CHARACTERIZATION AND MODELING OF FLUCTUATING HYPOXIA IN BREAST CANCER

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biomedical Engineering in the Graduate School of Duke University

2008
ABSTRACT

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Tumor hypoxia is an enduring problem for traditional cancer therapies such as radiation and chemotherapy. This obstacle has traditionally been attributed to the widespread presence of chronic, diffusion-limited hypoxia in solid tumors; recent data suggests that tumor hypoxia may also be spatially and temporally variable. In this work we characterize the presence of spatial and temporal fluctuations in hypoxia, as well as use mathematical modeling to predict the impact of fluctuations on the hypoxic cytotoxin, tirapazamine, and examine potential mechanisms of fluctuations in tumor oxygenation. Using phosphorescence lifetime imaging on preclinical tumors, we show that instabilities in tumor oxygenation are a prevalent characteristic of three tumor lines and that previous characterization of tumor hypoxia as being primarily diffusion-limited does not accurately portray the tumor microenvironment. Then, using a one-dimensional theoretical model, we examine the effects of fluctuating hypoxia on metabolized tirapazamine concentration; we find that fluctuating O₂ reduces the concentration of metabolized tirapazamine at distances farther from the source, thereby limiting its ability to reach and kill the most hypoxic cells. Finally, we use a three-dimensional Green’s function oxygen transport model to explore the effects of temporal fluctuations in hemoglobin saturation, blood flow, and overall perfusion on tumor tissue oxygenation. Results from the model predict that hemoglobin saturation has a
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1. Introduction

Tumor hypoxia is classically depicted as developing as a result of two independent phenomena: chronic hypoxia caused by limitations of oxygen diffusion, and fluctuating hypoxia caused by unsteady red cell flux and/or vascular stasis in tumor vasculature. Studies looking at tumor oxygenation have traditionally focused on methods to decrease tumor hypoxia through a paradigm focused on decreasing diffusion-limited hypoxia; my work will focus on the implications of fluctuating hypoxia on treatment.

Previous studies measuring oxygen fluctuations have only been able to examine either the temporal characteristics of fluctuating hypoxia at a single/few discrete location(s) or the spatial characteristics of fluctuating hypoxia at a single time point within a tumor (Baudelet and Gallez, 2003; Bennewith et al., 2002; Braun et al., 1999; Brown, 1979; Brurberg et al., 2004; Brurberg et al., 2003; Brurberg et al., 2005; Cardenas-Navia et al., 2003; Chaplin and Hill, 1995; Chaplin et al., 1987; Dewhirst et al., 1998; Durand and LePard, 1995; Ljungkvist et al., 2000; Minchinton et al., 1990; Minchinton and Fryer, 1996; Trotter et al., 1989). Thus, the extent of fluctuating hypoxia in tumors remains unresolved. This gap in knowledge provides a strong rationale for studying fluctuating hypoxia as instability in tumor oxygenation has implications for radiotherapy fractionation (Kirkpatrick et al., 2004), oxygen-sensitive chemotherapeutics...
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1.1 Background

1.1.1 Clinical Implications of Tumor Hypoxia

Tumor hypoxia has become one of the most studied physiological phenomena in cancer research due to its complexity and pervasiveness in solid tumors. Tumor hypoxia has been shown to be prognostically significant in many clinical studies, independent of treatment type (Hockel et al., 1996; Hockel et al., 1998; Rofstad et al., 2000; Sundfor et al., 1997; Vaupel et al., 1991; Walenta et al., 2000). Patients with hypoxic tumors have lower overall survival, decreased response rates, and higher rates of tumor reoccurrence and metastases (Figure 1) (Brizel et al., 1996; Hockel et al., 1996; Hockel et al., 1998; Rofstad et al., 2000; Sundfor et al., 1997; Vaupel et al., 1991; Walenta et al., 2000).

The causes for poorer prognosis are multi-fold. Initially, hypoxia-related resistance in tumors was believed to be primarily a problem for radiotherapy, because hypoxic cells have increased radio-resistance compared with normoxic cells (Hall and Giaccia, 2005). At O2 concentrations below 10 mm Hg, radiosensitivity of cells decreases.
Figure 1: Disease-free survival for patients with well oxygenated tumors vs. poorly oxygenated tumors. Tumors with a median pO2 > 10 mm Hg have a significantly better prognosis (Brizel et al., 1997).

Under anoxic conditions, a 3-fold higher dose of radiation is necessary to kill the same fraction of cells compared with irradiating them under normoxic conditions (Hall and Giaccia, 2005). In the clinical setting, fewer tumor cells in hypoxic regions are killed than in normoxic regions when given the same dose. The increased sensitivity of cells to radiation in the presence of oxygen occurs because molecular oxygen reacts with radiation induced damage in DNA, rendering changes in DNA base structure that is difficult for cells to repair (Hall and Giaccia, 2005).

Hypoxic tumor cells are also chemoresistant. Several factors contribute. First, the decreased rate of proliferation of hypoxic cells causes resistance to drugs that are cell cycle specific (Durand, 1994; Teicher et al., 1990; Teicher et al., 1981). Second, the same deficiencies in perfusion that lead to hypoxia contribute to inefficient drug transport to hypoxic cells (Durand, 1994). Third, hypoxic cells often have set up defenses to protect
them from their environment, such as elevations in glutathione, that lead to multidrug resistance (Durand, 1994).

In addition to treatment resistance, it has been shown that hypoxic cells also demonstrate other adaptations. Hypoxia leads to increased tumor cell invasiveness and increased angiogenesis. Both of these adaptations contribute to increased propensity for metastasis. Additionally, tumor cells are also known to develop a glycolytic phenotype, often altering their metabolism to utilize glucose when low oxygen levels are limiting aerobic respiration. While not contributing directly to treatment resistance, these alterations in metabolism favor tumor cell survival under noxious environmental conditions.

In summary, the plethora of effects caused by tumor hypoxia provides a strong rationale for trying to understand and potentially exploit this feature of tumors.

1.1.2 Definition of Hypoxia

Before going into detail on the causes and consequences of tumor hypoxia it is necessary to define what is meant by this term. For the purposes of this text, we will define this as a threshold of 10 mmHg. We chose this value because it is the threshold below which radioresistance increases (Hall and Giaccia, 2005). Second, below this value, changes in tumor cellular function tend to occur (Jiang et al., 1996), leading to the phenotypic changes described above. Additionally, below this value there are changes in
red cell fluidity that increase blood viscosity, leading to alterations in tumor perfusion (Kavanagh et al., 1993).

1.1.3 Determinants of Tumor Hypoxia

O₂ concentration in tumor tissue, as in all tissues, is the result of a balance between O₂ delivery and consumption. In normal tissues, this balance is tightly regulated to prevent hypoxia, even at times of peak O₂ metabolism. This balance is largely controlled by evenly distributed arteriolar-capillary networks. In the event that hypoxia does occur in normal tissues, balanced signaling cascades lead to vascular remodeling, or angioadaptation, until the tissue pO₂ is back within its normal range (Pries et al., 2001; Pries et al., 1998). In normal tissues, the supply of oxygen is sufficient to meet the demands of the tissue.

Tumors are unable to regulate their O₂ levels because they are unable to strictly control O₂ delivery or consumption. This results in regions of hypoxia within the tumor which are spatially and temporally variable. No standard treatment has been developed which successfully and significantly decreases tumor hypoxia, although many have been proposed. To understand the difficulty behind alleviating tumor hypoxia, a clear portrayal of the physiologic and metabolic characteristics of tumors is necessary.

There are two types of oxygen gradients in tumors: (1) radial gradients, or decline in oxygen concentration when moving radially away from a microvessel and (2) longitudinal gradients, or decline in vascular oxygen concentration when moving
afferently along the vasculature. These two features are not independent. The lower the vascular oxygen concentration, the shorter the radial oxygen diffusion distance is. These same types of gradients can be found in normal tissues, but in the case of normal tissues, one rarely observes hypoxia. Thus, the gradients are much more subtle in magnitude.

O\textsubscript{2} availability in tumors is limited due to physiological constraints, particularly irregularities in tumor vasculature. The irregularities are caused by imbalances in angiogenic cytokines that regulate angiogenesis and vascular maturation. Four unique traits of tumor blood vessels have been described: abnormal branching structures and uneven distribution of microvessels; steep longitudinal oxygen gradients along afferent vasculature; decreased quantity of arterioles; and unsteady red cell distribution at bifurcations, leading to unstable red cell flux (number of cells passing through a microvessel per unit time). Abnormalities in the shape and distribution of microvessels clearly result in regions with an overabundance of microvessels, which can be connected by short shunts, and regions with a scarcity of microvessels. In the regions in which microvessels are scarce, large inter-microvessel distances result in regions of tumor tissue which are beyond the radial diffusion distance of O\textsubscript{2}, and are chronically hypoxic (remain below 10 mm Hg for long periods of time).

Steep longitudinal oxygen gradients can also result in large areas of chronically hypoxic tumor tissue, although this is not due to the limited radial diffusion of O\textsubscript{2}. Rather, axial O\textsubscript{2} gradients are seen in afferent flow. This phenomenon is further
perpetuated by the reduced number of arterioles in tumors, as compared with comparable normal tissue. Lack of sufficient arteriolar supply and steep longitudinal gradients can result in tumor vessels which are themselves hypoxic, even when they are perfused. Consequently regions of tumor tissue adjacent to blood vessels can also be hypoxic.

Oxygen consumption rates of tumors are not exceedingly high compared with most normal tissues (Vaupel et al., 1987). This leads one to the conclusion that it is the deficiencies in oxygen delivery that are most responsible for hypoxia, as opposed to oxygen consumption rates. Nevertheless, at the microregional level, variations in oxygen consumption rate could contribute to hypoxia. For example, the oxygen consumption rate of proliferating cells averages 3-5 times that of Go cells (Freyer, 1994). Additionally, activated macrophages, which can be found in tumors, have very high oxygen consumption rates during periods of production of reactive oxygen species.

1.1.4 Evidence for fluctuating Hypoxia

In addition to the deficiencies in oxygenation caused by vascular architecture, it is now well established that the oxygenation state of tumors is not stable (Figure 2). This concept was introduced in the late 1970s by two different groups. Yamaura and Matsuzawa irradiated hepatomas grown in window chambers, and determined that tumor re-growth/radioresistance was the greatest at the outermost regions of the tumors (Yamaura and Matsuzawa, 1979). They suggest that this phenomenon was caused by
cell cycle effects due to the higher pO$_2$ levels likely to be on the periphery of the tumor; however, they also suggest that transient hypoxia might cause this radioresistance, as they had observed transient vascular stasis in the tumor periphery. Brown first

![Diagram](image)

**Figure 2:** Diagrammatic illustration of fluctuations in tumor hypoxia. Each image represents a different time point. At location A, the tumor tissue is undergoing diffusion limited hypoxia at all time points, the red cell flux in nearby vessels has a negligible effect at this location. At location B, the tumor tissue is well-oxygenated at all time points, although increased red cell flux at the first and last time points do have a minor effect on the oxygen concentration. At location C, the tumor tissue is undergoing fluctuations in tumor pO$_2$. As the red cell flux decreases in nearby vessels at different time points, less O$_2$ is available to diffuse out to this location and it becomes hypoxic, even though the tissue immediately near this same vessel stays normoxic. At location D, the O$_2$ concentration fluctuates with time, but ranges from normoxic to intermediately hypoxic as the red cell flux in several nearby vessels changes (Cardenas-Navia et al., 2007a).

demonstrated the existence of a population of tumor cells which were transiently hypoxic; 24 hrs after EMT6 tumors were treated with misonidazole, a hypoxic cytotoxin, a population of cells was found to be radioresistant (Brown, 1979).
The next series of studies examining fluctuating hypoxia were formulated under the assumption that cessation in blood flow or vascular stasis was responsible for fluctuating oxygenation in tumors. These studies used perfusion markers intravenously injected, either simultaneously or a few minutes apart, and mismatch in perfusion was attributed to vascular shut-down. Initial studies were done with a time difference of 20 min between administration of perfusion markers (Minchinton et al., 1990; Trotter et al., 1989; Trotter et al., 1991; Trotter et al., 1990; Tufto and Rofstad, 1995); Durand and LePard later determined that perfusion mismatch was significant for time differences of perfusion marker administration greater than 15 min, and suggested that the periodicity of transient flow changes was between 15-25 min (Durand and LePard, 1995). This

![Figure 3: Example of six traces of blood flow from human patient number 4 (Pigott et al., 1996).](image-url)
study also suggests, in contrast to previous studies which found the vessel mismatch to occur in fewer than 10% of all vessels, that up to half of all tumor vessels were subject to changes in perfusion in the SCCVII tumors studied.

In the mid-1990s a paper showing fluctuations in red cell flux in experimental tumors was published (Chaplin and Hill, 1995). Over a 1-hour period, laser Doppler flowmetry was used to measure changes in blood flow in up to four sites per tumor in murine adenocarcinomas and sarcomas. The results from these studies showed large changes in flow, with the durations of change ranging from 6 to 45 min. The four probes placed into different locations of the same tumor also showed distinctly different patterns of change in blood flow, suggesting spatial heterogeneity for flow fluctuations. The next year that same group used laser Doppler flowmetry probes with 300μm diameters in seven human patients (Pigott et al., 1996). Six probes were injected in each tumor. In 54% of all traces, blood flow was seen to change by at least a factor of 1.5. The median periodicity for the traces (19%) which were seen to have cyclic fluctuations was 13 min (range 4-44 min). The example patient data shown in this paper showed distinctly heterogeneous patterns of blood flow fluctuations in 4 of the 6 probes in the tumor (Figure 3).

During this time our group published the first direct measurements of fluctuations in tumor pO2 using tumor microelectrodes (tip diameter ~10μm) (Kimura et al., 1996). Kimura et al showed that fluctuations in microvessel red cell flux and
perivascular pO$_2$ were temporally coordinated (Figure 4), suggesting instabilities in red cell flux were directly responsible for instabilities in tumor pO$_2$ (Kimura et al., 1996). A second study examining fluctuations in red cell flux and pericellular pO$_2$ confirmed the results of the previous one; this study also examined vascular stasis in the microvasculature and found that it occurred in less than 4% of vessels, strongly

Figure 4: Composite graph showing temporal relationship between RCF and perivascular pO$_2$ for all 11 experiments (Kimura et al., 1996).
suggesting that cessation in blood flow was not the dominant mechanism for fluctuating hypoxia (Dewhirst et al., 1996a). Instead the authors propose that arteriolar vasomotion, which was measured to occur at the same slow frequency as the red cell flux fluctuations, was responsible for fluctuating pO\textsubscript{2}. Three further studies from our group showed that the period of fluctuations corresponding to the dominant magnitude of pO\textsubscript{2} was less much less than 1 cycle/min (period of 10s of minutes), and that the magnitude of the fluctuations was tumor type dependent (Braun et al., 1999; Cardenas-Navia et al., 2004; Dewhirst et al., 1998).

More recently, Røfstad’s group has used OxyLite pO\textsubscript{2} probes to examine pO\textsubscript{2} fluctuations in human xenografts (Brurberg et al., 2004; Brurberg et al., 2003; Brurberg et al., 2006). These studies showed the same slow frequency of fluctuations in the two melanoma lines examined (less than 0.1 cycles/min); however, the magnitudes of the fluctuations of the two tumor types were found to be significantly different. Additionally, the two pO\textsubscript{2} probes inserted into different locations within the same tumor were generally not temporally coordinated. In 2006 Røfstad’s group published a paper showing the first clinical evidence of fluctuating hypoxia (Brurberg et al., 2005). They measured pO\textsubscript{2} fluctuations in six spontaneous canine tumors and found that for at least one OxyLite probe in each tumor the pO\textsubscript{2} fluctuated across the radiobiologically relevant threshold of either 3 or 10 mm Hg (Figure 5). They also showed that the period corresponding to the dominant magnitude of the fluctuations in these spontaneous
Figure 5: Eppendorf pO2 histograms (left) and OxyLite pO2 traces (right) for canine patients 5–6 recorded during fractionated radiation therapy. Tissue pO2 measurements were performed immediately prior to radiation fraction 4, 7, and 10 (Brurberg et al., 2005).

tumors was on the order of 10s of minutes, and that the fluctuations were unchanged after radiation therapy.

