Non-protein sulphydryl expression and relationship to hypoxic microenvironments in cervical carcinomas

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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The non-protein sulfhydryl (NPSH) glutathione has been associated with increased tumor resistance to therapy by mechanisms that include conjugation and excretion of cytostatic agents, direct and indirect scavenging of reactive oxygen species and maintenance of the intracellular redox state. Tumor hypoxia, caused by aberrant structure and function of the tumor vasculature, is also associated with therapy-resistant and biologically aggressive malignant disease. Oxidative stress, commonly found in regions of intermittent hypoxia, has been implicated in regulation of glutathione metabolism, thus linking increased NPSH levels to tumor hypoxia. Therefore, the objective of the work presented was to characterize NPSH expression and the relationship to tumor hypoxia in cervical carcinoma using multiparameter fluorescence microscopy and advanced image analysis techniques.

The expression of NPSH glutathione and cysteine was studied in frozen sections from cervical carcinomas using a highly sensitive HPLC assay and semi-quantitative fluorescence microscopy. The major findings of this study were (i) the presence of milimolar cysteine concentrations in some tumors, (ii) lack of association of glutathione and cysteine concentrations in individual tumors and (iii) intertumoral heterogeneity in glutathione and cysteine levels was higher than the intratumoral heterogeneity.
The spatial relationship between tumor hypoxia and NPSH levels was assessed in cervical carcinoma xenografts using multiparameter fluorescence microscopy and image analysis. Higher NPSH levels were found in hypoxic regions of ME180 and SiHa xenografts, consistent with our previous findings. Treatment with buthionine sulfoximine, a specific inhibitor of γ-glutamylcysteine synthetase, resulted in depletion of NPSH levels preferentially in hypoxic regions. This finding suggests that overexpression of γ-glutamylcysteine synthetase is the likely mechanism responsible for elevated NPSH levels in hypoxic regions of ME180 and SiHa xenografts.

In the following study, image analysis methods and algorithms were developed for spatial characterization of tumor hypoxia. Distances from blood vessels were mapped and used for classification of tumor hypoxia as intermittent and chronic. NPSH levels were significantly higher in areas of intermittent than in areas of chronic hypoxia or in better oxygenated tumor regions. This finding is consistent with ischemia-reperfusion and oxidative stress in regions of intermittent hypoxia leading to overexpression of γ-glutamylcysteine synthetase and elevation of NPSH levels.
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CHAPTER 1: Introduction

Since its discovery in the 1920s, the non-protein sulphydryl glutathione has been extensively studied for its many roles in cellular metabolic processes under physiological and pathophysiological conditions. The finding of an association between increased levels of glutathione and the therapeutic resistance of solid tumors has triggered a number of studies aimed at understanding its role in the development of resistance to chemo- and radiotherapy. Protective mechanisms mediated by reversible oxidation of glutathione include enzymatic and non-enzymatic conjugation of reactive electrophiles and direct or enzyme-mediated reductive detoxification of reactive oxygen species.

Recent work has identified the pivotal role of the cellular redox status in regulation of vital cellular functions, e.g., DNA transcription, cell cycle regulation and propensity to apoptosis. In concert with the thioredoxin and glutaredoxin pathways, glutathione plays a central role in maintenance of the intracellular redox status. Therefore, glutathione is directly involved in regulation of cellular responses to conditions imposed by the tumor microenvironment and contributes to the development of specific tumor phenotypes.

Our laboratory has recently reported on the spatial colocalization of non-protein sulphydryls (NPSH) and hypoxia in cervical carcinoma xenografts (Moreno-Merlo et al., 1999). This observation is of potential clinical significance, since both factors have been independently associated with increased therapeutic resistance in the clinical setting. Previous reports on NPSH levels in solid tumors are conflicting and have not addressed mechanisms that underlie the observed heterogeneity in tumor NPSH content; studies in experimental and patient tumors indicate the presence of intra- and intertumoral
heterogeneity of tumor oxygen levels. The overall objective of the work presented in this thesis was to characterize NPSH expression in cervical carcinomas and to relate their levels to tumor hypoxia. In the first data chapter, the levels and distribution of NPSH were determined in multiple biopsies from cervical carcinoma patients. The relationship between tumor hypoxia and NPSH was investigated in cervical carcinoma xenografts. The study presented in the following chapter attempted to identify potential mechanisms responsible for the previously observed elevation of NPSH levels in hypoxic regions of ME180 and SiHa tumors. The γ-glutamyl analogue buthionine sulfoximine was used to characterize the inhibition of glutathione de novo synthesis in hypoxic and non-hypoxic tumor regions. In the study presented in Chapter 4, tumor hypoxia was classified as intermittent and chronic, based on the distance from blood vessels and tissue perfusion, and NPSH levels were determined in intermittently and chronically hypoxic regions. Therefore, analytical approaches and tools were developed that allow for spatial analysis and colocalization studies in images acquired by multiple parameter fluorescence microscopy.
1.1 Glutathione in solid tumors

1.1.1 Glutathione synthesis

Glutathione (L-γ-glutamyl-L-cysteinylglycine) is synthesized intracellularly, by the sequential action of γ-glutamylcysteine synthetase (γ-GCS) [1] and glutathione synthetase [2] (Meister and Anderson, 1983).

\[
\begin{align*}
[1] & \quad \text{L-Glutamate} + \text{L-Cysteine} + \text{MgATP} \\
& \quad \rightarrow \quad \text{L-γ-Glutamyl-L-cysteine} + \text{MgADP} + \text{Pi}
\end{align*}
\]

\[
\begin{align*}
[2] & \quad \text{L-γ-Glutamyl-L-cysteine} + \text{Glycine} + \text{MgATP} \\
& \quad \rightarrow \quad \text{Glutathione} + \text{MgADP} + \text{Pi}
\end{align*}
\]

γ-GCS, the rate limiting enzyme in glutathione de novo synthesis, is a heterodimer in mammalian cells. It is comprised of a heavy subunit (γ-GCS_H) that binds the substrate and is catalytically active, and a light subunit (γ-GCS_L) that modulates the binding affinity of the heavy subunit. The subunits are linked into the dimer via reversible disulfide bonds that are reduced in response to increased intracellular glutathione concentration (Misra and Griffith, 1998; Huang et al., 1993). This finding exemplifies the importance of disulfide bond formation in regulation of protein function; the protein is switched ‘on’ under conditions of oxidative stress (Aslund and Beckwith, 1999), and is turned ‘off’ at physiologic redox equilibrium. Mammalian glutathione synthetase is a homodimer (M_r ~52,000) (Oppenheimer et al., 1979); dimer stability is apparently
maintained via noncovalent bonds, since no disulfide bonds between the subunits have been detected.

