represent the average discrepancy. Greater average discrepancy was observed in A549 tumors in all BOLD measurements except $\Delta SI(\%)$. Wider limits of agreement were found in A549 in all four BOLD measurements.

Figure 4. Parametric maps of an H460 lung tumor showed high similarity in spatial patterns. Far left SI(%) map overlaid on sagittal image of orthotopic lung tumor. Color images show expanded parametric maps: from left to right: SI(%), $R_2^*(\text{air})$, $R_2^*(\text{oxygen})$ and R_2^* . From top to bottom: maps with fs, without fs, difference maps (non-fs minus fs), Bland-Altman analysis on voxels from the maps. In Bland-Altman plot, y-axis is the difference of measurements from the two settings and x-axis is the average of the two settings. Solid blue lines show the limits of agreement which is defined at 95% confidence interval and the dotted orange lines represent the average discrepancy.

Figure 5. Parametric maps of an A549 lung tumor showed high similarity in spatial patterns with greater discrepancy compared to H460.

Far left SI(%) map overlaid on sagittal image of orthotopic lung tumor. Color images show expanded parametric maps: from left to right: SI(%), $R_2^*(\text{air})$, $R_2^*(\text{oxygen})$ and R_2^* . From top to bottom: maps from fs setting, non-fs setting, difference maps (non-fs setting – fs setting), Bland-Altman analysis on voxels from the maps above. In Bland-Altman plot, y-axis is difference of measurements from the two settings and x-axis is the average of the two settings. The solid blue lines show the limits of agreement and the dotted orange lines represent the average discrepancy.

Figure 6. Higher flow-related discrepancy in spatial patterns was observed in A549 than H460 tumors (H460: n=20; A549: n=18).

Greater average and standard deviation of discrepancy was found in A549 compared to H460 tumors. Left panel: Average discrepancy of BOLD $\Delta SI(\%)$, $R_2^*(\text{air})$, $R_2^*(\text{oxygen})$ and R_2^* from fs and non-fs settings. Zero discrepancy (*i.e.*, no difference) is marked with dotted lines. Right panel: standard deviation of discrepancy of BOLD $\Delta SI(\%)$, $R_2^*(\text{air})$, $R_2^*(\text{oxygen})$ and R_2^* from fs and non-fs settings. Smaller values indicate better agreement. * indicates $p < 0.05$. The Tukey boxplots indicate median, and inter quartile range and whiskers extend 1.5x further providing about 99% of distribution. Outliers are indicated in red.

Figure 7. Histology of H460 and A549 tumors.

H&E staining of whole mount slices and expanded regions. (A) The H460 tumor appeared to be rather avascular with extensive desmoplastic reaction and some necrotic regions. (B) No vessels can be identified in the expanded region. Arrows indicate fibroblasts. (C) The A549 tumor appeared to be very well vascularized in most parts of the tumor with some necrotic areas in the center. (D) Expanded region. Arrows indicate blood vessels.

Table 1.
Summary of fs and non-fs BOLD measurements in H460 and A549 tumors.

From A to D: SI(%), $R_2^*(\text{air})$, $R_2^*(\text{O}_2)$ and R_2^* . Values in the cells are mean $\pm$ std (range: minimum to maximum). p-values were calculated from unpaired two-sample Student's t-test for H460 vs. A549 and paired t-test for fs vs. non-fs.

A. SI(%)			
	f_s	non- f_s	p-value (f_s vs non- f_s)
H460	2.1 $\pm$ 9.4 (–27.1 to 16.9)	3.4 $\pm$ 8.9 (–20.0 to 19.5)	0.048 *
A549	8.7 $\pm$ 12.9 (–9.5 to 43.1)	10.0 $\pm$ 10.9 (–9.1 to 32.2)	0.45
p-value (H460 vs A549)	0.076	0.046 *	

B. R_2^* air(s ^{–1})			
	fs	non-fs	p-value (fs vs non-fs)
H460	86.2 $\pm$ 19.8 (55.9 to 128.5)	87.3 $\pm$ 20.0 (56.4 to 129.3)	0.0009 *
A549	116.4 $\pm$ 21.9 (85.0 to 171.6)	119.4 $\pm$ 22.2 (83.8 to 166.7)	0.017 *
p-value (H460 vs A549)	8 $\times$ 10 ^{–5} *	4 $\times$ 10 ^{–5} *	

C. R_2^* O ₂ (s ^{–1})			
	fs	non-fs	p-value (fs vs non-fs)
H460	86.3 $\pm$ 21.7 (54.5 to 133.1)	86.7 $\pm$ 21.8 (51.2 to 133.2)	0.4
A549	109.4 $\pm$ 20.1 (79.1 to 146.3)	115.1 $\pm$ 22.5 (80.7 to 145.9)	1 $\times$ 10 ^{–4} *
p-value (H460 vs A549)	0.002 *	0.0003 *	

D. R_2^* (s ^{–1})			
	f_s	non- f_s	p-value (f_s vs non- f_s)
H460	0.1 $\pm$ 5.5 (–10.1 to 11.9)	–0.7 $\pm$ 5.7 (–9.8 to 12.4)	0.12
A549	–7.1 $\pm$ 8.1 (–25.3 to 4.3)	–4.3 $\pm$ 7.9 (–21.2 to 9.2)	0.007 *
p-value (H460 vs A549)	0.03 *	0.11	

* indicates p<0.05.