Although the studies directly measuring pO2 have resulted in a wealth of knowledge regarding the temporal characteristics, their insight into the spatial prevalence and heterogeneity of fluctuating hypoxia is limited. Baudelet et al. partially addressed this topic using an oxygen-sensitive functional MRI technique called BOLD (blood oxygen level-dependent). They found that T2* weighted gradient-recalled echo images are able to monitor change in tumor oxygenation and/or perfusion (Baudelet et al., 2004; Baudelet and Gallez, 2003). Over a 30 min period, Baudelet et al. found that an
area of up to 60% of murine fibrosarcomas showed spontaneous fluctuations in oxygenation and/or perfusion which were greater than fluctuations due to the natural noise in the imaging system. In areas which showed fluctuations in oxygenation and/or perfusion, the fluctuations occurred with frequencies less than 0.33 cycles/min. Sorg et al. examined fluctuations in hemoglobin saturation along a network of blood vessels (Sorg et al., 2008). Temporal fluctuations in hemoglobin saturation tended to be perpetuated along a vessel and its downstream branches, although regions which were spatially closer generally showed more temporal coordination.

![Graph](image)

**Figure 6:** Fraction of SiHa tumor cells labeled only with pimonidazole (pimo) after tumor-bearing mice consumed pimo-containing water *ad libitum* before tumor excision. Note the labeling pattern (*dashed line versus solid line*) (Bennewith et al., 2002).

Using a hypoxia marker-mismatch technique (Ljungkvist et al., 2000) directly analogous to the dye-mismatch technique, Bennewith et al. have suggested that there is a longer time-scale over which tumors are undergoing fluctuating hypoxia (Bennewith et al., 2002). This technique uses mismatch in pimonidazole, a hypoxia marker, and CCI-103F, a pimonidazole analogue, administered at time points greater than 3 hours apart
(Ljungkvist et al., 2000). A perfusion marker is injected immediately before the tumor is excised and cells are sorted according to intensity of perfusion marker staining, with the assumption that cells closest to a vessel will have the highest intensity of staining (Ljungkvist et al., 2000). Using pimonidazole put in the drinking water for continuous administration and an intraperitoneal injection of CCI-103F 3 hours before excising the tumor, Bennewith et al. showed a consistent increase in the mismatch of these two hypoxia markers, up to 96 hrs (Bennewith et al., 2002). The largest mismatch occurred for cells which were stained most dimly with perfusion marker, suggesting cells farthest from the vasculature are more likely to be impacted by fluctuating hypoxia. By 96 hrs up to 30% of all cells were shown to have hypoxia marker mismatch, although most of this mismatch was found to occur within the first 6-12 hours (Figure 6). This two-phase mismatch curve suggests that fluctuating hypoxia is occurring with two different time scales, perhaps through two different mechanisms.

This slower timescale was recently explored in head and neck cancer patients who received fluorine-18 labeled misonidazole, a hypoxia marker, and whose tumors were then imaged twice with PET-CT scans separated by three days (Lin et al., 2008). The fractional hypoxic volume was found to correlate strongly only in 6 out of 13 tumors; the other 7 patients showed clear changes in their spatial distribution of hypoxia. This study presents the first evidence that spatio-temporal fluctuations in hypoxia are occurring in human tumors (Figure 7).
Figure 7: Transaxial, coronal, and sagittal views of fluorodeoxyglucose target volume, and hypoxic volumes in $^{18}$F-fluoromisonidazole (FMISO)1 and FMISO2 as defined by tumor/blood (TB) ratio of ≥1.2. Large variability between patients’ repeat FMISO scans illustrated for patient with poorest (Patient 6, Top) and best (Patient 5, Bottom) correlation between FMISO1 and FMISO2. Images show transaxial, coronal, and sagittal views (Lin et al., 2008).

Many excellent investigators have worked diligently to characterize and understand fluctuating hypoxia. Nevertheless, many significant questions about fluctuating tumor pO$_2$ remain unanswered: (1) what is the spatial prevalence of oxygen fluctuations in tumors? (2) what are the dominant mechanisms causing oxygen fluctuations in tumors? (3) what impact does fluctuating hypoxia have on traditional anti-tumor therapies? This body work attempts to offer insight into the last three questions through a combination of experimental work and mathematical modeling.
2. The Pervasive Presence of Fluctuating Oxygenation in Tumors

2.1 Introduction

Tumor hypoxia is one of the most studied physiological phenomena in cancer research and has been shown to be prognostically significant in many clinical studies, independent of treatment type (Hockel et al., 1998; Rofstad et al., 2000; Vaupel et al., 1991). Since the discovery of diffusion limited tumor hypoxia in the 1960s, most strategies for overcoming or targeting hypoxic areas in tumors have been developed using a paradigm of chronically hypoxic areas in tumors, or areas of hypoxia that are oxygen-deficient because they are beyond the typical diffusion limit of O2. Although many investigators have measured the presence of so-called “intermittent” or “acute” hypoxia in preclinical (Baudelet et al., 2004; Bennewith et al., 2002; Braun et al., 1999; Brurberg et al., 2003; Cardenas-Navia et al., 2004; Chaplin et al., 1987; Durand and LePard, 1995; Trotter et al., 1989) and clinical tumors (Brurberg et al., 2005), instabilities in tumor oxygenation were presumed to be temporally rare, spatially isolated events attributed to transient episodes of vascular stasis (Durand and LePard, 1995; Minchinton et al., 1990; Trotter et al., 1989; Trotter et al., 1991; Trotter et al., 1990; Tufto and Rofstad, 1995).
Our group previously demonstrated that vascular stasis was not necessary for tissue to experience transient hypoxia – alternatively, fluctuations in red cell flux could easily cause this effect (Dewhirst et al., 1996a). Based on a series of studies we have advocated the concept that instabilities in red cell flux are the norm within tumors and that this condition could lead to widespread instability in oxygenation throughout a tumor. Since oxidative stress has been shown to cause a differential cellular response for intermittent vs. chronic hypoxia in tumor and endothelial cells (Cairns and Hill, 2004; Cairns et al., 2001; Graeber et al., 1996; Martinive et al., 2006; Prabhakar, 2001; Reynolds et al., 1996; Yuan et al., 2004; Yuan et al., 2005), the pervasive presence of fluctuating oxygenation in tumors has consequential implications for our understanding of tumor progression, stress response, and signal transduction; in all studies intermittent hypoxia has been shown to increase molecular or physiological responses in a manner consistent with more malignant tumor phenotypes (Cairns and Hill, 2004; Cairns et al., 2001; Graeber et al., 1996; Martinive et al., 2006; Prabhakar, 2001; Reynolds et al., 1996; Yuan et al., 2004; Yuan et al., 2005).

In this study we show data directly measuring temporal fluctuations in vascular pO$_2$ in three rat tumors, characterizing the spatial as well as temporal differences. We show that O$_2$ delivery to tumors is constantly fluctuating, resulting in reoxygenation events throughout the tumor.
2.2 Materials and Methods

2.2.1 Dorsal Skin Flap Window Chamber

Tumors were grown in the dorsal skin flap window chambers in female Fischer 344 rats (150-175g). The rats were surgically implanted with titanium window chambers as previously described (Papenfuss et al., 1979). A small piece of tumor ~1 mm$^3$ from a donor animal was transplanted onto the fascia at the time of surgery, and glass windows were placed on both sides of the chamber. Three rat tumor lines were studied: R3230Ac mammary adenocarcinoma (n=6), FSA fibrosarcoma (Grant and Wells, 1974) (n=6), and 9L glioma (Wheeler et al., 1975) (n=6).

At 2-4 days after surgery, animals were shipped from Duke University Medical Center to the University of Pennsylvania, and allowed to acclimate for 4-7 days before any measurements were taken. All experimental protocols were approved by Duke University Medical Center and University of Pennsylvania Institutional Animal Care and Use Committees.

2.2.2 Phosphoresence Lifetime Imaging (PLI)

Phosphorescence lifetime imaging was performed on the tumors in the dorsal window chamber using a previously described imaging set-up (Erickson et al., 2003).

PLI utilizes phosphorescence quenching to measure pO$_2$ within biological systems. Since Wilson’s group developed the technique in the 1980s(Rumsey et al.,
1988), it has been used to measure O₂ levels both in vitro (Robiolio et al., 1989; Rumsey et al., 1990; Wilson et al., 1988) and in vivo (Rumsey et al., 1994; Rumsey et al., 1988; Wilson and Cerniglia, 1992; Wilson et al., 1998; Wilson et al., 2005). It is particularly well-suited to measure temporal changes pO₂ levels within tumor vasculature or tissue due to 1) superb temporal resolution (milliseconds) allowing for real-time measurements of pO₂; 2) high precision of measurement over a large pO₂ range (0-155 mm Hg); 3) lack of tissue damage allowing for repeated measurements of a single region; 4) pO₂ measurement independence from phosphor concentration allowing for measurements over an extended time period; 5) pO₂ measurement independence from pH for the pH range of tumor tissues; and 6) ability to measure pO₂ over a large region (several mm in diameter).

Phosphorescence quenching requires the presence of an oxygen-sensing probe. The O₂ dependence of the phosphorescence of the probe can be described by the Stern-Volmer relationship:

\[
\frac{\tau^0}{\tau} = 1 + k_\text{Q} \tau^0 pO_2
\]

or

\[
pO_2 = \frac{\tau^0/\tau - 1}{k_\text{Q} \tau^0}
\]
where $k_Q$ is the quenching constant, $\tau^0$ is the phosphorescence lifetime in a anoxic environment, and $\tau$ is the phosphorescence lifetime at the measured environment. The values for $k_Q$ and $\tau^0$ are determined experimentally in plasma, and are found to be stable over a large pH range (6.4 to 7.8) (Dunphy et al., 2002). Once a phosphor is characterized, it does not need to be recalibrated for each experiment, and pO$_2$ measurements have been found to be highly reproducible (within 1 mm Hg) (Dunphy et al., 2002; Ziemer et al., 2005). Once the probe is present, the specimen is exposed in an excitation light specific to the probe, and the emission light is collected by a camera. The lifetime distributions are used to calculate a pO$_2$ map for the sample, according to the equations above.

2.2.3 Experimental Protocol

Tumors were selected for imaging as they reached a diameter ~3 mm. Rats were anesthetized with 50 mg/kg pentobarbital i.p. for imaging; this dose is known to maintain stable heart rate and blood pressure within a range reported for unanesthetized rats (Smith et al., 1985) over a period of several hours (Cardenas-Navia et al., 2004) and we have previously shown that blood oxygen content is equivalent to unanesthetized rats using this method (Dewhirst et al., 1996b). Before taking any measurements, the femoral vein was cannulated and the rat was placed in lateral recumbancy on a temperature controlled heating pad onto the stage with the tumor side of the window chamber facing the camera. The window chamber was secured using a
custom-made holder attached directly to the stage so that the glass window laid flat. Once secured and immobile, neither the rat nor the microscope stage were touched in order to maintain the same location for sequential images on the camera.

0.3 mL of 8 mg/kg Phosphor G2 (Dunphy et al., 2002) was then injected i.v. into the animal and imaging began. The field of view for the image was chosen randomly within the tumor, although care was taken to ensure that the entire field of view was tumor tissue and not normal tissue. Images were taken every 2.5 min for 60-90 min before the animal was sacrificed with an overdose of pentobarbital i.v. and a final image was taken. Experiments in which the pO$_2$ did not drop to near zero after the animal was sacrificed were discarded (n=2, these experiments are not included in the n=6 for each tumor type).

Phosphor G2 was excited using light at 450 nm, which penetrates ~50 μm into the tumor tissue (Dunphy et al., 2002); therefore PLI images reflect the oxygenation status of vessels near the surface of the tumor. Additionally, phosphor G2 is large (2400 Da) and has a high negative charge (-16), largely restricting it to the vascular space.

### 2.2.4 Image Analysis

Each PLI image yielded a 2.85 mm x 2.2 mm (480 x 752 pixels) map of vascular pO$_2$ at each time point. The maps of vascular pO$_2$ were imported into MATLAB, and all further analyses were done in MATLAB. Pixels from the original maps were averaged over every 4 x 4 pixels and a new, averaged map of vascular pO$_2$ was created (120 x 188
pixels). This averaged map of vascular pO$_2$ was used for all subsequent analyses. Analyses are presented in terms of pixel sizes; the light signal from the tissue scatters laterally before it is captured by the PLI camera, which is several centimeters away from the surface of the window. Therefore the signal from any given pixel contains a contribution from the surrounding pixels.

In an initial exploratory analysis, sequential time images were subtracted, i.e., time point 1 subtracted from time point 2, for all images. The results for each pixel were then plotted as either a positive change greater than 1 mm Hg, a negative change greater than 1 mm Hg, or no change. This analysis was also conducted on the difference images using a threshold of change of 5 mm Hg.

### 2.2.5 Spatial Statistics

The central consideration of spatial statistics is that data that are close together in space are more often alike than those that are not; the field often examines data to determine if the locations of the points or areas to which data refer may provide some information about the spatial pattern of variation in these data.

The scientific approach attempts to minimize or control variability through experimental design; nonetheless, nearly every data set contains some variability. This is highly descriptive of the oxygenation state of tumors, which is impacted locally and globally by many factors, as detailed earlier. Applying spatial statistics to two- or three-dimensional measurements of pO$_2$ allows for the quantification of variability in this
measurement for which control is not possible. Analysis of spatially coordinated data provides advanced methods for comparing pO₂ distributions, relationships, and exploring intra- and intertumoral differences.

Although spatially located data is critical to the underlying physiology of tumors (e.g., immunohistochemical analysis on biopsies), there are few of these type of analyses applied in this discipline. In this study two widely-used spatial analyses from geology and ecology are used to further understand fluctuations in tumor pO₂: watershed segmentation and Moran’s I autocorrelation.

To more robustly examine spatial patterns of vascular pO₂, watershed segmentation analysis for spatial change detection was conducted for each experiment (see Figure 8). First, the absolute difference between the time-averaged pO₂ for the entire experiment and each individual time point was calculated. The median value of each individual absolute difference image was then subtracted from the image, and watershed segmentation was performed on a pixel by pixel basis using an 8-connected neighborhood. Watershed analysis detects gradients in pO₂ values and segments an image along those gradients; segmented regions can be thought of as pO₂ isobars.

Global Moran’s I analysis (Cressie, 1991; Moran, 1948) was performed on all time points for all images to examine if spatial autocorrelations were consistently occurring within an image for disk-shaped spatial ranges with radii of 1, 4, 8, 12, 20, 40, and 100 pixels using a binary weighing function (see Figure 9). Moran’s I is a spatial
Figure 8: Spatial change analysis and watershed segmentation. The time-averaged pO₂ value for an entire data set is subtracted from PLI pO₂ data at each time point. Then the median of the absolute value of this difference is subtracted from the absolute difference image; this step is required for watershed segmentation, which begins defining regions at local minimums and moves outward from them, defining segment boundaries when a change in the pO₂ gradient is detected. Thus segmentation can be thought of as defining pO₂ isobars.

autocorrelation measurement which detects departures from spatial randomness by examining the variability within a defined region surrounding a pixel (the size of the region is defined by a weighing function) and comparing it to variability within the entire image. Calculation of Moran’s I is done for each individual pixel (local Moran’s I) and can then be normalized and average over all the local Moran’s I values to compute a single value for an entire image (global Moran’s I).

Local Moran’s I is calculated using
\[
I_i = \frac{N(x_i - \bar{x}) \sum_{j=1, j \neq i}^{N} w(i, j)(x_j - \bar{x})}{\sum_{j=1}^{N} (x_j - \bar{x})^2}
\]

and

\[
\bar{x} = \frac{\sum_{i=1}^{N} x_i}{N}
\]

where \( I_i \) is the local Moran’s I, \( N \) is the total number of pixels, \( w(i, j) \) is the weighing function for the pixel located at coordinates \((i,j)\), and \( x_i \) is the \( i^{th} \) pixel with pO2 value \( x \).

High local Moran’s I values indicate positive spatial autocorrelation (less variability within the region surrounding the pixel than the variability of the entire image), and low values of local Moran’s I indicate a lack of spatial autocorrelation (no difference in variability within the region surrounding the pixel compared to the variability of the entire image). Finding regions within an image with high local Moran’s I values indicate “hotspots” or regions of spatial correlation.