Net loss of glutathione measured as reducing equivalents occurs as (i) export from cells and (ii) oxidation to the disulfide form GSSG. Glutathione disulfide is reduced back to glutathione by the action of GSSG reductase, thus maintaining the glutathione/GSSG ratio > 100 (Akerboom et al., 1982). Under physiologic conditions (i.e., adequate energy metabolism), redox cycling between GSSG and glutathione usually does not have a major impact on cellular glutathione levels. De novo glutathione synthesis is rather determined by factors such as the expression and activity levels of γ-GCS, the availability of substrates, in particular L-cysteine, and feedback inhibition of γ-GCS activity by glutathione.

1.1.2 Regulation of γ-GCS expression

Regulation of glutathione synthesis via γ-GCS expression occurs at the level of transcription as well as through post-transcriptional and post-translational modifications. The absolute amounts and the relative ratios of γ-GCS_H and γ-GCS_L mRNA transcripts are heterogeneous in various normal human tissues (Gipp et al., 1995; Cai et al., 1997). There is evidence that in some tumor cells (Tomonari et al., 1997) the expression of γ-GCS_H can exceed that of γ-GCS_L; however, in other tumor cells both subunits are overexpressed (Galloway et al., 1997). A number of chemical and physical factors, i.e., heavy metals (Cd^2+, Cu^2+, Zn^2+, Hg^2+), H_2O_2, redox cycling drugs (menadione), ionizing radiation, oxidized low density lipoprotein, glutathione depletion by buthionine sulfoximine, diethyl maleate (DEM) and ethacrynic acid, and tumor necrosis factor-α
(TNF-α) are known to cause upregulation of γ-GCS expression (Gipp et al., 1995; Cai et al., 1997; Tomonari et al., 1997; Galloway et al., 1997; Sekhar et al., 1997; Mulcahy et al., 1997; Rahman et al., 1996a and 1996b; Shi et al., 1994; Tian et al., 1997; Urata et al., 1996). The common denominator of these factors is generation of oxidative stress; signaling induced by these stimuli can be transduced via redox-sensitive transcription factors, i.e., nuclear factor κB (NFκB), activating proteins 1 and 2 (AP-1 and AP-2), antioxidant and electrophile response elements (ARE/EpRE) and signal protein 1 (SP-1) (Mulcahy et al., 1997; Rahman et al., 1996a and 1996b), resulting in upregulation of γ-GCS expression (discussed in more detail in section 1.5).

Exposure of pneumocytes to DEM or 4-hydroxy-2-nonenal has been reported to result in stabilization of γ-GCS mRNA (Sekhar et al., 1999), but the mechanisms underlying this observation are not yet identified. Phosphorylation of γ-GCSH by protein kinases A and C and by Ca++/calmodulin-dependent protein kinase II (CMK) on serine and threonine residues, leads to γ-GCS inactivation without holoenzyme dissociation and to reduction in glutathione synthesis (Sekhar et al., 1999; Lu et al., 1991). All three kinases phosphorylate the same residues on γ-GCSH; the γ-GCSL, however, is not phosphorylated, indicating that the heavy subunit is the main subject of post-translational regulation (Sun et al., 1996). Various cytokines can induce expression of nitric oxide (NO) synthase, resulting in increased NO synthesis and subsequent inhibition of γ-GCS; inhibitors of NO synthase have been shown to prevent these effects (Goss et al., 1994).
1.1.3 Regulation of glutathione levels by substrate availability

Cellular L-cysteine levels are significantly lower than levels of the other glutathione precursors, L-glutamate and glycine. Tateishi et al. (1974) have found cysteine concentrations to be ~10x lower than concentrations of glutamate and glycine in both starved and refeed rats; they have concluded that cysteine is the rate-limiting glutathione synthesis precursor. In the liver, the main source of cysteine is the metabolic conversion of methionine (via transsulfuration of homocysteine); in addition, cysteine is obtained through uptake of cyst(e)ine from diet and/or recapture of cyst(e)ine originating from biliary glutathione and GSSG (Chang and Chang, 1994). Therefore, the liver acts as the central glutathione synthesis and distribution organ in the body. For other tissues, including most tumors, the main sources of cysteine are degradation of plasma glutathione and GSSG and uptake of the resulting cyst(e)ine.

1.1.4 Modulation of Glutathione levels

Increased glutathione levels have been associated with increased resistance of tumor cells to chemotherapeutic reagents (Ball et al., 1966; Suzukake et al., 1982; Begleiter et al., 1983; Kramer et al., 1988) and effects of ionizing radiation (Shrieve et al., 1985; van der Schans et al., 1986; Edgren et al., 1985). Therefore, modulation of glutathione levels is expected to result in increased therapeutic efficacy. Generally, two strategies have been employed for modulation of cellular glutathione levels: (i) metabolic depletion by glutathione conjugation and excretion (ii) inhibition of glutathione synthesis. GSH-transferase substrates, e.g., diethyl maleate (DEM) and N-ethylmaleate (NEM) are enzymatically coupled to glutathione and the resulting complexes are excreted from cells.
These agents have been used successfully for rapid depletion of glutathione levels in rodent cells; however, DEM and NEM are less potent in human cells, presumably because of the lower specificity of human GSH-transferases (reviewed in Hedley and Chow, 1994). The γ-glutamyl analogue buthionine sulfoximine (BSO) is a specific inhibitor of γ-glutamylcysteine synthetase (Griffith and Meister, 1979), the rate-limiting enzyme in glutathione de novo synthesis (see illustration [1], p.11). BSO has been widely used for depletion of glutathione levels (Meister, 1991) in vitro (Biaglow et al., 1983), experimental (Yu and Brown, 1983) and patient tumors (Bailey et al., 1994, Bailey, 1998). Early studies indicated that in vivo treatment with BSO did not result in profound depletion of glutathione levels (<10% of control levels), as often seen in cultivated cells. More recent results from a clinical phase I study indicate that the likely explanation for this observation was suboptimal BSO administration (Bailey et al., 1997).

1.1.5 Glutathione recycling

Gamma-glutamyl transpeptidase (γ-GT) is a peptidase located at the external surface of epithelial cells. γ-GT is involved in the maintenance of cellular glutathione homeostasis by hydrolyzing the γ-glutamyl bond in glutathione, thus generating precursors for glutathione synthesis (see Figure 1).

Analogous to γ-GCS, a variety of agents that generate reactive oxygen species can induce upregulation of γ-GT expression (Markey et al., 1998). In addition, several consensus sites for the binding of transcription factors AP-1, AP-2, ARE and NFκB have been identified in the promoter regions of rat and mouse γ-GT genes.
Extracellular glutathione is hydrolyzed by γ-GT. γ-Glu is conjugated to cystine and taken up into cells. (adapted from Meister and Andersen).