Global Moran’s I is then defined as the normalized average of all the local Moran’s I values:

\[
I = \frac{N \sum_{i=1}^{N} \left( \sum_{j=1, j \neq i}^{N} w(i, j)(x_i - \bar{x})(x_j - \bar{x}) \right)}{\sum_{i=1}^{N} \left( \sum_{j=1}^{N} w(i, j)(x_i - \bar{x})^2 \right)}
\]
The resulting I values from global Moran’s I are between -1 and 1. A value of zero for I indicates spatial randomness. As the value approaches 1, it suggests that for the image that there is more similarity due to location than can be accounted for by randomness, i.e., there is some underlying pattern/order; as the value approaches -1, it suggests that for the image that there is more variability due to location than can be accounted for by randomness.

Global Moran’s I analysis was also performed on differences in sequential time images using the same spatial ranges and weighing function as described above.

A two-way ANOVA test was conducted on the resulting Moran’s I values to determine if different tumor types had different patterns of spatial autocorrelations. Multiple comparison of the means was done to determine which groups were significantly different using the Bonferroni critical value of p<0.05.

Spatial autocorrelation within a single image was examined using local Moran’s I (Anselin, 1995). Each time point was checked for local spatial autocorrelations within disk-shaped regions with radii of 1, 4, 8, 12, 20, and 40 pixels using a binary weighing function. Additionally, subsequent time points were subtracted and changes in pO₂ between two time points were examined for local spatial autocorrelations using local Moran’s I.

Finally, the averaged map of vascular pO₂ was subjected to discrete Fourier transform to determine the dominant period of fluctuations for each pixel over time.
Figure 9: Moran’s I detects departures from spatial randomness within a figure. To calculate local Moran’s I for each pixel (A), a disc-shaped spatial range (B) is chosen and the variance of the values within area B is compared to the variance of the values in the entire image; high I values indicate that the values within area B are spatially associated within the image. In this figure local Moran’s I for pixel A and region C would be expected to be lower, and show less spatial autocorrelation, than pixel D and region E because region E contains more homogenous pO2 values. Global Moran’s I averages of all the local Moran’s I values and normalizes them to the variance in the image. As values of global Moran’s I approach 1, they indicate increased spatial correlation for the entire figure; that is, there is more similarity in pO2 with an examined disc-shaped region than would be expected from the distribution of pO2 of the entire image. Several sizes (1, 4, 8, 12, 20, 40, and 100 pixel radius) of disc-shaped regions were examined in the Moran’s I analysis.

Results from high frequency fluctuations (<5 min) were discarded as the sampling rate of once every 2.5 min could not resolve these high frequencies.
2.3 Results

The median vascular pO\textsubscript{2} for all 9L images was 38(26-40); all values reported are medians and 25-75\% mm Hg; 30(26-35) mm Hg for FSA; and 22(19-23) mm Hg for R3230. There was no clear trend over time of increasing or decreasing average pO\textsubscript{2} of the entire image for all the experiments. The representative experiments shown had a median pO\textsubscript{2} of 34(27-41) for 9L, 21(16-25) for FSA, and 18(16-21) mm Hg for R3230 (Figure 10). These values are higher than previously measured tissue pO\textsubscript{2} values in these same tumor lines (Cardenas-Navia et al., 2004), consistent with the imaging phosphor largely remaining in the vasculature. Perivascular pO\textsubscript{2} measurements have been made previously in the R3230 tumor using recessed tip oxygen microelectrodes. The data presented here match very well with these microelectrode measurements, which were performed independently (Dewhirst et al., 1992).

Although the median pO\textsubscript{2} for a single experiment generally changed only 1-2 mm Hg between any 2.5 min measurement interval, plots of randomly selected individual pixel pO\textsubscript{2} vs. time reflect significant spatial and temporal variability (Figure 10) in vascular pO\textsubscript{2}. This spatiotemporal variability strongly suggests that sampling a few spatially or temporally discrete pO\textsubscript{2} measurements may not be representative of tumor oxygenation.
Figure 10: Median pO$_2$ vs. time for A) 9L; B) FSA; and C) R3230 for representative experiments. Median values of the entire image are plotted as solid lines, and dots show the 25-75% range at each time point. For each example tumor, 10 pixels were randomly selected and pO$_2$ vs. time for each of these pixels was plotted on the same graph as a dashed line.
Figure 11: Representative images of PLI data for A) 9L; B) FSA; and C) R3230. Five time points at 0, 15, 30, 45, and 60 min show pO₂ values for each tumor line.

2.3.1 O₂ Delivery is Constantly Fluctuating

Examination of the raw phosphorescence lifetime imaging (PLI) images of a representative set of tumors showed different patterns of pO₂ fluctuations for each tumor line (Figure 11; same representative set of tumors used as examples throughout manuscript). 9L images had different areas of the images with their pO₂ fluctuating independently of each other; in the example shown in Figure 11, pO₂ on the left side of the image decreases with time, while pO₂ on the right side increases. Both FSA and R3230 seemed to have a distinct pattern of pO₂ at the initial time point, and pO₂ fluctuations with time maintained these patterns. The FSA example has an inverted triangular area at lower pO₂ values than the rest of the image. This area initially shrinks,
and then increases to almost encompass the entire image by the 60 min time point. The R3230 tumor in Figure 11 has an oval-shaped outline visibly at a lower pO$_2$ at the first time point, and over time this oval-shaped outline remains visibly at a lower pO$_2$.

These observations of differences in vascular pO$_2$ pattern between tumor types was reinforced through an exploratory analysis looking at the sign of pO$_2$ changes greater than 1 mm Hg for each pixel from one time point to the next. Not a single pixel remained unchanged over three sequential time points in all the experiments, and fewer than 5% of the pixels changed less than 1 mm Hg between any two subsequent time points. These patterns were also visible in the difference images when examining the sign of pO$_2$ changes greater than 5 mm Hg for each pixel. Although there are 2.5 min intervals where only a small percentage of the image shows changes in vascular pO$_2$ greater than 5 mm Hg, many of the 2.5 min intervals show 30-50% of the image fluctuating more than 5 mm Hg.

**2.3.2 Tumor Type Impacts Spatial Distribution of Oxygen**

Watershed segmentation (Figure 12) showed distinctly different oxygen isobar patterns for different tumor types. At most time points, 9L was highly segmented, with regions of very different pO$_2$ values next to each other. R3230 also showed areas which were highly segmented, however, many of the images contained one or two large spatial pO$_2$ segments. FSA had a great deal of segmentation, but segments with similar pO$_2$ were almost always conjoined.
Figure 12: Representative watershed segmentation results for each 2.5 min time point for A) 9L; B) FSA; and C) R3230. Time increased from left to right, top to bottom, in 2.5 min increments. Watershed segmentation creates boundaries at sharp gradients in pO₂; segmented regions can be thought of as pO₂ isobars. Segments are color-coded by their deviations from the median pO₂ of the image; red indicates high deviations from the median, blue indicates no deviation from the median.
Global Moran’s I analysis showed significant differences in patterns of spatial autocorrelation for the different tumor types (Figure 13A). Generally, spatial autocorrelation decreased as the size of the region examined increased for all tumor types. I values for 9L and FSA were significantly different from R3230 for the smallest spatial range examined (1 pixel radius; Table 1); both 9L and FSA had several time points which showed no autocorrelation at this spatial range, suggesting that in these images adjoining pixels had pO$_2$ values which were independent of each other. Within and between tumor types, I values for different spatial ranges tended to be significantly different from each other (Table 1). Combined with the knowledge that spatial autocorrelation decreased as the area examined increased, this analysis emphasizes the local quality of O$_2$ delivery in tumors.

Global Moran’s I was also used to examine patterns of change in pO$_2$ between time points (Figure 13B). There was much less spatial autocorrelation in the change in pO$_2$ values between time points than within a single time point, although all tumor types had some difference images which showed a high degree of spatial autocorrelation. Spatial autocorrelation further decreased in the difference images as the spatial range examined increased. However, differences in spatial autocorrelation were evident for different tumor types. 9L and R3230 showed significantly less spatial autocorrelation than FSA at spatial ranges higher than a 1 pixel radius (Table 2), and I values for these two tumor types were more tightly clustered than FSA at all spatial
Figure 13: Global Moran’s I values A) all pO₂ time points for all experiments; and B) all pO₂ difference images for all time points for all experiments. Median values are solid lines, boxes contain 25% and 75%, error bars show 1.5x interquartile range, and x’s are I values which fall outside 1.5x interquartile range. Moran’s I values approaching 1 indicate high spatial autocorrelation of pO₂ values, Moran’s I values of zero indicates spatial randomness. For each tumor type, Moran’s I is shown for disk-shaped spatial ranges of radii 1, 4, 8, 12, 20, 40, and 100.
Table 1: Significance table for global Moran’s I. Between and within tumor types, Moran’s I is examined for disk-shaped spatial ranges of radii 1, 4, 8, 12, 20, 40, and 100. Significance is indicated with a “Y” if $p<0.05$ (multiple comparison of means).

<table>
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<th>9L</th>
<th>F3A</th>
<th>R3230</th>
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<td>1</td>
<td>Y</td>
<td>Y</td>
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</tr>
<tr>
<td>4</td>
<td>Y</td>
<td>Y</td>
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</tr>
<tr>
<td>8</td>
<td>Y</td>
<td>Y</td>
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<tr>
<td>12</td>
<td>Y</td>
<td>Y</td>
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<tr>
<td>20</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>40</td>
<td>Y</td>
<td>Y</td>
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</tr>
<tr>
<td>100</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
</tbody>
</table>

Table 2: Significance table for global Moran’s I of difference images. Between and within tumor types, Moran’s I is examined for disk-shaped spatial ranges of radii 1, 4, 8, 12, 20, 40, and 100. Significance is indicated with a “Y” if $p<0.05$ (multiple comparison of means).

<table>
<thead>
<tr>
<th></th>
<th>9L</th>
<th>F3A</th>
<th>R3230</th>
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<tr>
<td>1</td>
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<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>4</td>
<td>Y</td>
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<td>40</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>100</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
</tbody>
</table>
ranges. FSA and R3230 tend to have significant decreases in Moran’s I values within each tumor type for small increases in spatial ranges (1 and 4 pixel radii for FSA, 1 and 8 pixel radii for R3230), suggesting changes in pO₂ values over time were spatially coordinated only for small regions in these tumor types.

Global Moran’s I for an image is an average of normalized local Moran’s I values for each pixel within that image. Therefore, an examination of local Moran’s I values for an image offers insight into the areas within an image which have the greatest spatial autocorrelation.

The example images shown are local Moran’s I values for a spatial range of an 8 pixel radius (Figure 14). The 9L tumor had the same general pattern of spatial autocorrelation at all time points, with a large region of spatial autocorrelation on the lower right hand side. The example FSA showed an area of high spatial autocorrelation in the inverted triangular region, which increased and decreased in size with time. R3230 showed high spatial autocorrelation at all time points in the oval-shaped outline visible in Figure 11; this area of high spatial autocorrelation seemed to increase in size with time. Consistent with the global Moran’s I results for this spatial range, the example 9L, FSA, and R3230 tumors had a similar proportion of the image showing high spatial coordination.

Figure 15, which shows local Moran’s I values for difference images for the same example tumors in Figure 11, offers insight into the spatial autocorrelation of changes in
Figure 14: Representative local Moran’s I results (8 pixel radius spatial range) for each 2.5 min time point for A) 9L; B) FSA; and C) R3230. Time increased from left to right, top to bottom, in 2.5 min increments. I values reflecting high spatial autocorrelation for a pixel are red; I values reflecting no spatial autocorrelation, or spatial randomness, are blue.
pO$_2$ between time points. The 9L example shows that there is almost no spatial coordination in changes between 2.5 min time points. The example FSA tumor has noticeably more spatial autocorrelation in pO$_2$ changes, with regions of high spatial autocorrelation appearing first along the top and lower right side of the image, with a few later time points showing a large region of spatial autocorrelation along the left side. Most time points for the example R3230 difference images showed a few, highly diffuse spots of high autocorrelation, with most of the image reflecting spatial randomness of pO$_2$ values. There was a recurring region with high spatial pO$_2$ autocorrelation in the top left corner of the R3230 example. The trends in spatial autocorrelation seen in Figure 15 are consistent with global Moran’s I values for each tumor type.

For all of the example tumors shown, the areas of spatial autocorrelation at a single time point are not the same areas which show spatial autocorrelation for pO$_2$ changes between time points. The distinct pattern of the inverted triangle visible in Figure 14B for FSA is not apparent in Figure 15B; similarly, the oval-shaped outline boldly apparent in Figure 14C for R3230 is almost entirely missing in Figure 15C.

### 2.3.3 All Tumors Fluctuate with the Slow Periodicities

Fourier analysis showed the periodicities with the largest pO$_2$ fluctuation magnitudes were greater than 10 min for all three tumor types. Generally the three slowest periodicities (ranging from 10-40 min) for a pixel have a similar order of magnitude contribution to the overall pO$_2$ fluctuation magnitude; faster fluctuation
periodicities have $10^2$-$10^4$ lower order of magnitude contribution to the overall $pO_2$ fluctuation magnitude.

**2.4.3 Discussion**

This study proves our hypothesis that fluctuating vascular oxygenation is a prevalent characteristic of these three tumor lines. The main result of this study is one that has perhaps been intuitively understood but not previously explicitly shown: $O_2$ delivery to tumor tissue is constantly changing. Previous characterization of hypoxia as perfusion-limited or diffusion-limited are simply the extreme cases of $O_2$ delivery dominant or $O_2$ metabolism dominant areas in tumors; most tumor tissue does not distinctly fall into either category and the local $pO_2$ is heavily influenced by both.

This study demonstrates that up to 50% of tumor vascular $pO_2$ can change more than 5 mm Hg within a 2.5 min interval; previous theoretical predictions have calculated that a change in vascular $pO_2$ of 10 mm Hg can cause a considerable increase (~30%) in the proportion of severely hypoxic tumor tissue (< 3 mm Hg) (Kimura et al., 1996). Although these predictions cannot be directly applied to the data in this study, changes in vascular $pO_2$ of greater than 5 mm Hg, such as those seen in this study, would certainly be expected to alter the hypoxic fraction of the surrounding tissue.

Perhaps the universal characteristic of tumor $pO_2$ is that the vascular $pO_2$ fluctuations are occurring at low frequencies, or periods on the order of 10s of minutes.
Figure 15: Representative local Moran’s I results (8 pixel radius spatial range) for each pO$_2$ difference image for A) 9L; B) FSA; and C) R3230. Time increased from left to right, top to bottom, in 2.5 min increments. I values reflecting high spatial autocorrelation for a pixel are red; I values reflecting no spatial autocorrelation, or spatial randomness, are blue.
This idea is not a new one: the seminal work of Chaplin et al. using perfusion markers showed that the injection of dyes 20 min apart resulted in marker mismatch in vessels (Chaplin et al., 1987). Later studies showed that the significant time scale was at least 15 min for perfusion marker mismatch (Durand and LePard, 1995), a time scale which has been reinforced with direct measurements of pO₂ fluctuations (Braun et al., 1999; Brurberg et al., 2003; Brurberg et al., 2005; Cardenas-Navia et al., 2004). This study is the first to show that these direct, single-point studies of tumor pO₂ can be generalized to describe the dominant period of pO₂ fluctuations for the entire tumor. A recent publication has shown that pO₂ fluctuations are occurring at dominant periods of 10s of minutes in spontaneous canine tumors (Brurberg et al., 2005), suggesting the characteristic behavior of fluctuating pO₂ is not limited to murine tumors or xenografts grown in rodents and may have a common mechanism across all tumors. Studies measuring pO₂ fluctuations in normal tissue (muscle) in rats and mice have not observed significant fluctuations (Braun et al., 1999; Brurberg et al., 2003).

Spatiotemporal analysis reveals some clues regarding the underlying mechanism(s) of fluctuating hypoxia. Fluctuations in red cell flux, possibly due to vasomotion, are known to cause fluctuations in tumor tissue pO₂ (Dewhirst et al., 1996a; Kimura et al., 1996; Lanzen et al., 2006). This proposition of fluctuations in red cell flux within tumor vessels is consistent with the analysis shown in Figure 14: while pO₂ for individual pixels show temporal fluctuations throughout the image, the continuous
presence over time of the inverted triangle and oval-shaped outline visible in two of the
tumors suggest a network of vessels through which red cells are moving. This result is
not unexpected; in prior work with microelectrodes, we found significant fluctuations in
tissue pO$_2$ of regions of 200-300 μm in diameter in which small networks of 5-6
microvessels were involved (Lanzen et al., 2006). However, in Figure 15 autocorrelation
of changes in pO$_2$ between time points is generally not occurring in the locations of the
“networks” in Figure 14. One potential mechanism for these localized areas of change in
pO$_2$ may be vascular intussusception, which has been shown to locally alter blood flow
in experimental tumors with a similar timescale to the one measured in our study (Patan
et al., 1996). Alternatively, changes in red cell deformability and viscosity due to lower
pH values, in the same range as those seen in the tumor microenvironment, have also
been shown to alter tumor blood flow and oxygenation (Kavanagh et al., 1993); spatially
heterogeneous pH profiles in tumors (Helmlinger et al., 1997) could result in localized
heterogeneity in tumor oxygenation.
3. Effects of Fluctuating Oxygenation on Tirapazamine Efficacy: Theoretical Predictions

3.1 Introduction

Hypoxia is known to play an important role in altering the efficacy of radiation and chemotherapy. A variety of strategies have been used to improve oxygen delivery, with varying degrees of success. An alternative approach has been to develop therapeutic agents that selectively kill hypoxic cells.

Lin et al. first proposed this strategy in the 1970s, suggesting that prodrugs that were selectively metabolized in hypoxic cells could be used with little systemic toxicity (Lin et al., 1972). These drugs would complement traditional therapies which predominantly target well-oxygenated cells. Since the concept of bioreductive drugs was first proposed, several different prodrugs have been developed and have shown efficacy in clinical and preclinical studies (for review see (McKeown et al., 2007)).

One of the most promising agents has been the development of a hypoxic cytotoxin, tirapazamine (TPZ), a 1,2,4-benzotriazine 3-amine 1,4-dioxide bioreductive compound (Figure 16). Once in the cell, TPZ undergoes an enzymatic one-electron reduction to a radical anion (Patterson et al., 1995; Patterson et al., 1998; Patterson et al., 1997; Saunders et al., 2000a; Saunders et al., 2000b). In the presence of oxygen, this anion is oxidized back to its parent form; in the absence or at low levels of oxygen, the anion is highly reactive and produce cytotoxic oxidizing species (Wang et al., 1992). These
radicals then react with DNA, resulting in DNA strand breaks (Peters and Brown, 2002; Wang et al., 1992). TPZ has been shown in vitro to be 40 to 200 (Zeman et al., 1986; Zeman et al., 1988) times more lethal to hypoxic than normoxic cells. Due to this selectivity, TPZ is believed to be able to target hypoxic cells at distances far from the vasculature (70-100 μm).

**Figure 16:** TPZ is activated via reduction to an O₂-sensitive radical (TPZ•), which decays for form a DNA-oxidizing radical (TPZ*). A second molecule of TPZ further oxidizes the DNA radicals; complex DNA lesions may be forms by interaction with additional TPZ radical and/or TopoII.

Clinical trials using TPZ have shown limited efficacy as a monotherapy, but some promise in combination with radiation and/or chemotherapy. Phase I and II trials found several dose-limiting toxicities, including neutropenia, nausea, vomiting,
diarrhea, and skin rash; reversible deafness and muscles cramping were also observed (Covens et al., 2006; Doherty et al., 1994; Le et al., 2004; Lee et al., 1998; Rischin et al., 2005; Rischin et al., 2001; Senan et al., 1997). The most promising clinical studies involved patients whose tumors were examined to determine the presence of hypoxia as measured by \(^{18}\)FMISO PET (Hicks et al., 2005; Rischin et al., 2006). In head and neck cancer patients with hypoxia, overall survival was significantly increased with TPZ+cisplatin+RT vs. cisplatin+RT alone (Rischin et al., 2006). This study suggests that TPZ is a highly promising adjuvant therapy for targeting hypoxic cells in the clinic.

Several investigators have previously addressed the issue of TPZ diffusion into solid tumors using well characterized transport equations developed for TPZ diffusion, metabolism, and efficacy in vitro in multi-cell layers (Durand and Olive, 1992; Hicks et al., 1998; Hicks et al., 2003; Hicks et al., 2004; Kyle and Minchinton, 1999). Previous simulations have focused on TPZ metabolism and efficacy while holding the \(O_2\) concentration constant. These studies suggested that TPZ has a limited ability to diffuse into hypoxic regions far from tumor vessels (Durand and Olive, 1992; Hicks et al., 1998; Hicks et al., 2003; Hicks et al., 2004; Kyle and Minchinton, 1999); these data do not correspond completely with in vivo data which have shown higher TPZ efficacy than predicted by models (Durand and Olive, 1997; Minchinton and Brown, 1992). Previous simulations did not account for potential fluctuations in pO\(_2\), which have been seen to be present in every solid tumor type examined (Baudelet and Gallez, 2003; Bennewith et al.,
The effects of fluctuating hypoxia on intracellular TPZ concentration using normalized sinusoidal functions as well as experimentally measured O$_2$ fluctuations are here predicted using a theoretical model. The results suggest that fluctuations in pO$_2$ can have a large influence on the spatial distribution of TPZ and overall TPZ efficacy.

**3.2 Materials and Methods**

**3.2.1 Coupled O$_2$ and TPZ transport and metabolism simulations**

One-dimensional transport of O$_2$ and TPZ was simulated according to the equations developed by Hicks et al (Hicks et al., 2004).

\[
\frac{\partial [O_2]}{\partial t} = D_{O_2} \frac{\partial^2 [O_2]}{\partial x^2} - \frac{V_{O_2} [O_2]}{K_{mO_2} + [O_2]}
\]

and

\[
\frac{\partial [TPZ]}{\partial t} = D_{TPZ} \frac{\partial^2 [TPZ]}{\partial x^2} - \dot{M}
\]

where
\[ M = \phi \left( \frac{K_O}{K_{O_2} + [O_2]} \right) \times \left( k_{\text{met}}[TPZ] + \frac{V_{\text{max}}[TPZ]}{K_m + [TPZ]} \right) \]

is the rate of intracellular metabolism of TPZ. The amount of metabolized TPZ at each location was obtained by taking the time integral of eq. (3). All variables and parameters were taken from Hicks et al. (Hicks et al., 2004) and are defined in Table 3.

Using the same transport parameters, one-dimensional transport was also simulated for O\(_2\) and TPZ in cylindrical coordinates:

\[ \frac{\partial [O_2]}{\partial t} = \frac{1}{r} \frac{\partial}{\partial r} \left( D_{O_2} r \frac{\partial [O_2]}{\partial r} \right) - \left( \frac{V_{O_2}[O_2]}{K_{mO_2} + [O_2]} \right) \]

and

\[ \frac{\partial [TPZ]}{\partial t} = \frac{1}{r} \frac{\partial}{\partial r} \left( D_{TPZ} r \frac{\partial [TPZ]}{\partial r} \right) - \dot{M} \]

The equations were solved for a 10-hr period within a 50-, 100-, or 200-μm domain extending from a source microvessel, using the MATLAB 6.5 (The Mathworks, Inc.) `pdepe` function solver. At \( t = 0 \), the TPZ and O\(_2\) concentrations throughout the domain were set to zero and to the average \( O_2 \) concentration for the 10-hr period,
respectively. At $x = 0$ or $r = 5$ (which corresponds to the microvessel wall), $O_2$ concentration was specified as a function of time $t$, and TPZ concentration was assumed to remain at a maximum concentration of 40 μM for 150 min and then to decrease exponentially with $t_{1/2} = 46.4$ min (Graham et al., 1997) to account for clearance from the bloodstream. Zero-flux boundary conditions for both $O_2$ and TPZ were imposed at the outer boundary of $x=50$, 100, or 200-μm or $r=55$, 105, or 205-μm (resulting in $r$ domains of 50, 100, or 200-μm).

### 3.2.2 TPZ survival simulations

Cell survival with TPZ was predicted using the empirical relationship developed by Hicks et al. between surviving fraction ($SF$), TPZ concentration, and rate of TPZ metabolism (Hicks et al., 2004):

$$\frac{d \ln SF}{dt} = \gamma [TPZ] M$$

where $\gamma$ is an empirically derived constant (see Table 3).

### 3.2.3 RT Survival Simulations

Cell survival following a single dose of 2 Gy was predicted according to the linear-quadratic model developed by Wouters and Brown (Wouters and Brown, 1997):
\[
\begin{align*}
\text{(7) } & \quad - \ln SF = \alpha OER_\beta D + \beta [OER_\beta D]^2 \\
\text{where} \\
\text{(8) } & \quad OER_\alpha = \frac{OER_{\alpha m}[O_2] + K_{ms}}{[O_2] + K_{ms}} \\
\text{and} \\
\text{(9) } & \quad OER_\beta = \frac{OER_{\beta m}[O_2] + K_{ms}}{[O_2] + K_{ms}}.
\end{align*}
\]

The fractionated dose was delivered during a time period spanning the minimum \(O_2\) concentration (see Table 3 for variable definitions). No interaction was assumed between RT and TPZ and the SF resulting from each therapy was calculated independently.

### 3.2.4 Sensitivity Analysis

Prior experimental observations by our group (Braun et al., 1999; Cardenas-Navia et al., 2003) and others (Brurberg et al., 2004; Brurberg et al., 2003; Brurberg et al., 2005) have shown that oxygenation of tumors fluctuates with a dominant periodicity of 2-3 cycles per hour, although some fluctuations of lower magnitude occur at higher
frequencies. For the initial simulations, therefore, sinusoidal fluctuations of the pO$_2$ at $x = 0$ were specified. The fluctuations were varied with respect to period (1, 10, 30 min), amplitude (1, 5, 10, 15 mm Hg), and time-averaged pO$_2$ (1, 5, 10, 15 mm Hg). These values are in the range of experimentally measured values. Constant, non-fluctuating cases were also simulated using pO$_2$ values of 1, 5, 10 and 15 mm Hg. pO$_2$ values were converted to O$_2$ concentrations assuming a solubility of 1000 μM/760 mm Hg at 37° and simulations for O$_2$ and TPZ concentration were conducted as described above. TPZ SF at the end of the 10 hrs was calculated, as described above.

3.2.5 RT+TPZ Simulations

Additional simulations of combined RT and TPZ therapy were conducted based on seven previously published time-courses of perivascular pO$_2$ observed in rat R3230Ac mammary adenocarcinomas. These time-courses were used to set the O$_2$ conditions at $x = 0$. Since the perivascular pO$_2$ was measured every 5 min, the data were linearly interpolated every 10 s between measurement time points. Equations 1 and 2 were solved for 90 or 120 min, as described above, except that TPZ concentration at $x = 0$ was initially set to 40 μM and then immediately began to decrease exponentially with $t_{1/2} = 46.4$ min (Graham et al., 1997). RT and TPZ SF were calculated using the methods described above. The RT dose was assumed to be delivered first, followed by the TPZ dose (both simulated as described above). Simulations of this combined therapy were conducted in Cartesian coordinates for three cases: 1) **forward** case: the condition at $x = 0$
Table 3: TPZ and oxygen variables and parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Description</th>
<th>Value</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>( f_m )</td>
<td></td>
<td>SR 4317 yield as a fraction of TPZ consumed</td>
<td>0.73</td>
<td>(Hicks et al., 2003)</td>
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<tr>
<td>( k_{net} )</td>
<td>min(^{-1})</td>
<td>First-order rate constant for TPZ consumption</td>
<td>0.78</td>
<td>(Hicks et al., 2003)</td>
</tr>
<tr>
<td>( V_{\text{max}} )</td>
<td>( \mu M \text{ min}^{-1} )</td>
<td>Maximal rate for Michaelis-Menten component of TPZ metabolism</td>
<td>8.5</td>
<td>(Hicks et al., 2003)</td>
</tr>
<tr>
<td>( K_m )</td>
<td>( \mu M )</td>
<td>Michaelis constant for TPZ metabolism</td>
<td>3.5</td>
<td>(Hicks et al., 2003)</td>
</tr>
<tr>
<td>( \Gamma )</td>
<td>( \mu M^2 )</td>
<td>Proportionality constant in pharmacodynamic model</td>
<td>( 5.35 \times 10^{-5} )</td>
<td>(Hicks et al., 2003)</td>
</tr>
<tr>
<td>( D_{TPZ} )</td>
<td>( cm^2 s^{-1} )</td>
<td>Diffusion coefficient of TPZ in tumor</td>
<td>( 0.40 \times 10^{-6} )</td>
<td>(Hicks et al., 2003)</td>
</tr>
<tr>
<td>( \Phi )</td>
<td></td>
<td>Cell volume fraction</td>
<td>0.517</td>
<td>(Hicks et al., 2003)</td>
</tr>
<tr>
<td>( K_{O_2} )</td>
<td>( \mu M )</td>
<td>Solution ( O_2 ) concentration at which the rate of TPZ metabolism and cytotoxicity is half maximal</td>
<td>1.21</td>
<td>(Hicks et al., 2004)</td>
</tr>
<tr>
<td>( D_{O_2} )</td>
<td>( cm^2 s^{-1} )</td>
<td>Diffusion coefficient of ( O_2 ) in tumor</td>
<td>( 25 \times 10^{-6} )</td>
<td>(Secomb et al., 1998)</td>
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<tr>
<td>( V_{\text{mO}_2} )</td>
<td>( \mu M min^{-1} )</td>
<td>Rate of ( O_2 ) consumption</td>
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<td>(Secomb et al., 1998)</td>
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<tr>
<td>( K_{mO_2} )</td>
<td>( \mu M )</td>
<td>Michaelis constant for ( O_2 ) consumption</td>
<td>1.0</td>
<td>(Secomb et al., 1998)</td>
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<td>( \alpha )</td>
<td>( Gy^{-1} )</td>
<td>Parameter of LQ radiation survival model under anoxia</td>
<td>0.08</td>
<td>(Wouters and Brown, 1997)</td>
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<td>( \beta )</td>
<td>( Gy^{-1} )</td>
<td>Parameter of LQ radiation survival model under anoxia</td>
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<td>(Wouters and Brown, 1997)</td>
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<td>( D )</td>
<td>( Gy )</td>
<td>Assumed radiation dose</td>
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<td>(Wouters and Brown, 1997)</td>
</tr>
<tr>
<td>( K_{ms} )</td>
<td>( \mu M )</td>
<td>( O_2 ) concentration for half maximal radiation sensitization</td>
<td>4.3</td>
<td>(Wouters and Brown, 1997)</td>
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<td>( OER_{\alpha m} )</td>
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<td>Maximum OER for ( \alpha ) component of radiation response</td>
<td>2.5</td>
<td>(Wouters and Brown, 1997)</td>
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<tr>
<td>( OER_{\beta m} )</td>
<td></td>
<td>Maximum OER for ( \beta ) component of radiation response</td>
<td>3</td>
<td>(Wouters and Brown, 1997)</td>
</tr>
</tbody>
</table>
follows the observed values; 2) **reverse** case: condition at \( x = 0 \) follows the reversed time course of the observed values; and 3) **constant** case: the prescribed \( O_2 \) condition at \( x = 0 \) is set equal to the time-average of the observed values. A tissue domain of 200 \( \mu m \) was used in these simulations to determine if TPZ treatment complements RT by targeting hypoxic regions distant from the vasculature.

### 3.2 Results

#### 3.2.1 Sensitivity Analysis

Figures 17, 19, 21, 23, 25, and 27 show the dependence on distance from the microvessel of the amount of metabolized TPZ for several combinations of average \( pO_2 \) and fluctuation amplitude and period; Figures 18, 20, 22, 24, 26, 28 and 29-32 show the dependence of cell kill on distance from microvessel.