The binding of these transcription factors is known to be regulated in a redox-sensitive manner (Sen et al., 1996; Tacchini et al., 1995). Therefore, both glutathione synthesis and recycling are regulated by the cellular redox state in response to oxidative stress. Overexpression of γ-GT has been related to accelerated growth of prostate carcinoma xenografts and their chemoresistance to cisplatin (Hanigan et al., 1999). In addition, γ-GT overexpression has been associated with increased resistance to the effects of ionizing radiation in human melanoma cells by a mechanism that involves the maintenance of cellular glutathione levels (Prezioso et al., 1994). These findings illustrate the clinical significance of γ-GT-mediated glutathione recycling processes.
1.1.6 Role of glutathione in determining radiation sensitivity

Radiotherapy is a major treatment form for many human malignancies; therefore, characterization of mechanisms involved in the development of radioresistance is of particular clinical interest. Despite some recent reconsideration, DNA is still regarded as the most important cellular target for ionizing radiation (Radford, 1999). Tumor hypoxia and non-protein sulphydryls, i.e., glutathione and cysteine, have been associated with reduced sensitivity of cells to the cytotoxic effects of ionizing radiation; this relationship has been formalized as the Oxygen Competition Model for thiols. In the Oxygen Competition Model, Alper et al. (1956) postulated that molecular oxygen can compete with NPSH for binding to DNA radicals formed in the course of irradiation of cells (Figure 2).

![Diagram](image)

Figure 2: The oxygen competition model

Competition of oxygen and thiols for reaction with DNA radicals.
The effects of oxygen and NPSH on radiation sensitivity have been extensively characterized in cell-free systems and cultivated cells. The vast majority of these studies suggest that radioprotective effects of NPSH are maximized under low oxygen conditions, as predicted by the Oxygen Competition Model for NPSH (Biaglow et al., 1983). However, a number of observations provide evidence that chemical repair of DNA radicals may not be the only radioprotective mechanism mediated by NPSH. Van der Schans et al. (1986) have observed increased radiosensitization of aerobic cells upon glutathione depletion. Clark et al. (1986) confirmed this finding; in addition, they have also observed in vitro radioprotective effects of extracellular GSH. Saunders et al. (1991) observed impeded repair of DNA strand breaks by post-irradiation depletion of glutathione. Although some of these findings could be explained by the radioprotective effects of γ-GT-mediated glutathione recycling (Prezioso et al., 1994), these observations are generally consistent with an important role for glutathione in regulating enzymatic DNA repair and/or mechanisms that determine the cellular propensity for apoptosis.

1.2 Tumor hypoxia

The presence of hypoxia has been directly demonstrated in experimental (Kallinowski et al., 1990) and human tumors (Hockel et al., 1991). Tumor hypoxia has been associated with overall poor patient outcome (Hockel et al., 1993 and 1996; Brizel et al., 1994; Fyles et al., 1998), irrespective of the treatment modality employed. These findings indicate that tumor hypoxia contributes to the emergence of both therapy-resistant and biologically aggressive tumor phenotypes.
1.2.1 Diffusion-limited (chronic) hypoxia

Thomlinson and Grey found areas of necrosis in human lung cancers at distances from blood vessels that exceed the expected oxygen diffusion depth in tissue, thus implying the existence of a transitional zone in which tumor cells are poorly oxygenated but still viable. This type of tumor hypoxia is commonly referred to as chronic or diffusion-limited hypoxia (see Figure 3). Tannock (1968) demonstrated that proliferation occurs predominantly in better oxygenated tumor cells, usually found at distances less than the expected oxygen diffusion distance in tissue. This work has provided further evidence for the presence of a population of metabolically deprived but still viable tumor cells. Since oxygen diffusion constants in tumors seem to be similar to those in normal tissues (Grote et al., 1977), tumor oxygenation is determined by the structural and functional characteristics of the tumor vasculature and oxygen consumption by tumor cells (Dewhirst, 1998). Variability in the proliferation rate and the metabolic requirements of tumor cells are the probable cause of the wide range in effective oxygen diffusion distances in tumors. Using physiological modeling (Tannock, 1968; Grote et al., 1977) and markers of tissue hypoxia (Olive et al., 1992), the oxygen diffusion distance in tumors has been estimated at 100-200 μm, depending on the tumor type investigated. The oxygen diffusion distance in patient tumors is generally estimated at 150-200μm. In animal tumors or xenografts the typical oxygen diffusion distance is significantly lower, 100-150μm. These differences may also reflect the higher tumor growth rates of experimental tumors in comparison to patient tumors.
1.2.2 Perfusion-limited (intermittent) hypoxia

Circulation in tumors is characterized by dynamic changes in blood flow; factors such as rheologic effects on erythrocyte deformability in the tumor vasculature, stasis of flow in tumor blood vessels, and longitudinal gradients of partial pressure of oxygen (pO₂) along the vascular tree (Dewhirst et al., 1999) have been postulated to contribute to overall tumor hypoxia. Intermittent episodes of hypoxia (Figure 4) have been demonstrated in experimental tumors using electrode measurements of oxygenation (Dewhirst et al., 1998; Figure 5) and fluorescence labeling of perfusion (Durand and LePard, 1995). In those studies, transient flow was demonstrated in up to 50% of all tumor blood vessels. Similar observations were made in breast cancers and their nodal metastases using non-invasive laser Doppler flowmetry (Hill et al., 1996; Pigott et al., 1996). Therefore, the
oxygenation status of solid tumors is characterized by the presence of both chronic and intermittent (perfusion-limited) hypoxia; however, current clinically applicable diagnostic methods do not discriminate between these physiological entities.