#### 3.2.1.1 Effects of fluctuating hypoxia

Over all, the effect of fluctuating oxygen levels is to increase the amount of metabolized TPZ at points close to the vessel. Generally, this effect is modest, but this effect is most prominent in cases where the fluctuation magnitude equals the average value. In such cases, the \( pO_2 \) adjacent to the microvessel is close to zero during part of each oscillation, allowing increased TPZ metabolism. For all cases the concentration of metabolized TPZ reaches a peak within 50-100 \( \mu m \) from the vessel. At points closer to the microvessel, TPZ metabolism is limited by the relatively high oxygen levels, while
Figure 17: Dependence of intracellular metabolized TPZ on distance from microvessel for several combinations of average perivascular pO$_2$, fluctuation amplitude, and fluctuation period. Curves for different cases are similar and may overlap. Simulations are for Cartesian coordinates with a domain of 200 μm.
Figure 18: Dependence of cell kill, expressed as $-\log(\text{survival fraction})$, on distance from microvessel, for several combinations of average perivascular $pO_2$, fluctuation amplitude and fluctuation period (greater $-\log SF$ means greater cell kill). Surviving fraction for different cases is similar and the curves may overlap. Simulations are for Cartesian coordinates with a domain of 200 $\mu$m.
Figure 19: Dependence of intracellular metabolized TPZ on distance from mirovessel for several combinations of average perivascular pO$_2$, fluctuation amplitude, and fluctuation period. Curves for different cases are similar and may overlap. Simulations are for cylindrical coordinates with a domain of 200 μm.
Figure 20: Dependence of cell kill, expressed as \(-\log(\text{survival fraction})\), on distance from microvessel, for several combinations of average perivascular pO$_2$, fluctuation amplitude and fluctuation period (greater \(-\log SF\) means greater cell kill). Surviving fraction for different cases is similar and the curves may overlap. Simulations are for cylindrical coordinates with a domain of 200 \(\mu\text{m}\).
Figure 21: Dependence of intracellular metabolized TPZ on distance from mirovessel for several combinations of average perivascular pO$_2$, fluctuation amplitude, and fluctuation period. Curves for different cases are similar and may overlap. Simulations are for Cartesian coordinates with a domain of 100 μm.
Figure 22: Dependence of cell kill, expressed as $-\log$(survival fraction), on distance from microvessel, for several combinations of average perivascular pO$_2$, fluctuation amplitude and fluctuation period (greater $-\log$ SF means greater cell kill). Surviving fraction for different cases is similar and the curves may overlap. Simulations are for Cartesian coordinates with a domain of 100 $\mu$m.
Figure 23: Dependence of intracellular metabolized TPZ on distance from mirovessel for several combinations of average perivascular pO$_2$, fluctuation amplitude, and fluctuation period. Curves for different cases are similar and may overlap. Simulations are for cylindrical coordinates with a domain of 100 μm.
Figure 24: Dependence of cell kill, expressed as $-\log(\text{survival fraction})$, on distance from microvessel, for several combinations of average perivascular pO$_2$, fluctuation amplitude and fluctuation period (greater $-\log$ SF means greater cell kill). Surviving fraction for different cases is similar and the curves may overlap. Simulations are for cylindrical coordinates with a domain of 100 μm.
Figure 25: Dependence of intracellular metabolized TPZ on distance from microvessel for several combinations of average perivascular pO₂, fluctuation amplitude, and fluctuation period. Curves for different cases are similar and may overlap. Simulations are for Cartesian coordinates with a domain of 50 μm.
Figure 26: Dependence of cell kill, expressed as $-\log$ (survival fraction), on distance from microvessel, for several combinations of average perivascular pO$_2$, fluctuation amplitude and fluctuation period (greater $-\log$ SF means greater cell kill). Surviving fraction for different cases is similar and the curves may overlap. Simulations are for Cartesian coordinates with a domain of 50 μm.
Figure 27: Dependence of intracellular metabolized TPZ on distance from mirovessel for several combinations of average perivascular pO$_2$, fluctuation amplitude, and fluctuation period. Curves for different cases are similar and may overlap. Simulations are for cylindrical coordinates with a domain of 50 μm.
Figure 28: Dependence of cell kill, expressed as $-\log(\text{survival fraction})$, on distance from microvessel, for several combinations of average perivascular $pO_2$, fluctuation amplitude and fluctuation period (greater $-\log SF$ means greater cell kill). Surviving fraction for different cases is similar and the curves may overlap. Simulations are for cylindrical coordinates with a domain of 50 μm.
Figure 29: Dependence of cell kill, expressed as $-\log$ (survival fraction), on distance from microvessel, for several combinations of average perivascular $pO_2$, fluctuation amplitude and fluctuation period (greater $-\log$ SF means greater cell kill). Surviving fraction for different cases is similar and the curves may overlap. Simulations are comparing Cartesian vs. cylindrical coordinates with a domain of 200 $\mu$m. Constant cases are solid line, fluctuating case with a 30 min period is dotted line.
Figure 30: Dependence of cell kill, expressed as $-\log(\text{survival fraction})$, on distance from microvessel, for several combinations of average perivascular $\text{pO}_2$, fluctuation amplitude and fluctuation period (greater $-\log \text{SF}$ means greater cell kill). Surviving fraction for different cases is similar and the curves may overlap. Simulations are comparing Cartesian vs. cylindrical coordinates with a domain of 100 $\mu$m. Constant cases are solid line, fluctuating case with a 30 min period is dotted line.
Figure 31: Dependence of cell kill, expressed as $-\log(\text{survival fraction})$, on distance from microvessel, for several combinations of average perivascular pO$_2$, fluctuation amplitude and fluctuation period (greater $-\log$ SF means greater cell kill). Surviving fraction for different cases is similar and the curves may overlap. Simulations are comparing Cartesian vs. cylindrical coordinates with a domain of 50 μm. Constant cases are solid line, fluctuating case with a 30 min period is dotted line.
Figure 32: Dependence of cell kill, expressed as \(-\log(\text{survival fraction})\), on distance from microvessel, for several combinations of average perivascular pO\(_2\), fluctuation amplitude and fluctuation period (greater \(-\log\ SF\) means greater cell kill). Surviving fraction for different cases is similar and the curves may overlap. Simulations are comparing Cartesian vs. cylindrical coordinates for domains of 50, 100, and 200 \(\mu\)m. Constant cases are solid line, fluctuating case with a 30 min period is dotted line.
beyond this point (for long intervascular distances, x=200 μm or r=200 μm) the amount of available TPZ declines due to diffusion limitations. For small intervascular distances (50 μm), the constant O₂ conditions is also predicted to decrease the peak of the maximum intracellular metabolized TPZ, but not the distance of the peak from the vessel.

The effects of fluctuating pO₂ on cell kill due to TPZ seemed to be highly dependent on the fluctuation magnitude. Larger fluctuation magnitudes (5, 20, 15 mm Hg) showed decreased cell kill for the constant case; the periodicity of the fluctuations had almost no effect.

3.2.1.2 Effects of intervascular distance

Intervascular distance had a sizeable impact on TPZ metabolism. Longer intervascular distances shifted the concentration peak or plateau closer to the microvessel. Simulations with a 100 μm domain had the highest amount of metabolized TPZ at distances higher than 20 μm when compared to the 50 μm or 200 μm domain simulations.

The effects of intervascular distance on TPZ cell kill mirror those of TPZ metabolism: a longer intervascular distance resulted in the peak or plateau which is closer to the microvessel and the highest cell kill overall is predicted to occur when the domain is 100 μm (Figure 32). The shift in the location of the peak is particularly evident when comparing the 50 μm and 200 μm domain simulations in cylindrical coordinates;
the highest cell kill in the 200 μm domain simulations tends to occur immediately adjacent to the microvessel whereas in the 50 μm domain simulations it occurs at 50 μm.

3.2.1.3 Effects of Cartesian vs. cylindrical coordinates

Simulations using Cartesian coordinates yielded distinctly different resulted than those using cylindrical coordinates (Figures 29-31). For simulations with a domain of 200 μm, cylindrical coordinates had a decreased maximum amount of metabolized TPZ and cell kill compared to Cartesian coordinates. Simulations with domains of 50 μm or 100 μm, however, had increased overall and peak metabolized TPZ levels and cell kill in cylindrical coordinates. The location of the peak or plateau concentrations and peak or plateau cell kill relative to the microvessel was also affected by the different coordinate systems; for cylindrical coordinates they were shifted closer to the microvessel for 100 μm or 200 μm domains, but not the 50 μm domain.

3.3.2 RT+TPZ Simulations

Simulations using experimentally observed time-courses of perivascular pO₂ yielded results consistent with the sensitivity analysis. For all seven time-courses, the SF near the O₂ source was altered for the forward and reverse cases compared to the constant case. A representative example is shown in Figure 33. In this example, the reverse case has the highest cell kill for tissue within about 50 μm of the vessel. This reflects the fact that oxygen levels in the time-reversed case are low during the initial
period of high TPZ availability. In the forward case, less TPZ is metabolized in the tissue close to the vessel, resulting in slightly higher cell kill in the tissue 100-200 μm from the vessel.

![Figure 33: A) Experimental perivascular O2 time-course used in RT+TPZ simulation. Original data replotted from Kimura et al. (Kimura et al., 1996) B) Dependence of cell kill, expressed as \(-\log(\text{survival fraction})\), on distance from microvessel, for O2 time-course shown in A (forward case), reversed time course (reverse case) and corresponding constant average value (constant case) (Cardenas-Navia et al., 2007b).](image)

3.4 Discussion

The simulations presented here show that fluctuations in pO2 can have a significant effect on TPZ pharmacodynamics. The impact of fluctuations in pO2 was highly dependent on the average pO2 value, the magnitude of the fluctuations, and the
intervascular distance between vessels. Fluctuations in pO\(_2\) also altered the amount of free TPZ in the tissue available for diffusion into regions more distant from the O\(_2\) source. For larger fluctuation magnitudes, the position in the tissue with highest cell kill was shifted towards the vessel when effects of fluctuations were included. This result reflects the occurrence of transient periods of hypoxia close to the vessel, during which TPZ metabolism was increased.

The results support the basic rationale for using hypoxic cytotoxins. The amount of metabolized TPZ increases with increasing distance in the tissue and a peak 50 to 100 μm from the vessel, thus preferentially delivering TPZ to hypoxic regions. This is a consequence of the model, specifically equation (3), in which TPZ metabolism increases with decreasing pO\(_2\). (Hicks et al., 2004) In the model, the rate of cell kill depends on the product of the local TPZ concentration and the rate of TPZ metabolism.

The vessel to vessel distance, most easily seen in the cylindrical coordinate simulations which assume a Krogh’s cylinder geometry, is critical in analyzing TPZ as a hypoxic cytotoxin. For large domains (200 μm), the vessel to vessel distance is 400 μm, which is quite large; for these simulations, TPZ is not predicted to be very efficacious at distances farther than 80-100 μm from the vessel, and does not seem to particularly target regions (those farther from the vessel) that would complement RT. However, for shorter domains, such as 50 and 100 μm, the efficacy of TPZ increases as distance from the vessel increases, and reaches a peak at the edge of the domain. These simulations
represent intervascular distances of 100 to 200 μm, which are more prevalent in
tumors (Hardee et al., 2008); these simulations in cylindrical coordinates best represent
the true physiology of tumors. These simulations predict that TPZ is well optimized to
work as a hypoxic cytotoxin in tumors. Nonetheless, three-dimensional modeling and *in vivo*
experimentation by Hicks et al. has shown that TPZ analogues with higher diffusion
coefficients are more efficacious (Hicks et al., 2006), suggesting that TPZ is not reaching
distances far from the vessel, complementary to RT. Alternatively, even for shorter
intervascular distances, TPZ analogues with higher diffusivity could be more effective if
the vessels themselves are poorly oxygenated (Dewhirst et al., 1996a; Kimura et al., 1996;
Lanzen et al., 2006); even with the same $K_{O2}$ the faster diffusivity of the TPZ analogues
would result in decreased metabolism of the analogues immediately near the vessel
compared with TPZ, leaving more available to target the most hypoxic regions of the
tumors.

Previously published *in vivo* data support the limited ability of TPZ to diffuse
away from the vessel. Minchinton et al. conducted experiments in SCCVII and SiHa
tumors, both of which have been measured as having fluctuating oxygen levels, treated
with radiation, with or without TPZ and/or with or without carbogen breathing
(Minchinton et al., 2002). In both tumor types, RT+carbogen decreased SF, consistent
with an increase in tumor oxygenation. In SCCVII tumors, TPZ+RT had a large effect on
SF, but this effect was not enhanced with the addition of carbogen breathing. In SiHa
tumors, TPZ+RT also had an effect on SF and this effect was enhanced with the addition of carbogen breathing; however this enhancement was equal to that of RT+carbogen alone. These results suggest that the increase in pO₂ in the tumor tissue due to carbogen breathing had no impact on TPZ efficacy; in other words, increases in tumor pO₂ had no effect on TPZ diffusion or distribution in hypoxic areas distant from microvessels which were resistant to RT killing, suggesting it was metabolized at regions close to the vessels and consistent with the results from these simulations. Carbogen breathing was not shown to significantly alter plasma TPZ pharmacodynamics, or distribution and metabolism of TPZ in liver, kidney or SCCVII tumor tissue, although animals with SiHa tumors were not examined for possible effects. (Minchinton et al., 2002)

An assumption of the model is that TPZ may accumulate without limit within each cell, according to eq. (3). In reality, the amount of TPZ that can be metabolized by a single cell may be limited. In such a case, uptake of TPZ by metabolism near the vessel would potentially be limited, and the distance to which TPZ could diffuse into the tissue would be increased. This would alter the distribution of metabolized TPZ simulated in this paper, particularly at very low average pO₂s. However, no information is currently available to determine whether such a limitation applies.

Two in vivo studies support the key result seen in the simulations: fluctuating pO₂ increases the efficacy of TPZ. Masunaga et al. recently published a study in murine tumors in which the same dose of TPZ was delivered either in a single bolus dose i.p. or
delivered subcutaneously over 24 hrs using a pump (Masunaga et al., 2005). The results from this study showed a decreased SF for tumors in which TPZ was delivered over 24 hrs. Although we cannot exclude the possibility that these different delivery methods have altered the amount of TPZ available to the tumor, our simulations suggest that this difference in SF is due to the longer time frame of TPZ delivery during which slow fluctuations in pO$_2$ occurred, increasing the efficacy of TPZ. When TPZ is infused over a longer period of time, many more pO$_2$ fluctuation cycles are covered, resulting in higher TPZ metabolism over a larger range of distances from the vessel, as pO$_2$ fluctuations alter the distance over which TPZ is metabolized.

More evidence of the increased efficacy of TPZ during fluctuating pO$_2$ may also be seen in experiments combining tumor electroporation with TPZ administration. In these experiments, Maxim et al. delivered eight electric pulses to SCCVII flank tumors shortly following i.p. administration of TPZ (Maxim et al., 2004). These pulses have been shown to significantly decrease O$_2$ saturation in the tumors for a period of approximately 10 min. Tumor growth delay was significantly increased in tumors receiving this electroporation+TPZ treatment compared to TPZ treatment alone. This increased growth delay is consistent with the results from our simulations, however, it cannot be directly attributed to the decrease in O$_2$ saturation from the electroporation since no control experiments were done to examine the anti-tumor effects of electroporation alone. Additionally, electroporation may have an effect on the delivery
of TPZ through the tumor microenvironment and/or into the tumor cells, which was not examined in the study.

Huxham et al. recently showed that TPZ can cause vascular dysfunction in the central regions of HCT-116, SiHa, SCCVII, and C3HBA tumors. (Huxham et al., 2006) Although pimonidazole binding was not visible around the non-perfused vessels in the center of these tumors, our simulations have shown that there can be a significant TPZ pharmacodynamic effect at a perfused microvessel due to fluctuating pO$_2$ even though the average pO$_2$ value of that microvessel is greater than 10 mm Hg or above that needed to cause pimonidazole staining. The vascular dysfunction seen by Huxham et al. (Huxham et al., 2006) 24 hrs after TPZ administration could be due to TPZ effects on tumor endothelial cells.

The results presented include one-dimensional simulations for Cartesian and cylindrical coordinates; these results have implications regarding TPZ efficacy in a real, three-dimensional tumor environment. Predictions using cylindrical coordinates showed decreased efficacy compared to those using Cartesian coordinates; this is because in cylindrical coordinates the same amount of TPZ must diffuse into a larger area when compared to Cartesian coordinates. In the three-dimensional case, TPZ diffuses in several dimensions instead of along a single dimension; this will reduce the amount of TPZ available to individual cells located at distances farther away from blood vessels. Fluctuations in pO$_2$ seemed to shift the peak for TPZ metabolism toward the
blood vessel. This effect could be exaggerated in three dimensions, further reducing the
amount TPZ available to cells distant from blood vessels.