![Figure 4: Perfusion-limited (intermittent) hypoxia](image)

C: Blood vessels labeled with anti-CD31 mAb. D: EF5 staining within the typical oxygen diffusion distance in tissue (adapted from Evans et al., 2000)

Transient fluctuations in tumor circulation/perfusion can result in intermittent hypoxia and oxidative stress (Parkins et al., 1995), commonly referred to as ischemia-reperfusion injury. A central role of nitric oxide (NO) in mediating the pathophysiological effects of ischemia-reperfusion injury has emerged from studies in endothelial cells. The current understanding of these processes is formalized as the Nitric oxide-superoxide imbalance theory. In the initial ischemic phase, the NO flux exceeds the rate of superoxide production, resulting in formation of DNA-reactive peroxynitryl radicals.
In the reperfusion-reoxygenation phase, however, increased superoxide production and decreased NO synthesis change the NO–superoxide balance, causing an increase in arteriolar tone, increased adhesive interactions between leukocytes and the endothelial cell surface, and increased platelet aggregation and thrombus formation. Furthermore, the accumulated superoxide is increasingly dismutated to hydrogen peroxide; oxidative stress mediated by reactive oxygen metabolites can trigger redox-sensitive transcription factors, i.e., NFκB and AP-1, that mediate transcription of endothelial adhesion molecules and subsequent inflammatory responses in endothelial
cells (reviewed in Carden and Granger, 2000). One of the consequences of ischemia-reperfusion injury is an increase in the rate of capillary filtration; the likely cause is increased hydraulic conductivity of the endothelial barrier rather than an increased intracapillary pressure (Harris, 1997). This mechanism might lead to increases in interstitial fluid pressure and further compromise tumor perfusion. Summarized, oscillations in tumor perfusion can evoke significant biological responses that include transient episodes of hypoxia and exposure to oxidative stress. Therefore, intermittent hypoxia may be more relevant than chronic hypoxia in determining biologically aggressive tumor phenotypes.

1.2.3 Methods for measurement of hypoxia

A number of invasive and non-invasive methods are utilized for measurement of oxygenation in tumors. These methods can further be subdivided into measurements of oxygen (pO₂) and measurements of tissue hypoxia.

Invasive methods involve inserting a probe into the tumor and measuring directly the oxygen concentration; a method that has found wide clinical acceptance is the use of the polarographic O₂ Eppendorf histograph. In the presence of oxygen, a current proportional to O₂ concentrations flows between the platinum cathode and an AgCl anode. By stepping the sensor needle through the tissue, a series of oxygen measurements from different tumor regions can be obtained. Another method that uses sensor needles is based on the property of oxygen to quench luminiscence of dyes (OxyLite™); an optical microsensor embedded into a probe can be inserted into the tissue region of interest for continuous quantitative monitoring of microregional pO₂. The OxyLite™ probe is more
sensitive than the Eppendorf probe at low $pO_2$, since less oxygen is consumed for the measurement. Another potential advantage is that fibre-optic devices, unlike the Eppendorf probe, can be used in simultaneous MRI studies. A direct comparison of the polarographic sensor method and the optical microsensor measurements yielded very similar estimates of the hypoxic fraction in tumors (Collingridge et al., 1997).

The alternative to direct oxygen concentration measurements in tissue is the determination of tissue hypoxia. The functional oxygenation status in tissue can be characterized with markers that localize in hypoxic tissue regions. Nitroimidazole derivatives EF5 (Evans et al., 2000, Lord et al., 1993), pimonidazole (Arteel et al., 1995) and NITP (Webster et al., 1995) have been used extensively to label hypoxia in experimental and in patient tumors. At low oxygen concentrations ($pO_2 < 1$ mm Hg), these compounds can bind to macromolecules upon bioreduction by cellular nitroreductases; the detection of tissue-bound nitroimidazole derivatives is done in tissue specimens using labeled Mabs. Another approach to measurements of tumor hypoxia is the determination of intracapillary $HbO_2$ saturation by cryospectrophotometry (Fenton et al., 1999), in combination with spatial modeling of oxygen diffusion. This method is similar to measurements of hypoxia using nitroimidazole derivatives in the sense that it is based on microscopic evaluation of tissue sections.

All of the aforementioned methods have advantages and potential problems. While measurements of oxygenation using probes provide absolute values of oxygen concentration and therefore are considered the ‘gold standard’, they do not provide information about the type of tissue in which the measurements are made (i.e., tumor cells, stroma, necrotic regions). Another important consideration is the resolution of
measurements in heterogeneous microenvironments. The Eppendorf probe is stepped in 700 µm increments (1000 µm step forward and 300 µm retraction) through the tissue and therefore may not register steep spatial gradients in oxygen concentrations that often exist in solid tumors. For example, the oxygenation of tumor cells in vascular cords varies as a function of distance, and is significantly reduced at distances of 100-200 µm from the supplying blood vessel. In addition, the current probe technologies are designed to provide either a spatial or temporal profile of oxygenation. Therefore, they have limited ability to provide information about the variability in oxygenation levels often associated with solid tumors.

Indirect microscopic measurements of oxygenation/hypoxia in tissue specimens using hypoxia markers have a much better resolution (<1 µm) and allow for spatial correlates with other parameters of biological significance. Furthermore, measurements of hypoxia provide information about functional oxygenation in situ, a parameter that is potentially more relevant than the oxygen concentration per se. However, the inherent problems associated with quantification of such measurements are calibration and the uncertainty about adequate sampling of tumor tissue, thus potentially limiting their clinical applicability.

While many of the aforementioned problems associated with invasive methods could be minimized by combining different techniques and approaches, the ultimate goal should be the development of non-invasive methods for measurements of tumor oxygenation in patients. The obvious reasons are minimized health risks, feasibility of measurements in many tumor types and repeat measurements. Ultrasound imaging and a variety of magnetic resonance imaging (MRI) techniques are currently being developed
for non-invasive determinations of blood flow and the tumor oxygenation status in the clinical setting.

Ultrasound techniques such as color Doppler and power Doppler sonography are utilized for quantification of tumor vascularity and flow. The resolution of ultrasound-based techniques is directly related to the frequency of the ultrasound and can be enhanced by the use of contrast agents. However, increased frequency results in decreased penetration depth in tissue. Ultrasound methods are currently used clinically for detection of micrometastases based on alterations in blood flow patterns (Moehrle et al., 1999; Leen, 1999). Although these methods do not measure directly tumor oxygenation or the presence of tumor hypoxia, they have been utilized for detection of variability in tumor blood flow. Evans et al. used power Doppler ultrasound to pinpoint regions of reduced blood flow in 9L gliomas. Tumor regions identified as poorly perfused generally stained positively with the hypoxia marker EF5, thus indicating a good agreement of these methods (Evans et al., 1997).

MRI is more versatile than ultrasound imaging, since different techniques can be used to image changes in tumor perfusion and blood oxygenation. Gradient recalled echo MRI (GRE-MRI) is used to determine spatial and temporal changes in tumor oxygenation, blood flow and perfusion by measuring the changes in deoxyhemoglobin concentration and by detecting motion of water molecules (Robinson et al., 1998). The contribution of individual components to the GRE MRI image is estimated using another flow-sensitive technique, spin echo MRI. Tumor oxygenation can be determined directly using fluorinated oxygen probes (perfluorocarbons) and GRE-MRI (Dardzinski and Sotak, 1994; Al-Hallaq et al., 2000). Fluorinated bioreductive drugs, e.g. misonidazole
and SR-4554, have been used as probes for tumor hypoxia with $^{19}$F magnetic resonance spectroscopy (MRS) detection. The principle of imaging of hypoxia with these probes is the longer retention times in tumors with large hypoxic fractions than in non-malignant tissues of non-hypoxic tumors (Maxwell et al., 1988; Aboagye et al., 1997). A comparison of SR-4554 imaging and electrode oxygen measurements in tumor models has shown that SR-4554 tumor retention is associated with low tumor pO$_2$ values (Aboagye et al., 1998).

In summary, ultrasound techniques do not provide direct information on the tumor oxygenation status; therefore, they should be used in combination with or as an adjunct to other method(s). The major strengths of ultrasound are high spatial resolution and low cost. MRI techniques are more versatile, since they can directly measure flow, oxygenation and hypoxia in tumors. The general problem with MRI techniques is relatively low sensitivity, resulting in an inverse relationship between spatial and temporal resolution. Generation of high-resolution MR images capable of delineating the heterogeneous tumor microenvironment in reasonable detail requires acquisition times that may be difficult to achieve in the clinical setting.

1.3 Adaptive response of hypoxic tumor cells

1.3.1 Elevation of tumor NPSH levels

Recent work in Dr. David Hedley's laboratory has shown that NPSH levels are increased in hypoxic areas of human cervical carcinoma xenografts (Moreno-Merlo et al., 1999). This finding is of particular clinical significance, since it demonstrates spatial correlation
of the radioprotective factors hypoxia and NPSH in solid tumors. The renewed interest in characterizing cellular responses to conditions of reduced oxygenation has identified molecular mechanisms that may explain the link between tumor hypoxia and increased levels of NPSH.

As discussed above, tumor hypoxia can occur in regions that are located beyond the oxygen diffusion distance in tissues. O’Rourke et al. (1997) have found that the redox sensitive transcription factor hypoxia-inducible factor 1 (HIF-1) can mediate transcription of its target genes in response to reduced oxygen concentration. The β subunit of the heterodimer HIF-1 is constitutively expressed in cells, while the α subunit is induced in response to reduced oxygenation (see Figure 6). Fluctuations in circulation along with aberrations in tumor blood flow can result in intermittent perfusion of distinct tumor regions. The ischemia-reperfusion injury is the consequence of cyclical changes in the availability of oxygen caused by multiple rounds of hypoxia and reoxygenation of tumor cells. In addition to evoking cellular adaptive response to hypoxia, intermittent hypoxia also induces cellular responses to oxidative stress. Parkins et al. (1995, 1997) demonstrated that ischemia-reperfusion injury and the resulting oxidative stress have cytotoxic effects on tumor cells. Jessup et al. (1999) showed that oxidative stress generated in the course of ischemia-reperfusion injury results in selection of tumor phenotypes that are highly metastatic. Reynolds et al. (1996) have shown that exposure of tumor cells to hypoxia in vitro results in DNA mutation rates and profiles similar to those observed when the cells were grown as xenografts. In a more recent study from the same laboratory, Yuan et al. (2000) have shown that a combination of hypoxia and low pH, as
often found in solid tumors, potentiates the effects of hypoxia on mutagenesis and contributes to inhibition of DNA repair in cultivated cells.

The spatial co-localization of hypoxia and oxidative stress can lead to overexpression of HIF-1 and NFκB. Haddad and Land (2000) have recently shown that upregulation of HIF-1 and NFκB expression by hypoxia/oxidative stress results in upregulation of γ-GCS and glutathione synthase expression in perinatal lung epithelium, with subsequent increase of glutathione levels. Therefore, it seems likely that increased NPSH levels in hypoxic areas observed by Moreno-Merlo et al. were caused by oxidative stress-mediated upregulation of γ-GCS and glutathione synthase expression. NFκB-mediated upregulation of γ-GT activity can additionally augment the elevation in cellular NPSH levels by increasing the efficiency of glutathione recycling (Moellering et al., 1999).
Figure 6: HIF-1 regulation by cellular oxygen levels

Reduced O₂ concentration results in increased HIF-1α levels, its translocation into the nucleus and heterodimerization with HIF-1β. Binding of the HIF-1 heterodimer to the hypoxia response element (HRE) is facilitated by the transactivational activator/histone acetyltransferase protein p300 and phosphorylation by p42/p44 MAPK; p53 exerts negative regulation of HIF-1α. HIF-1 induces the transcription of VEGF, EPO etc. Under normoxic conditions, HIF-1α is targeted by the von Hippel-Lindau protein (pVHL), part of an E3 ligase, for proteosomal degradation. (adapted from Richard et al., 1999).
1.3.2 Hypoxia-induced angiogenesis

The renewed interest in tumor hypoxia has resulted in a better understanding of molecular mechanisms involved in the response of tumor cells to hypoxic microenvironments. Several studies have investigated the adaptive responses of cells exposed to low oxygen conditions (Carmeliet et al., 1998; Forsythe et al., 1996; Iyer et al., 1998; Ryan et al., 1998); expression of HIF-1 was shown to induce angiogenesis via the upregulation of VEGF expression (Mazure et al., 1996), with subsequent induction of endothelial cell proliferation and the formation of new blood vessels (Flamme et al., 1997). Kuroki et al. (1996) have shown that reactive oxygen intermediates, i.e., superoxide and hydrogen peroxide, can increase VEGF expression in vitro and in vivo. Ravi et al. (2000) have recently demonstrated that functional inactivation of p53 leads to uncoupling of HIF-1α regulation by oxygen levels, resulting in increased neovascularization and tumor growth. This work is of particular relevance for the clinical situation, indicating that loss of functional p53 is associated with two major determinants of tumor aggressiveness, reduced predilection to apoptosis and an increased potential for angiogenesis. It may also provide the molecular basis for the correlation of tumor hypoxia with increased vascularity and poor patient outcome.

In the pioneering work by Folkman (1971) it was proposed that growth and metastatic spread of tumors are dependent on angiogenesis. The main hypothesis was that tumors could not grow beyond a size that is determined by the diffusion limits for oxygen and nutrients, unless they establish a functional vasculature. A growing number of factors that regulate angiogenesis at the molecular level have been identified (Carmeliet, 2000). Their characterization reveals an increasingly intricate and carefully balanced system of
pro- and antiangiogenic stimuli that modulate the structure and function of vascular networks in tumors. Due to their deregulated growth, tumors often show irregularities in the morphologic appearance of blood vessels; these irregularities include non-hierarchical vascular networks, vascular rings and sinusoids with dilated and narrowed segments, elongation and coiling, as well as an abnormal structure of the vessel wall, that often lacks a muscular layer (Less et al., 1991). Furthermore, the spatial arrangement of the tumor vasculature is often perturbed, resulting in a clustered distribution of blood vessels. Weidner et al. (1991) have found that intratumoral microvascular density (MVD), i.e. the average density of blood vessels in areas of highest vascularity in histological tumor sections (vascular hot-spots), was strongly correlated with metastasis in breast cancers. This observation has been confirmed in a number of common human malignancies, including carcinoma of the breast (Toi et al., 1993), prostate carcinoma (Weidner et al., 1993), cervical squamous carcinomas (Wiggins et al., 1995), head-and-neck squamous carcinomas (Albo et al., 1994), malignant melanomas (Fallowfield and Cook, 1991), gastrointestinal carcinomas (Takebayashi et al., 1996) and central nervous system tumors (Leon et al., 1996). At present, however, little information is available on the mechanisms that underlie regional increases in tumor blood vessel density.