The diffusion of TPZ into tumor tissue may also be greater than simulated in this
study when given as part of a full therapy. The simulations were for a single dose of
TPZ, whereas in clinical trials TPZ is usually administered several times (Aquino et al.,
2004; Covens et al., 2006; Le et al., 2004; Rischin et al., 2005; Rischin et al., 2001). Between
administrations of TPZ in patients, the tumor microenvironment may change to alter the
transport properties of drugs in tumor tissue. As cells closest to the vessel are killed by
TPZ or RT, subsequent doses of TPZ may diffuse further into tissue than predicted in
this study. Additionally, fluctuating pO₂ may have an impact on other O₂ dependent
antitumor agents, such as doxorubicin. (Teicher, 1994; Teicher et al., 1981)
4. Green’s Function Analysis of Fluctuations in $O_2$ Delivery to Tissue

4.1 Introduction

Many investigators have previously measured both spatial and temporal heterogeneity in the tumor microenvironment oxygenation in both tumor tissue and tumor vasculature. Unfortunately, due to technical limitations, the ability to experimentally manipulate individual hemodynamic variables potentially involved in spatio-temporal fluctuations in oxygen delivery and tumor tissue has not been possible in clinical or pre-clinical tumor models. In order to examine oxygen transport in the tumor microenvironment, investigators have relied on theoretical methods.

Beginning with the classical model of Krogh, which represents tissue microvasculature as a set of parallel, evenly distributed capillaries, investigators have developed increasingly complex and realistic models of microvascular networks (for reviews see Popel (Popel, 1989), Hellums et al (Hellums et al., 1996), and Pittman (Pittman, 2005)). In particular, more recent models have attempted to incorporate the spatial heterogeneities that arise from microvascular geometry, blood flow, and oxygen delivery (Ellsworth et al., 1988; Goldman and Popel, 2000; Goldman and Popel, 2001; Hoofd and Turek, 1996; Secomb et al., 1998; Secomb et al., 1993; Secomb et al., 2004).
Simulating O₂ transport in experimentally derived networks can be very computationally demanding. Finite difference methods require a uniformly spaced computational mesh; high O₂ consumption rates can result in steep gradients near the vessel, requiring fine spatial resolution and increasing the number of unknowns which must be solved. Finite elements analysis, which allows unequal spacing in its mesh, offers a promising alternative to finite differences, although it can also be very computationally intensive. However, Hsu and Secomb have developed an elegant Green’s function approach to O₂ transport which can accurately determine an O₂ field with a relatively small number of unknowns (Hsu and Secomb, 1989). Their approach, which is described below, has been successfully applied to experimentally determined microvascular regions with a volume of up to 0.246 mm³, and is extremely well suited to examine the complexities of O₂ transport in the tumor microenvironment (Hsu and Secomb, 1989; Kimura et al., 1996; Secomb and Hsu, 1988; Secomb et al., 1998; Secomb et al., 2004). In this study, we apply this approach to further explore how temporal changes vascular pO₂, blood flow rate, and discharge hematocrit alter the oxygenation of tumor tissue in a tumor microvascular network.
4.2 Theoretical Analysis

4.2.1 Governing Equations

The governing equation for steady state O\textsubscript{2} transport in tumor tissue three-dimensions is given by the partial differential equation (section 4.2 is adapted from Secom et al. (Secomb et al., 2004)):

\[ D \nabla^2 u = R(u) \]

where \(D\) is the O\textsubscript{2} diffusion constant, \(u\) is the O\textsubscript{2} concentration, and \(R(u)\) is the O\textsubscript{2} consumption rate in the tissue.

Oxygen consumption in tissue is often described by the Michaelis-Menten equation:

\[ R(u) = \frac{v_{\text{max}} u}{k_{\text{met}} + u} \]

where \(v_{\text{max}}\) is the O\textsubscript{2} consumption rate when O\textsubscript{2} is freely available and \(k_{\text{met}}\) represents the O\textsubscript{2} concentration at \(\frac{1}{2}v_{\text{max}}\).

Convective O\textsubscript{2} transport along vessel segments, \(f(u_b)\), can be written as:

\[ f(u_b) = Q(Hct_d u_b S + \alpha_{\text{eff}} u_b) \]

where \(Q\) is the blood flow rate, \(Hct_d\) is the discharge hematocrit, \(S\) is the oxyhemoglobin saturation, \(u_b\) is the oxygen concentration in the blood, \(u_0\) is the concentration of
hemoglobin-bound oxygen in a fully saturated red blood cell, and $\alpha_{\text{eff}}$ is the effective solubility of O$_2$ in blood. $S$ is also dependent on $u_5$, represented by the Hill equation:

$$S(u_b) = \frac{u_b^n}{u_b^n + u_{50}^n}$$

where $u_{50}$ is the O$_2$ concentration at 50% hemoglobin saturation and $n$ is a constant.

Conservation of mass implies that:

$$\frac{df(u_b)}{dz} = -q_v(z)$$

in each vessel segment, where $z$ is distance along the segment and $q_v$ is the rate of diffusive O$_2$ efflux per unit length.

The diffusive O$_2$ efflux and the O$_2$ concentration must be continuous at the interface between blood and tissue; we can relate these using:

$$u_v(z) = u_b(z) - Kq_v(z)$$

where $u_v(z)$ is the tissue O$_2$ concentration averaged around the circumference of the vessel and $K$ is the intravascular resistance to radial O$_2$ transport.

### 4.2.2 Green’s Function Analysis

The central concept of the Green’s function approach to solving the O$_2$ transport equations is the representation of the blood vessels as a set of discreet O$_2$ sources and the O$_2$ field in the tissue as a superposition of field resulting from the discreet O$_2$ sources.
Furthermore, the tissue is represented as a set of discrete O\textsubscript{2} sinks whose strengths are unknowns to be determined as part of the solution.

Thus the Green’s function, G, for a given domain may be defined as the O\textsubscript{2} concentration at a point \(x\) resulting from a unit point source \(x^*\):

\[
D\nabla^2 G = -\delta(x - x^*)
\]

where \(\delta\) is the three-dimensional delta function. The O\textsubscript{2} concentration can then be described by:

\[
u(x) = \int_{\text{sources}} G(x; x^*)q(x^*)dx^*
\]

where \(q(x)\) represents the distribution of the sources. For tissue nestled within an infinite domain, the solution is:

\[
G = \frac{1}{4\pi D|x - x^*|}
\]

### 4.3 Materials and Methods

#### 4.3.1 Modeling and Simulations

This Green’s function method has previously been applied to simulated oxygen transport in tumors, including a three-dimensional tumor network derived from an
R3230 mammary adenocarcinoma (R3230) grown in a dorsal window chamber and imaged using confocal microscopy (Secomb et al., 1998) (Figure 34). The details of this network are described in Table 4.

This Green’s function analysis of oxygen transport has been implemented for this R3230 tumor network in a FORTRAN program developed by Hsu and Secomb, and is available for use at http://www.physiology.arizona.edu/people/secomb/greens.html. This implementation from the website was used in all these simulations and run using a Lahey FORTRAN 95 compiler. The network geometry for the tumor network as well as

![Image of vascular network configuration](image_url)

**Figure 34: Vascular network configuration from observations in the R3230 rat tumor, used in simulations. Dimensions are indicated in μm.**

the flow rates relative to the input vessels were provided by Drs. Hsu and Secomb, and changes to the input file for boundary conditions were made as described below.
Results from the program were then imported into MATLAB for further analyses and visualization.

Using this same network, physiologically relevant temporal fluctuations in vascular O\textsubscript{2} content (Braun et al., 1992; Sorg et al., 2008), blood flow rate (Braun et al., 1999; Cardenas-Navia et al., 2004; Hill et al., 1996; Pigott et al., 1996), and overall perfusion were simulated to examine their effects on tumor tissue oxygenation. Our group and others have previously demonstrated that the frequency of temporal changes in O\textsubscript{2} delivery to tissue is very slow (Braun et al., 1999; Brurberg et al., 2003; Brurberg et al., 2005; Cardenas-Navia et al., 2004); therefore, a single periodicity of 40 min was chosen for all O\textsubscript{2} delivery simulations, and temporal fluctuations in O\textsubscript{2} delivery were applied discretely, changing every minute. Since O\textsubscript{2} diffuses very quickly in tumor tissue (Secomb et al., 1993) and the volume of tissue in which the simulations are applied is small (Secomb et al., 1998), tissue oxygenation reaches an equilibrium in our network within several seconds. This allows us to use the Green’s function model, which is for steady-state O\textsubscript{2} transport, to solve the O\textsubscript{2} transport equation after each discrete change in O\textsubscript{2} delivery.

O\textsubscript{2} consumption parameters remained constant for all simulations.

4.3.1.1 Fluctuations in Vascular O\textsubscript{2} Content Simulations

Fluctuations in inlet pO\textsubscript{2} for all three inlet vessels were determined using a sinusoidal function with an average pO\textsubscript{2} of 30 mm Hg, with fluctuation magnitudes and.
periodicities of $10 \text{ mm Hg}$ and $40 \text{ min}$, respectively. The blood flow rate entering the tissue region was always $2.8 \times 10^6 \text{ cm}^3/\text{s}$. In one case, pO$_2$ fluctuations at the inlet were synchronized for all three vessels. In another simulation, the pO$_2$ fluctuations at the inlet were shifted $\pm \pi/2$ in two of the vessels with respect to the third vessel, although all vessels maintained an average pO$_2$ of $30 \text{ mm Hg}$ and $40 \text{ min}$ periodicity. The value of $30 \text{ mm Hg}$, with a range of $\pm 10 \text{ mm Hg}$, for inlet vascular pO$_2$ was chosen from the vascular pO$_2$ values from the phosphorescence lifetime imaging data in the same tumor (chapter 3); the range in average vascular pO$_2$ varied from 14-30 mm Hg. A value at the high end, $30 \text{ mm Hg}$, was chosen as the baseline for inlet pO$_2$ since the Green’s function model incorporates longitudinal gradients in vascular pO$_2$; previous simulations using vessel in the network (Kimura et al., 1996). The fluctuation amplitude of $10 \text{ mm Hg}$ was chosen based on temporal fluctuation magnitude from average pO$_2$ from the phosphorescence lifetime imaging experiments and microelectrode perivascular measurements, which were $8 \text{ mm Hg}$ and $7 \text{ mm Hg}$ (Kimura et al., 1996), respectively.

4.3.1.2 Fluctuations in Blood Flow Simulations

The effects of fluctuations in blood flow rate for the inflowing vessels were also examined. In these simulations, the blood flow rate for all the incoming vessels this inlet pO$_2$ value with the same blood flow rate and O$_2$ consumption rate in this microvascular network predicted a decrease of $10 \text{ mm Hg}$ in vascular pO$_2$ in the largest remained constant at $2.8 \times 10^6 \text{ cm}^3/\text{s}$ for the entire $40 \text{ min}$ simulated. The inlet pO$_2$ for all vessels
<table>
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<tr>
<th><strong>Parameter</strong></th>
<th><strong>Units</strong></th>
<th><strong>Description</strong></th>
<th><strong>Value</strong></th>
<th><strong>Source</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>$Q$</td>
<td>cm$^3$ s$^{-1}$</td>
<td>Blood flow rate into microvascular region</td>
<td>$2.8 \times 10^{-6}$</td>
<td>(Secomb et al., 2004)</td>
</tr>
<tr>
<td>$D$</td>
<td>cm$^2$ s$^{-1}$</td>
<td>Diffusion coefficient of O$_2$ in tumor</td>
<td>$25 \times 10^{-6}$</td>
<td>(Secomb et al., 2004)</td>
</tr>
<tr>
<td>$v_{\text{max}}$</td>
<td>cm$^3$ O$_2$/min/100 cm$^3$</td>
<td>Rate of O$_2$ consumption</td>
<td>1.5</td>
<td>(Secomb et al., 2004)</td>
</tr>
<tr>
<td>$k_{\text{met}}$</td>
<td>mm Hg</td>
<td>Michaelis constant for O$_2$ consumption</td>
<td>1.0</td>
<td>(Secomb et al., 2004)</td>
</tr>
<tr>
<td>$u_0$</td>
<td>cm$^3$ O$_2$/mm Hg/cm$^3$</td>
<td>O$_2$ binding capacity for blood</td>
<td>0.2</td>
<td>(Secomb et al., 2004)</td>
</tr>
<tr>
<td>$\alpha_{\text{eff}}$</td>
<td>cm$^3$ O$_2$/cm tissue</td>
<td>Effective solubility of O$_2$ in blood</td>
<td>$3.1 \times 10^{-5}$</td>
<td>(Secomb et al., 2004)</td>
</tr>
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<td>$n$</td>
<td></td>
<td>Hill coefficient for O$_2$ binding to hemoglobin</td>
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<td>(Secomb et al., 2004)</td>
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<tr>
<td>$H_{\text{ctd}}$</td>
<td></td>
<td>Discharge hematocrit</td>
<td>0.40</td>
<td>(Secomb et al., 2004)</td>
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<tr>
<td>$u_{50}$</td>
<td>mm Hg</td>
<td>O$_2$ concentration at which hemoglobin saturation is half-maximal</td>
<td>38</td>
<td>(Secomb et al., 2004)</td>
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<tr>
<td>domain size</td>
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<td></td>
<td>550 x 520 x 230</td>
<td>(Secomb et al., 2004)</td>
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<td>$\mu$m</td>
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<td>30</td>
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<tr>
<td>number of vessel segments</td>
<td></td>
<td></td>
<td>104</td>
<td>(Secomb et al., 2004)</td>
</tr>
<tr>
<td>number of subsegments</td>
<td></td>
<td></td>
<td>517</td>
<td>(Secomb et al., 2004)</td>
</tr>
</tbody>
</table>

was held constant at 30 mm Hg. The vascular network used in these simulations consists of two separate blood flow networks with a total of three inlet vessels. Initially,
blood flow was equally divided between the three inlet vessels. To simulate fluctuations in blood flow rate, this fraction was shifted, increasing to 66.6% and then decreasing to 0.1% in the network with one inlet vessel during the first 20 min (the remaining fraction of flow was equally divided between the other two inlet vessels). After slowing the blood flow rate in this inlet vessel to near zero, the flow direction in this vascular network was reversed. Then the fraction of flow into this network was again increased to 66.6% to examine the effects of reversed flow, then decreased back to 0.1%, reversed back to its original direction, and increased back to 33.3%.

Relative flow rates in individual vessel segments (relative to input vessel) were initially calculated by Secomb et al.; fluctuations in flow rate were implemented by changing the blood flow rate sinusoidally in the inlet nodes and calculating the flow rates of the other vessels in the network. To calculate the new blood flow rates for each segment of the network, mass was balanced at each node using:

\[ Q_1 + Q_2 + Q_3 = J_1(p_1 - p_0) + J_2(p_2 - p_0) + J_3(p_3 - p_0) = 0 \]

where \( Q \) is the flow rate in a segment (shown here for 3 converging segments), \( p \) is the pressure at the node, and \( J \) is the hydraulic conductance, which is calculated from the vessel geometry using:

\[ J = \frac{\pi d^4}{128 \eta L} \]
where \( d \) is the diameter of the vessel segment, \( \eta \) is the Hct, and \( L \) is the length of the vessel segment (\( J \) is constant for each segment as the network geometry does not change in these simulations). Applying these equations to each vessel segment and node in the network yielded a system of linear equations (involving \( J \) and \( p \)) which was solved in MATLAB; the solutions were used to calculate the new flow rates.

### 4.3.1.3 Fluctuation in Overall Perfusion

Fluctuations in the blood flow rate entering the tissue region were determined using a sinusoidal function with a perfusion of \( 2.8 \times 10^6 \text{ cm}^3/\text{s} \), with a fluctuation magnitude and periodicity of \( 1.4 \times 10^6 \text{ cm}^3/\text{s mm Hg} \) and 40 min, respectively. The inlet \( \text{pO}_2 \) for all vessels was held constant at 30 mm Hg.