1.4 Redox-regulated cellular signaling

A number of vital functions are affected by the cellular redox state, e.g., metabolic processes, DNA synthesis and transcription, cell cycle regulation and proliferation (Meister and Anderson, 1983; Powis et al., 1995; Ziegler, 1985; Hutter et al., 1997; Mallery et al., 1993; Cotgraeve and Gerdes, 1998; Abate, 1990; Toledano, 1991).
Glutathione and the thioredoxin and glutaredoxin pathways are of central importance in regulation and maintenance of the cellular redox status.

![Diagram of the thioredoxin and glutaredoxin pathways](Figure 7)

Figure 7: The thioredoxin and glutaredoxin pathways
Flux of reducing equivalents in the thioredoxin (a) and glutaredoxin (b) pathways. Trx – thioredoxin, TrxR – thioredoxin reductase, Grx – glutaredoxin (adapted from Mustacich and Powis, 2000).

Thioredoxin and glutaredoxin are members of the thioredoxin superfamily; they mediate disulfide exchange via their Cys-X1-X2-Cys active site. While glutaredoxins mostly reduce mixed disulfides containing glutathione, thioredoxins are involved in the maintenance of protein sulphydryls in their reduced state via disulfide bond reduction (Prinz et al., 1997). The reduced form of thioredoxin is generated by the action of thioredoxin reductase; glutathione provides directly the reducing potential for regeneration of the reduced form of glutaredoxin (Figure 7).
Of particular significance is the role of redox factor 1 (Ref-1), a nuclear redox active protein, in regulating DNA transcription. A number of redox-sensitive transcription factors, such as NFκB, AP-1, p53, heat shock factor 1 (HSF-1) are activated under conditions of oxidative stress and subsequently translocated into the nucleus. Ref-1 facilitates the binding of transcription factors to their respective DNA sequences by reduction of cysteine residues in their DNA binding domains. Thioredoxin plays a regulatory role in mediating this thiol-disulfide exchange by supplying reducing
equivalents to Ref-1. An illustration of the regulation of redox-sensitive transcription factors by thiols is given in Figure 8.

1.5 Multiparameter wide-field fluorescence microscopy and image analysis

Fluorescence microscopy has found wide use in biology for spatial and temporal characterization of cellular structures and (patho)physiology. Difficulties in establishing and maintaining calibration procedures limit adequate quantification of the fluorescence signal; therefore, fluorescence microscopy is generally considered as a semiquantitative technique. Nevertheless, the capability to analyze multiple parameters of interest in their physiologic and structural context, and perform relative measurements makes it a technique of choice for studies of complex biological phenomena in individual cells and tissues.

1.5.1 Multiparameter fluorescence microscopy

The most commonly employed illumination system in fluorescence microscopy is the incident light or epi-illumination. The exciting light is reflected into the back aperture of the objective by a dichromatic beam-splitting mirror. Fluorescence is collected by the objective and the light forming the image passes through the dichromatic beam-splitting mirror to either the eyepieces or a camera. The dichromatic beam-splitting mirror reflects any initial or refracted exciting light, allowing only the emitted fluorescence light to pass (see Figure 9).
The main advantage of fluorescence over bright field microscopy is the ability to individually assess multiple parameters in the same specimen, since the emitted fluorescence light from a single fluorophore occupies only a segment of the light spectrum. By carefully choosing a combination of fluorophores and the appropriate optics, 4-5 different parameters can be assessed sequentially in a single specimen. This approach minimizes image registration problems that may occur if the same parameters were assessed in different tissue sections. Therefore, multiparameter fluorescence microscopy allows for (i) assessment of the relative distribution of multiple parameters, and (ii) the analysis of their respective spatial relationships.
1.5.2 *Wide-field microscopy*

The natural extension of *in vitro* studies in tumor biology is the use of *in vivo* models; however, the gain in physiological relevance is often accompanied by reduced ability to control the experimental conditions. For instance, intrinsic heterogeneity of tumor cell populations and the varying proportions of non-malignant tissue and/or necrosis in solid tumors often raise the issue of adequate tissue sampling. Furthermore, bulk measurements of tissue samples produce average values and limit the ability to assess the dynamic range of expression of a parameter of interest.

The use of a computerized microscope stage in combination with digitized image capture allows for the sequential acquisition of high magnification images comprised of several conventional fields of view. The individual images can then be composed into a 2D image array with dimensions that exceed by far those of an individual field of view, thus allowing for imaging of entire tissue specimens at submicrometer resolutions. This technique provides an effective link between micro- and macroscopic imaging, since structure and/or function in biological specimens can be studied at dimensions that span over several orders of magnitude (see Figure 10). The major strength of this approach is that distribution of parameters of interest can be assessed in their respective context; furthermore, the statistical evaluation of such measurements is facilitated by the fact that a large number of events can be determined and analyzed in a single specimen. In this respect, wide-field microscopy is similar to flow cytometry; however, the obvious advantage is the preservation of the spatial domain in the analysis.
Figure 10: Composite wide-field microscopic image

Using a computerized microscope stage, individual fields of view (10x objective) are digitally captured and arranged into a 2D image array.
Image processing and analysis

Image processing techniques are usually applied to improve deficits in image quality, such as illumination artifacts or poor signal-to-noise ratio. A typical example is the background correction, caused by variations in the illumination field intensity. To correct for this problem, an image of a calibration slide is captured, followed by determination of ratios of the mean pixel brightness value and the brightness value contained in individual pixels. These ratios are stored as a 2D brightness ratio template; subsequently, every captured image is convolved with this template, resulting in minimization of illumination gradients.