The choice to vary blood flow by \( \pm 50\% \) was made based on a study measuring relative changes in blood flow using laser Doppler flowmetry in the same tumor, R3230, as the vascular network used in these simulations (Braun et al., 1999). In that study, blood flow was measured at two different sites for nine different tumors for 36-125 min; the dominant fluctuations in blood flow were found to occur for very slow frequencies (0.03 cpm). The magnitude for these slow fluctuations in blood flow for individual traces was found to very greatly, generally from 40-50% from baseline. These relative changes in blood flow are consistent with those seen in clinical and preclinical tumor models (Cardenas-Navia et al., 2004; Hill et al., 1996; Pigott et al., 1996).
The effects of these simulations for perfusion cannot be directly compared with previous simulations in blood oxygenation and blood flow since the amount of blood entering the tissue region is different at each time point, and mass balance is not maintained. Nonetheless, blood perfusion for microregions of the tissue may not be temporally constant, so these simulations may offer some insight into their impact on oxygen delivery to tissue.

4.4 Results

4.4.1 Fluctuations in O\textsubscript{2} Delivery Results in Fluctuations in Tissue Hypoxic Fraction

Temporal fluctuations in inlet pO\textsubscript{2}, blood flow, and overall perfusion all resulted in temporal fluctuations in tumor tissue oxygenation and hypoxic fraction (Figure 35).

For both simulated cases, fluctuations in inlet pO\textsubscript{2} for the vessels had an appreciable impact on tumor tissue pO\textsubscript{2}. For the case in which all inlet pO\textsubscript{2} fluctuations were synchronized, changes in O\textsubscript{2} values in the tissue scaled linearly with temporal changes in inlet pO\textsubscript{2}. The fraction of tissue with pO\textsubscript{2} of less than 3 mm Hg was predicted to vary from ~10-65%.

Staggering the periodicity of the pO\textsubscript{2} fluctuations at the inlet of the three vessels also considerably altered tissue pO\textsubscript{2} values. However, this effect was decreased compared with the synchronized case, and the fraction of tissue less than 3 mm Hg was only predicted to vary from ~25-50%.
Fluctuations in blood flow and flow reversal had a very small effect on tissue pO$_2$. Surprisingly, dropping the flow rate to near-zero did not result in a sharp increase in hypoxic fraction, perhaps because there was an increased flow rate in the nearby vessel network. The change in flow direction also had a minimal impact on hypoxic fraction; the hypoxic fraction of tissue less than 3 mm Hg varied from ~32-38%.

Altering overall perfusion to the tissue by 50% had only a modest impact on tissue pO$_2$. Simulations did predict that decreases in overall blood flow rate had a slightly larger impact on tissue pO$_2$ than increases; overall the hypoxic fraction of tissue less than 3 mm Hg varied from ~30-40%.

Analysis of the distribution of pO$_2$ values at different time points within a simulation was examined using the Bartlett multiple sample test for equal variances. No significant differences in variance of pO$_2$ distributions was found for any of the simulations (p>0.05).

4.4.2 Unsynchronized Temporal Fluctuations Result in Spatio-temporal Variability in Tissue pO$_2$

The Green’s model simulations show that spatial heterogeneity is predicted to result in all simulations from the uneven distribution of the vasculature through the tissue. Tumor tissue which is near a vessel (<100 μm) have a visible gradient in pO$_2$ with distance from the vessel. Surprisingly, blood flow in opposite directions, which resulted in longitudinal vascular gradients in opposite directions, did not result in obvious
longitudinal tissue gradients in opposite directions under any of the conditions simulated here.

When examining temporal fluctuations along a single plane, out-of-plane vessels resulted in “intermittent” spatial heterogeneity some regions in pO$_2$ when the O$_2$ delivery in those vessels approached their maximum and minimum values.

Unsynchronized temporal fluctuations in vascular pO$_2$, even with the same average vascular pO$_2$ and same periodicity as the synchronized case, resulted in decreased hypoxic fraction and increased spatial heterogeneity at any single time point.

4.5 Discussion

The results from this analysis suggest that there are several potential mechanisms by which temporal fluctuations in O$_2$ delivery to a tumor microenvironment can cause temporal fluctuations in the surrounding tumor tissue. Using the Green’s function model has allowed us to independently examine the impact of each of these factors on fluctuations in tumor oxygenation.

Although results from the model suggest that, of the variables examined, changes in vascular oxygenation have the largest impact on tissue pO$_2$ values, it is unlikely that any one variable in O$_2$ delivery is changing independently in vivo. In fact, one would expect red cell flux and hemoglobin saturation to be influenced by blood flow rate and overall perfusion.
Previously, Kiani et al have used a network blood flow model to demonstrate that nonuniform axial distribution of red blood cells and disproportionate cell partitioning at bifurcations in rat mesentery networks can cause high frequency (3-6 cpm) fluctuations in blood velocity and Hct at in some vessels (Kiani et al., 1994). *In vivo* experiments in normal tissue have not reflected this prediction; studies measuring pO2 over time in rat and mouse muscle have shown that those tissues do not show significant temporal fluctuations in tissue pO2 occurring at these high frequencies (Braun et al., 1999; Brurberg et al., 2003). This discrepancy may be due to the regions over which the experimental data are taken; the predictions are for single microvessels, whereas microelectrode and oxyLite pO2 measurements may be influenced by several nearby microvessels (Braun et al., 2001).

This complex relationship between O2 delivery parameters and tumor tissue pO2 is seen in a study by Helmlinger et al. (Helmlinger et al., 1997), who measured blood flow, intravascular pO2, and perivascular pO2 in the tumor microcirculation in the dorsal window chamber. Blood flow measurements were taken before and after perivascular pO2 measurements. No correlation was found between perivascular pO2 and blood flow in tumor xenografts grown in window chambers. Interestingly, no correlation was seen even when comparing intravascular pO2 and flow rate (Helmlinger et al., 1997), emphasizing the importance of hemoglobin saturation levels in O2 delivery to tumors. Unfortunately, no experiments directly relating temporal changes in hemoglobin
saturation to changes in tumor tissue pO$_2$ have been conducted; future experiments examining these variables could offer further insight into the physiological causes of spatio-temporal fluctuations in pO$_2$.

Examinations of small networks of vessels have shown that both our synchronized and unsynchronized simulations of vascular pO$_2$ fluctuations are occurring *in vivo* (Lanzen et al., 2006). However, in the R3230 network simulated here, unsynchronized vascular fluctuations resulted in hypoxic fraction in the tissue which were more consistent with published results on R3230 tissue oxygenation (Braun et al., 2001) than the synchronized fluctuations at most time points. This suggests that vascular pO$_2$ fluctuations are not synchronized over entire tumors, consistent with the spatial heterogeneity in O$_2$ delivery previously seen in this tumor (Braun et al., 1999; Lanzen et al., 2006).

The transport simulations in three-dimensions also highlighted the influence of out-of-plane vasculature; temporal fluctuations in out-of-plane vasculature caused visible fluctuations on nearby tissue pO$_2$. This cautions against immunohistochemical analyses of hypoxia markers with reference to a vessel marker when only a one-dimensional tissue slice is examined.

Fluctuations in tumor oxygenation were implemented every minute based on the assumption for quasi-steady state; this timescale was determined from calculations examining the time it takes for an O$_2$ molecule to diffuse from one corner of the tissue
Figure 35: Effects of varying parameters of \( \text{O}_2 \) delivery on tumor tissue \( \text{pO}_2 \) distribution and hypoxic fraction. A) and B) are results from synchronized inlet \( \text{pO}_2 \) fluctuations; C) and D) are results from unsynchronized inlet \( \text{pO}_2 \) fluctuations; E) and F) are results from blood flow rate fluctuations and G) and H) are results from overall perfusion fluctuations. In A), C), E), and G) each line represents results at a single time point.
region to the opposite corner. Using an $O_2$ solubility value measured \textit{in vivo} in tumors of $1.79 \times 10^{-2}$ cm$^3$ O$_2$/mL/atm (Grote et al., 1977), the diffusion time was found to be 58.5 s.

However, $O_2$ solubility in tumor tissue may be altered with water content (Grote et al., 1977), cellular make-up (Grote et al., 1977), and perhaps by therapeutics; changes in solubility due to these factors would linearly affect the diffusion time. An upper bound of 2-fold in the diffusion time is a best estimate, which would assume that the solubility is the same as that for water (Mahler et al., 1985). In this case, the timescale for the quasi-steady state condition should be 2 min. This change in timescale would have no real impact on either the qualitative and quantitative results here since the quasi-steady state time step would be 2 min, which can still resolve the $O_2$ fluctuations of interest (fluctuations with a periodicity of 40 min).

The results from the model are limited to only the physiological parameters which the model incorporates; factors which might also impact spatial and temporal heterogeneity in tumor tissue oxygenation, such as arteriolar vasomotion (Dewhirst et al., 1996a; Dewhirst et al., 1996b; Goldman and Popel, 2001; Hundley et al., 1988; Intaglietta et al., 1977), changes in red cell viscosity (Kavanagh et al., 1993; Pries et al., 1994), changes in blood flow resistance (Sevick and Jain, 1989), vascular remodeling (Ji et al., 2006; Patan et al., 1996), and changes $O_2$ consumption rate (Erickson et al., 2003; Snyder et al., 2001), were not examined. Nonetheless, we feel that the results from the model offer some insight into the dominant mechanisms for fluctuations in $O_2$ delivery.
5. Conclusions and Future Directions

The concept of hypoxia being a source for radioresistance goes back to the seminal work of Thomlinson and Gray (Thomlinson and Gray, 1955). In this study the investigators carefully measured the width of the viable rim of biopsies taken from patients with lung tumors and determined that the maximum width tended to be 180-200μm, regardless of the size of the tumor nodule. They concluded that the viable rim width was consistent with the maximum diffusion distance of oxygen in tumor tissue. Thomlinson and Gray hypothesized that cells that reside near the necrotic edge would be hypoxic and therefore radioresistant. Although this observation spurred a number of clinical trials, it was nearly 30 years before it was definitively demonstrated that hypoxia was a cause for radioresistance in human tumors (Gatenby et al., 1988).

Vaupel was among the first investigators to show that human tumors contained hypoxic regions and that the presence of hypoxia was an important prognostic factor in cervix cancer (Hockel et al., 1993). Subsequently, similar reports were published involving a number of human tumors, including head and neck, sarcomas, and prostate cancer (Fyles et al., 2002; Movsas et al., 2002; Nordsmark et al., 2005). The work of Thomlinson and Gray alluded to and was based-upon the assumption of a steady-state gradient of oxygen from a source vessel, a concept that was further promulgated by the advent of hypoxia marker drugs, which typically exhibit increased binding in tumor cells as a function of their distance from the nearest microvessel. This feature of tumor
hypoxia has been termed “chronic,” implying that cells that located far from a microvessel reside in a constant state of hypoxia.

The notion that tumor cells might be transiently hypoxic as a result of instabilities in tumor blood flow was introduced in the late 1970s in three seminal papers. In one study Intaglia et al. observed arteriolar vasomotion in feeding vessels of tumors grown in window chambers (Intaglia et al., 1977). They reported the sinusoidal behavior of red blood cell movement in vessels, presumably caused by this vasomotor activity. Yamaura and Matsuzawa irradiated tumors growing in window chambers and carefully monitored the location of tumor regrowth (Yamaura and Matsuzawa, 1979). They observed that the tumors almost always regrew at the periphery of the tumors and suggested two potential mechanisms in explanation of this localized radioresistance. In one proposal cells in the tumor periphery are assumed to be better oxygenated, resulting in a higher growth fraction and a higher fraction of relatively resistant cells in S-phase at the time of irradiation. Yamaura and Matsuzawa also proposed that transient hypoxia could be responsible for radioresistance, as vascular stasis had occasionally been observed at the tumor periphery. Brown demonstrated the re-emergence of radiobiologically hypoxic cells 24 hr after administration of a hypoxic cytotoxin, Misonidazole, in the EMT6 tumor (Brown, 1979). This study was the first to demonstrate that transient hypoxia could be radiobiologically important.
Although no studies directly measuring fluctuating pO₂ have been done in human tumors, fluctuations in red cell flux have been measured in clinically in humans and were shown to occur with a median periodicity of 13 min (range 4-44 min) (Pigott et al., 1996). Measurements of fluctuating blood flow in different areas within these human tumors also revealed spatial heterogeneity in the fluctuations; spatial heterogeneity in temporal oxygen fluctuations has important implications for optimization of traditional therapies such as radiation. If areas of fluctuating hypoxia were able to be visualized, higher doses of radiation could be delivered to hypoxic areas at a time during which pO₂ values in that area were at the peak of their fluctuations. Further studies examining the spatiotemporal periodicity of human tumor oxygenation, over a longer timescale (days) relevant to treatment scheduling, need to be conducted.

This work has shown that fluctuations in vascular pO₂ are constantly occurring throughout tumors. Nearly all of the current understanding of tumors and tumor cell adaptation to hypoxia contains the underlying assumption of cellular exposure to chronic hypoxia. However, numerous publications have shown that intermittent hypoxia or hypoxia/reoxygenation events alter the behavior of tumor cells. Graeber et al. demonstrated that multiple rounds of hypoxia/reoxygenation selected for apoptosis-resistant tumor cells, with each hypoxic treatment increasing the percentage of p53-deficient cells by 2.4% (Graeber et al., 1996). Another study more broadly demonstrated the potential of intermittent hypoxia to act as a physiological selective agent for tumor
cell mutations: Reynolds et al. showed the mutation frequency of tumor cells increased with each cycle of hypoxia exposure (Reynolds et al., 1996). Whole-body exposure of tumor-bearing mice to intermittent hypoxia has been shown to result in increased spontaneous metastases in lungs (Cairns et al., 2001) and lymph nodes (Cairns and Hill, 2004). Recently a study has shown that fluctuating hypoxia promotes different phenotypes in endothelial and tumor cells than chronic hypoxia, further promoting cells to participate in tumor progression and treatment resistance (Dewhirst, 2007; Martinive et al., 2006).

The literature also increasingly suggests that the molecular effects of hypoxia will have to be revisited. Studies looking at oxidative stress have shown that cycles of intermittent hypoxia increased HIF-1α protein expression and transactivation function in tumor cells through molecular mechanisms which are distinct from those of chronic hypoxia (Yuan et al., 2005). Activation of HIF-1 was shown to increase with increasing number of hypoxia/reoxygenation cycles (Martinive et al., 2006; Yuan et al., 2005). A related study has also shown that intermittent hypoxia also increases c-fos mRNA in tumor and endothelial cells and increased activation of AP-1, both of which were abolished through the use of an SOD mimetic (Yuan et al., 2004). These studies strongly suggest that intermittent hypoxia is more potent in activating gene expression than chronic hypoxia. Additionally, one study has shown that more than 200 genes are selectively affected by intermittent, but not chronic, hypoxia (Prabhakar, 2001); this
presents a multitude of potential therapeutic targets which are currently overlooked due to a misunderstanding of the prevalence and importance of fluctuating oxygenation in tumors. Given the probable widespread presence of fluctuating hypoxia in human tumors, many more studies need to be done under this new paradigm of tumor physiology.

Mathematical modeling of biological processes offers an elegant alternative to direct experimentation to increase the understanding of their underlying physiology. Since the introduction of the Krogh cylinder (Krogh, 1919), mathematical modeling of O$_2$ transport has often led experimentation in advancing the understanding of the microcirculation and microenvironment. Although imaging tumor oxygenation and hypoxia is growing increasingly sophisticated, no current techniques have both the temporal and spatial resolution to directly measure fluctuations tumor tissue pO$_2$ (Manzoor et al., 2008); the development of a fully transient non-linear O$_2$ transport model could potentially further increase the understanding of the underlying mechanisms of fluctuating hypoxia.

Ideally, this mathematical model would take advantage of the benefits of increased computational power and used a finite element based approach. Finite element analysis is ideal for modeling the microenvironment, particularly tumor tissues, as it is well-suited for irregular geometries and the mesh size at which solutions are found can be adapted to better resolve steep gradients near vessels. This flexibility
might also allow for the ability to solve problems for larger spatial regions, as mesh size can be increased in regions of little change in tissue oxygenation. Additional benefits of finite element analysis include the ability to incorporate transient or steady spatially localized changes in O2 consumption, simulating cell cycle effects of contained regions of tumor cells.

Creating a mathematical model of the vascular networks, similar to the one created by Pries et al. (Pries et al., 1990), in tumors and coupling it with a mathematical model of oxygen transport in tumor tissue would allow the finite element model to truly reflect transient changes in blood flow in its boundary conditions. In particular, scale of the effects of discrete changes in vascular geometry on tumor tissue pO2 could be explored.