Image analysis involves the use of computer algorithms to extract numerical information from the image. An example from my own work characterizes morphometric features of the tumor vasculature. A grayscale image of blood vessels stained with the appropriate fluorescence labeled mAb is converted to a binary image by setting a threshold on the pixel brightness values (Figure 11, panels A and B). The features corresponding to individual blood vessels are labeled and counted; this information can be used to calculate the blood vessel density in the tissue. By counting the number of pixels in features corresponding to individual blood vessels, a profile of blood vessel sizes can be generated. The spatial relations of individual blood vessels in the vascular network can be determined by calculating the distances between neighboring blood vessels (Figure 11E); analysis of the nearest neighbor distance profiles can reveal patterns in spatial arrangements that are of biological relevance, such as clustering of blood vessels. It should be noted that 2D images of 3D objects represent only an approximation of the real object structure and may underestimate nearest neighbor distances. Therefore,
several sections from a tissue specimen should be analyzed to maximize the accuracy of measurements.

Figure 11: Multiparameter image analysis in a SiHa cervical carcinoma xenograft

Panel A: grayscale image of the tumor vasculature; B: binary form of A; C: EF5 fluorescence image; D: binary form of C; E: distances from blood vessels, bright – close, dark – distant; F: percent EF5 staining as a function of distance from blood vessels.
Of particular biological significance is the ability to determine spatial relationships between multiple parameters of interest. The extent of spatial colocalization and the characterization of relative expression levels of two or more parameters can be determined using image arithmetic operations and spatial modeling techniques. For instance, functional tissue oxygenation can be assessed using the nitroimidazole hypoxia marker EF5 (Evans et al., 2000; Lord et al., 1993) (Figure 11, panel C: EF5 fluorescence grayscale image, panel D: binary form of the EF5 grayscale image). Using blood vessels as anatomical landmarks (panel B), a map of distances from blood vessels to all points in a tumor can be constructed (panel E). Hypoxia can then be analyzed as a function of distance to blood vessels (panel F) by superimposing the distance map onto the EF5 fluorescence image.

Table 1 summarizes parameters that were analyzed in studies presented in Chapters 2-4, along with the respective detection technique.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Target</th>
<th>Detection</th>
<th>Fluorophore</th>
<th>Excitation (nm)</th>
<th>Emission (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPSH</td>
<td>-SH groups</td>
<td>SHg complexes</td>
<td>Mercury orange</td>
<td>555</td>
<td>610</td>
</tr>
<tr>
<td>Blood vessels</td>
<td>CD31</td>
<td>anti CD31 mAb</td>
<td>Cy3</td>
<td>552</td>
<td>568</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>EF5</td>
<td>anti EF5 mAb</td>
<td>Cy5</td>
<td>650</td>
<td>667</td>
</tr>
<tr>
<td>Tissue perfusion</td>
<td>DNA</td>
<td>binding to DNA</td>
<td>Hoechst 33342</td>
<td>350</td>
<td>461</td>
</tr>
</tbody>
</table>

Table 1: Summary of parameters assessed by fluorescence microscopy
1.6 Rationale for experiments and outline of thesis

Considering the importance of NPSH for many aspects of cellular physiology, it is not surprising to find an abundance of published literature on NPSH measurements in solid tumors. However, in the vast majority of studies only the levels of glutathione were determined; few studies report on measurements of NPSH other than glutathione, e.g., cysteine. Despite reports of significant intertumoral variations in NPSH levels, the relationship between intra- and intertumoral variability in NPSH levels has not been adequately addressed in the published literature. The experiments described in Chapter 2 address these two issues specifically: (i) determination of glutathione and cysteine levels and (ii) determination of the intra- and intertumoral heterogeneity of NPSH levels in human cervical carcinomas. A prospective clinical study was carried out in patients entering a clinical trial studying the prognostic value of oxygenation status in cancers of the uterine cervix for radiation treatment outcome. NPSH levels were determined using two independent techniques: HPLC measurements of glutathione and cysteine in single frozen tumor biopsy sections and image analysis of parallel tumor sections labeled with the sulfhydryl stain, mercury orange. This approach allows for the determination of NPSH levels directly in the tumor tissue using image analysis techniques, while relating these measurements to results obtained with a quantitative HPLC method. The intratumoral heterogeneity was additionally assessed using multiple tumor biopsies.

Previous work in our laboratory has indicated that NPSH levels are higher in hypoxic than in better oxygenated areas of cervical carcinoma xenografts. This finding is clinically relevant, since it demonstrates the spatial co-localization of two well-known determinants of tumor radioresistance: hypoxia and elevated NPSH levels. In
experiments described in Chapter 3, buthionine sulfoximine (BSO), a specific \( \gamma \)-GCS inhibitor was used to deplete glutathione levels in ME180 and SiHa cervical carcinoma xenografts. Hypoxic regions were labeled with the hypoxia marker EF5. Using image analysis in combination with HPLC measurements, NPSH levels were determined selectively in hypoxic and non-hypoxic regions of tumors from control and BSO-treated mice.

Solid tumors are characterized by the presence of diffusion-limited and perfusion-limited hypoxia. Tumor cells found in chronically hypoxic regions are likely to be metabolically less active, due to lack of adequate supply with oxygen and nutrients. In contrast, tumor cells in intermittently perfused regions are exposed to alternating cycles of hypoxia and reoxygenation. The resulting ischemia-reperfusion injury is a potential mechanism for selection of biologically more aggressive tumor phenotypes. Experiments aimed at classification of tumor hypoxia as chronic and intermittent, and understanding the implications for NPSH levels are described in Chapter 4. Using advanced image analysis techniques, the tumor microenvironment was characterized with respect to blood vessel distribution, perfusion and hypoxia. By analyzing the distribution of EF5 fluorescence as a function of distance from blood vessels, tumor hypoxia was classified as chronic and intermittent. Subsequently, NPSH levels were selectively determined in regions of chronic and intermittent hypoxia. Chapter 5 contains a summary of the experimental work, discussion of the results and the conclusions. In addition, directions for future work are proposed.
CHAPTER 2: Microregional Heterogeneity of Non-Protein Thiols in Cervical Carcinomas Assessed by Combined use of HPLC and Fluorescence Image Analysis

*This chapter is the modified text of a paper published in Clinical Cancer Research (2000 May;6(5):1826-32.). The authors of the paper are Vojislav Vukovic, Trudey Nicklee and David W. Hedley. All experimental work was done by the author of the thesis or under his direct supervision.*
2.1 Abstract