Vascular remodeling has been shown to occur in tumors over the same timescales that fluctuant hypoxia occurs, periods of 10-30 minutes (Patan et al., 1996). Even small changes in vessel geometry have an impact on blood flow and the overall distribution of red blood cells because of redistribution of blood pressure in each vessel segment. Although intussusception has been shown in tumor vasculature, previous studies have looked at 2-D images at several time points (Patan et al., 1996). Repeatedly capturing Confocal images of tumor vasculature (from a tumor grown in a dorsal window chamber), along with other rheological parameters such as red cell flux, red cell velocity, and hemoglobin saturation, taken either several times per min for one hour or
once every 30 to 60 min for several hours would offer excellent inputs to test the effects of angioadaptation on O₂ transport in tumors. Perturbing the microenvironment by the administration of agents which cause vascular shutdown, chemotherapeutic agents, hyperoxic or hypoxic gas, or antiangiogenic agents and model pre- and post-interventions could also offer significant insight into the dominant mechanism of action of these agents and their effects on fluctuating hypoxia.
Appendix 1: List of Abbreviations

pO$_2$: Oxygen concentration / partial pressure of oxygen

TPZ: Tirapazamine

1-D: one-dimension / one-dimensional

2-D: two-dimensions / two-dimensional

3-D: three-dimensions / three-dimensional

R3230: rat mammary adenocarcinoma R3230

FSA: rat fibrosarcoma

9L: rat 9L glioma

Pimo: pimonidazole

PLI: Phosphorescence Lifetime Imaging

DFT: Discrete Fourier Transform

SF: Surviving Fraction

OER: Oxygen Enhancement Ratio

RT: radiation

BF: Blood Flow

t$_{1/2}$: half-life

Hct$_d$: discharge hematocrit
Appendix 2: Oxygen Gradients in Tissue Culture

Introduction

It is generally considered that cultured monolayer cells are not exposed to the ambient oxygen environment as consequence of cellular respiration (Boag, 1969; Stevens, 1965), but the impact that this has on local oxygen levels at the cellular layer has not been evaluated. Variability in O₂ concentration has been shown to alter cellular growth rate, protein production, and gene expression, particularly under hypoxic conditions, suggesting a need for precise knowledge of pericellular pO₂. In this study pericellular pO₂ was measured and shown to be highly variable within and between four different cancer cell lines grown to plateau phase in monolayers under the same ambient O₂ conditions. These findings have important consequences for studies performed using cross-cell comparisons and highlight an important variable that is not considered in the interpretation of cell-based studies performed in vitro.

Materials and Methods

O₂ Gradient Measurements

4T1 mouse breast cancer, HCT 116 human colon carcinoma, R3230 rat mammary adenocarcinoma, and 786-0 human renal carcinoma cells were cultured in T25 flasks in an incubator at 37°C with 5% CO₂ and 95% air ambient gas mixture. 4T1, R3230, and
786-0 were cultured in DMEM+10%FBS+1% antibiotic/antimycotic and HCT 116 cells were cultured in RPMI+10%FBS+1% antibiotic/antimycotic.

Recessed-tip microelectrodes were made using a previously published technique(Linsenmeier and Yancey, 1987; Schneiderman and Goldstick, 1978). The electrodes had tip diameters of 9.2 (±0.8) μm (11 electrodes, mean±SEM) and recess lengths of 24.7 (±2.1) μm. Microelectrodes were polarized at −0.7 V using a commercial polarizing box and a picoammeter unit (Chemical microsensor no. 1201, Diamond General, Ann Arbor, MI). Electrodes were calibrated before and after experiments in a 37° C saline-filled tonometer alternately bubbled with 0%, 2.5%, 5%, or 15% O₂ (balance nitrogen). The average sensitivity of the electrodes was 1.20(±0.13) mm Hg/pA. Use of

Figure 36: Schematic of experimental setup for microelectrode measurements of pO₂ in T25 cell culture flasks. Not drawn to scale.
these microelectrodes minimizes perturbation of the oxygen field and its measurements reflect a region of only a few cells (Schneiderman and Goldstick, 1978).

Once cells were confluent, flasks were almost entirely submerged in a water bath at 37°C to maintain a uniform temperature in the flask medium. A small 1 cm² hole was made in the top of each flask for microelectrode insertion; a Ag/AgCl reference electrode was placed into the media. The entire water bath was covered tightly with polyvinylidene chloride (PVDC) film to help maintain temperature and humidity for the cells. Gas containing 5% CO₂ and 95% air was set to flow over the flask at a rate of 1 L/min, maintaining positive pressure within the set-up. A dissection microscope was placed to permit visualization of the cells through the hole in the flask (Figure 36).

After 4 hours, when pO₂ in the flask medium reached steady-state value, a 1 cm² hole was made in the PVDC film directly above the hole previously made in the flask. An O₂ microelectrode was introduced through the hole and positioned at the air-media interface. The electrode was then moved through the media in 5-100 μm steps until its tip was in the same focal plane as the cell layer, as viewed through the dissection scope (in most cases, the electrode tip was seen to bend slightly when the flask bottom was reached). A 10-30 second measurement was recorded at each step. Cells grown in two parallel T25 flasks were trypsinized and counted using a hemocytometer after the pO₂ measurements were finished. This experiment was repeated for all four cell lines, and for a control experiment in a flask containing no cells.
Figure 37: pO₂ vs. depth examples (0 is bottom of cell layer) for A) Hct116; B) Hct116; C) 4T1; D) 786-0; E) R3230; F) No cells; and G) NaCN poisoned 4T1. Open circles are measured data points, with O₂ consumption fits shown with dotted lines (k₀ and k₁ fits overlap).
In at least one experiment for each cell line 100 μL NaCN was added to yield a concentration of 10^-3 M. After 60-90 min, the electrode was stepped out of the medium in 5-100 μm intervals, with pO_2 measurements recorded at each step.

**Mathematical Model**

The one-dimension equation of change for O_2 concentration, C_{O_2}, at steady-state with diffusion and consumption is governed by Fick’s 2nd Law, which can be written

\[ D_{O_2} \frac{\partial^2 C_{O_2}}{\partial x^2} = f(C_{O_2}) \]

where \( f(C_{O_2}) \) is the O_2 consumption. The O_2 consumption in the cell layer was assumed either to be constant, \( f(C_{O_2}) = k_0 \), or to be directly proportional to local oxygen concentration, \( f(C_{O_2}) = k_1 C_{O_2} \). In the medium no O_2 consumption is assumed, i.e., \( f(C_{O_2}) = 0 \). The depth of the medium was measured for each experiment and the cell layer was assumed to be 20 μm deep. No net flux of oxygen was assumed along the length or width of the flask, reducing the problem to one dimension (depth). Additionally, no O_2 flux was assumed at the bottom of the flask,(Chapman et al., 1970) and the measured O_2 concentration at the air-media interface was held constant according to the experimental value. The fluxes out of the medium and into the cell layer were assumed to be equal at the cell-medium interface.
Values for $k_0$ and $k_1$ were determined by finding the smallest least squares residual between measured and predicted values for O$_2$ concentration in the media. Equation 1 was solved using MATLAB 6.5 (The Mathworks, Inc., Natick, MA).

To simulate oxygen consumption under hypoxic conditions, Michaelis-Menten kinetics were assumed to apply, i.e., recalling equation 1, $f(C_{O_2}) = kC_{O_2}/(k_{met}+C_{O_2})$. The value for $k_{met}=990\mu$M was taken from the literature,(Hicks et al., 2003) while $k$ was assumed to be equal to $k_0$, as determined from experiments at elevated pO$_2$ and reported in Table 1. Again, no net flux of oxygen was assumed along the length or width of the flask, reducing the problem to one dimension, and no flux was assumed at the bottom of the flask. Equation 1 was solved using MATLAB 6.5.

**Results**

Large variability in pericellular pO$_2$ values existed within and between tumor cell types, with individual experimental values ranging from 8 to 100 mm Hg; controlling ambient pO$_2$ did not predictably control pericellular pO$_2$. Both HCT116 and 4T1 cell lines had at least one flask with pericellular pO$_2$ less than 10 mm Hg, even with an ambient gas mixture containing 21% O$_2$ (Figure 37). The pO$_2$ at the cell layer was significantly lower ($p<10^{-6}$, t-test) than pO$_2$ at the media surface for all cell lines measured. O$_2$ profiles were linear in the media above the cell monolayer for all cell lines, consistent with the steady-state conditions during the pO$_2$ measurements. The average cell layer pO$_2$ values for three of the lines were not significantly different from each
Table 5: Pericellular pO₂ data with O₂ consumption and fitted O₂ rate constants. Data are shown as mean(±SEM); a \( p<10^{-6} \) compared to medium surface pO₂ within each cell line; b \( p<10^{-4} \) compared to cell layer pO₂ of no cells or cells+NaCN.

<table>
<thead>
<tr>
<th></th>
<th>Hct 116 (n=12)</th>
<th>4T1 (n=10)</th>
<th>786-0 (n=2)</th>
<th>R3230 Ac (n=4)</th>
<th>No cells (n=4)</th>
<th>Cells + NaCN (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium Layer Depth (μm)</td>
<td>1719 (±124)</td>
<td>1614 (±67)</td>
<td>1460 (-)</td>
<td>1443 (±186)</td>
<td>1889 (±48)</td>
<td>1568 (±118)</td>
</tr>
<tr>
<td>Medium Surface pO₂ (mm Hg)</td>
<td>138 (±7)</td>
<td>121 (±5)</td>
<td>127 (-)</td>
<td>130 (±8)</td>
<td>140 (±3)</td>
<td>121 (±6)</td>
</tr>
<tr>
<td>Cell layer pO₂ (mm Hg)</td>
<td>51(^{a,b}) (±9)</td>
<td>53(^{a,b}) (±8)</td>
<td>87 (-)</td>
<td>89(^{a,b}) (±7)</td>
<td>133 (±1)</td>
<td>112 (±3)</td>
</tr>
<tr>
<td>O₂ consumption rate (nmol O₂/h/10(^6)cell)</td>
<td>223 (±34)</td>
<td>212 (±25)</td>
<td>172 (-)</td>
<td>138 (±48)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rate Constant (</td>
<td>k_0</td>
<td>) (nmol O₂/L-s/10(^6)cell)</td>
<td>1570 (±241)</td>
<td>1480 (±175)</td>
<td>1210 (-)</td>
<td>981 (±340)</td>
</tr>
<tr>
<td>Rate Constant (</td>
<td>k_1</td>
<td>) (1/s/10(^6)cell)</td>
<td>62 (±21)</td>
<td>55 (±26)</td>
<td>11 (-)</td>
<td>9 (±4)</td>
</tr>
</tbody>
</table>

Other (\( p=0.06, \) ANOVA); only two experiments were done with the 786-0 cells, so statistical comparison could not be made for this cell line (Table 5).

O₂ profiles in medium of flasks without cells showed no gradient through the medium layer, and pO₂ at the flask bottom was significantly greater than flasks.
containing cells (p<10^4, ANOVA). No significant O_2 gradient was measured in flasks containing cells after addition of NaCN; pericellular pO_2 of these flasks was also found to be significantly greater than pericellular pO_2 of flasks with cells not treated with NaCN (p<10^4, ANOVA).

As described in the methods, constant and linear O_2 consumption terms were fitted to the microelectrode O_2 data. O_2 consumption rates were found to be consistent and comparable with previously measured O_2 consumption rates for these cell lines in vitro (Metzen et al., 1995; Snyder et al., 2001; Whillans and Rauth, 1980) (Table 5).

Figure 38: Pericellular pO_2 vs. change in medium depth from an initial depth of 1750 μm for Hct116, 4T1, 786-0, and R3230. In panel A) the ambient environment contains 21% O_2; in panel B) the ambient environment contains 7% O_2 for Hct116 cells; 5.5% O_2 for 4T1 cells; 4% O_2 for 786-0 cells; and 4% O_2 for R3230 cells.

The effects of a 10-500 μm change in media depth on pericellular pO_2 were simulated using a baseline medium depth of 1750 μm and O_2 consumption rates from
Table 5. At an ambient O$_2$ percentage of 21%, medium depth changes of 10 µm altered pericellular pO$_2$ ~1 mm Hg; however, medium depth changes of 500 µm were predicted to alter pericellular pO$_2$ up to 20 mm Hg (Figure 38). Precise control of medium volume may be particularly important in small cell-growth containers, such as 96-well plates, since small absolute errors in medium volume can have a large impact on pericellular pO$_2$.

Due to the hole created in the PVDC film required for microelectrode access to the medium, a stable hypoxic (<10 mm Hg) environment could not be maintained in the experimental setup, preventing measurement of pericellular O$_2$ concentration at low ambient pO$_2$. To simulate oxygen consumption under hypoxic conditions, Michaelis-Menten kinetics were assumed to apply. Michaelis-Menten kinetics simulations predicted pO$_2$ values <0.5 mm Hg at the cell level when medium depth was 1750 mm with 2.5% oxygen in the air above the medium (Fig. 21). Variations in media depth were predicted to have little influence on pericellular pO$_2$ when cells are grown under hypoxic conditions (Figure 39).
Figure 39: A) Prediction of relationship between pericellular pO$_2$ vs. percent O$_2$ in gas above media for Hct116, 4T1, 786-0, and R3230 cells. B) Prediction of relationship between pericellular pO$_2$ vs. percent O$_2$ in gas above media for each individual Hct116 experiment. Each line represents a single experiment.

Discussion

Oxygen availability affects a range of cellular processes and regulation of ambient O$_2$ concentrations to which cells grown in monolayer culture are exposed. To achieve the same O$_2$ concentration at the cell layer for different cell lines or within a cell line, the ambient gas mixture would have to be determined from independent measurements of oxygen consumption rates for each experiment. Figure 39A shows that the same ambient O$_2$ concentration of 10% O$_2$ is predicted to result in hypoxic conditions at the cell layer for 4T1 and Hct116 but not for 786-0 or R3230. Variability within a cell line is shown in Figure 38B, where only half of the Hct116 cells are exposed to hypoxic conditions with an ambient O$_2$ gas concentration of 10%. Maintaining a uniform pericellular pO$_2$ across experiments in cell culture is further complicated in dividing cell
cultures, which are known to have higher O$_2$ consumption rates than quiescent cells (Freyer, 1994) and modulate their total O$_2$ consumption from day to day (Pettersen et al., 2005).

The use of an O$_2$-permeable flask was also simulated. In this case, the no flux condition at the bottom of the flask was replaced with a constant O$_2$ concentration equal to that of the ambient O$_2$ percentage. Under both hypoxic (2.5% O$_2$) and normoxic (21% O$_2$) ambient conditions, pO$_2$ was predicted to decrease less than 1 mm Hg in the cell layer, and oxygenation in the entire flask was equal to the ambient oxygen concentration.

Culture dishes made of gas-permeable materials as a means of regulating pericellular pO$_2$ should be considered for more precise control of pO$_2$ in monolayer culture, as suggested by Wolff et al. (Wolff et al., 1993). Typical polystyrene cell-culture dishes, like the ones used in this study, have very low O$_2$ diffusivity, more than 1000-fold lower than that of water (Chapman et al., 1970). This results in virtually no O$_2$ flux through the surface of the flask on which the cells are growing. Some investigators have employed glass containers and vigorous stirring of the media layer above the cells to homogenize O$_2$ concentration in vitro. However, vigorous stirring may result in high shear stress on cell membranes, which can alter cell morphology and result in up-regulation of pathways such as ROCK, Ras, and VEGF (Chiarotto and Hill, 1999; Dong et al., 2002; Lawler et al., 2006; Slattery et al., 2005).
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Biography

Laura Isabel Cárdenas-Navia was born in Washington, D.C. She received a Bachelor of Science in Mechanical Engineering from Yale University in May 2000.

Honors

2008: Duke Comprehensive Cancer Center Annual Meeting Outstanding Poster Award
2005-present: Department of Defense Breast Cancer Predoctoral Trainee
2005, 2006: Junior Investigator Award for Tumor Microenvironment Workshop
2003, 2006, 2007: Scholar-in-Training Travel Award from Radiation Research Society
2002-2004: National Institutes of Health Cell and Biosurface Engineering Fellow

Professional Societies

American Society of Mechanical Engineers
Biomedical Engineering Society
Radiation Research Society
Sigma Xi

Publications