Under low oxygen conditions non-protein thiols (NPSH) can effectively compete for DNA radicals sites, and hence represent a potentially important cause of radiation resistance in the clinic. Intra- and intertumoral heterogeneity of glutathione (GSH) and cysteine were assessed in cryostat sections of multiple biopsies obtained from 10 cervical carcinomas by the combined use of a sensitive HPLC method and a fluorescence image analysis technique to examine the spatial distribution of NPSH in tumor tissue. Glutathione concentrations ranged from 1.98 to 4.42 mM; significant ($\geq 1\text{mM}$) concentrations of cysteine, a more effective radioprotector than GSH, were found in some tumors. By HPLC, intratumoral heterogeneity of NPSH was relatively small compared to the inter-tumoral heterogeneity. The histochemical stain 1-(4-chloromercuryphenoylazo)-2-napthol (mercury orange), which binds to GSH and cysteine, was used to determine the spatial distribution of NPSH in tumor tissue. A comparison of NPSH levels in serial cryostat sections showed a close correlation between NPSH values determined by HPLC and mercury orange fluorescence quantification. Using fluorescence image analysis, an approximately twofold increase of NPSH in tumor versus non-malignant tissue was observed in the same section. Because some cervical carcinomas contain radiobiologically important levels of cysteine, agents that target the biochemical pathways maintaining tumor cysteine have therapeutic potential as adjuncts to radiotherapy in cervix cancer patients.
2.2 Introduction

Glutathione (GSH), a cysteine-containing tripeptide, is the most abundant non-protein sulfhydryl (NPSH) in mammalian cells. It is involved in a wide range of biochemical processes (Meister and Anderson, 1983) but is particularly relevant to oncology because of its potential roles in resistance to chemotherapy and ionizing radiation (Biaglow et al., 1983b; Ozols et al., 1987). Glutathione is able to conjugate electrophilic drugs such as alkylating agents and cisplatin under the action of glutathione S-transferases. Recently GSH has also been linked to the efflux of other classes of agents such as anthracyclines via the action of the multidrug resistance associated protein, MRP. In addition to drug detoxification, GSH enhances cell survival by functioning in antioxidant pathways that reduce reactive oxygen species, and maintain protein sulfhydryls in their reduced states (Kigawa et al., 1998; Marbeuf-Gueye et al., 1998; Higashi et al., 1985; Ketterer, 1988; Holmgren, 1989; Nakamura et al., 1997).

Cysteine, another radiobiologically important NPSH, and glutathione are able to effect the chemical repair of DNA radicals produced by ionizing radiation, in competition with oxygen which stabilizes DNA radical sites. Cysteine concentrations are typically much lower than GSH when cells are grown in tissue culture, and the role of cysteine as an in vivo radioprotector is less well characterized. However, on a molar basis cysteine protects DNA from the effects of ionizing radiation much more effectively than GSH (Fahey et al., 1991; Bump et al., 1992; Aguilera et al., 1992). Furthermore, there is evidence that cysteine concentrations in tumor tissues can be significantly greater than those typically found in tissue culture (Koch and Evans, 1996; Guichard et al., 1990).
A number of studies have examined GSH levels in a variety of solid human tumors, often linking these to clinical outcome (Ghazal-Aswad et al., 1996; Lee et al., 1989; Honegger et al., 1988; Langemann et al., 1989; Cook et al., 1991; Parise et al., 1994; Jadhav et al., 1988; Berger et al., 1994; Hochwald et al., 1997; Chang et al., 1993; Murray et al., 1987). Wide ranges of tumor GSH concentrations have been reported, and in general these have been greater (up to 10-fold) in tumors compared to adjacent normal tissues. Most authors have assessed the GSH content of bulk tumor tissue using enzymatic assays, or GSH plus cysteine using HPLC. In general only one tumor biopsy specimen was examined, and the problem of tumor heterogeneity was not addressed.

The histochemical stain mercury orange (1-(4-chloromercuryphenoylazo)-2-naphthol) has been previously shown to have high specificity for GSH in tissue sections (Asghar et al., 1975), allowing semi-quantitative assessments of intra-tumoral heterogeneity of NPSH. We recently refined the mercury orange histochemical technique by the use of digital image analysis to quantify labeling intensities in extensive areas of tumor tissue, acquired using fluorescence optics and a computer-controlled microscope stage (Moreno-Merlo et al., 1999). By the combined use of this technique and a sensitive HPLC method based on electrochemical detection, we have shown that the intratumoral distribution of NPSH is relatively homogenous, whereas there is an approximately twofold range in values between individual tumors. Cysteine concentrations greater than 1mM were found in some samples; values that are potentially significant in terms of chemotherapy and radiation resistance.
2.3 Material and methods

2.3.1 Tumor biopsies

A total of 34 punch biopsies were taken from 10 cervix cancer patients who were entered into a clinical trial conducted at the Princess Margaret Hospital – Ontario Cancer Institute, studying the effects of oxygenation status on radiation treatment outcome (Fyles et al., 1998). Nine of the tumors were squamous cell carcinomas, and one was an adenocarcinoma. Informed consent was obtained in accordance with institutional ethical guidelines. The biopsies (2-4 per patient) were placed into cryovials containing Tissue-Tek OCT embedding medium (Sakura Finetek, Torrance, CA) and rapidly frozen in liquid nitrogen.

2.3.2 Preparation of tissue sections

Five-micron serial sections of tumor tissue were cut using a Tissue-Tek II Cryostat (Miles Laboratories, Naperville, IL). The first two sections were adhered to 3-aminopropyl triethoxysilane (Sigma, St Louis, MO) treated glass microscope slides. A third serial section was used for HPLC measurements of NPSH. The first section was stained for NPSH with the sulphydryl-reactive dye mercury orange (Sigma, St Louis, MO). Mercury orange was first dissolved in acetone, then distilled water was added to produce a final concentration of 75 μM in 9:1 (v/v) acetone-water. In order to minimize the loss of reduced thiols through oxidation, the sections were cut and rapidly placed in the mercury orange solution and stained on ice for 5 minutes, followed by two rinses with 9:1 acetone-water. After air-drying, the nuclei were counterstained with 1 μg/ml 4’, 6-
diamidino-2-phenylindole dihydrochloride (DAPI) for 10 minutes. After rinsing with PBS, the slides were mounted with the Vectashield medium for fluorescence (Vector Laboratories, Burlingame, CA). The second section was fixed in 3.7% neutral buffered formalin for 10 minutes, rinsed and stained with hematoxylin and eosin (H&E).

2.3.3 HPLC measurement of NPSH

NPSH were extracted using the method described by Koch and Evans (1996). Briefly, the tissue section was rapidly placed into a vial with extraction buffer containing 50 mM sulfosalicylic acid and 50 μM of each of the iron and copper chelators EDTA, sodium diethyldithiocarbamate and diethylenetriaminepentaacetic acid and kept at 4 °C for one hour. The samples were then centrifuged at 14000 g for 15 minutes and the optically clear supernatant was aliquoted and stored at 4 °C. Determinations of NPSH were carried out by HPLC-based electrochemical measurement, usually within 24 hours of extraction. The HPLC system consisted of a Waters 600E system controller, Waters Ultra WISP 715 sample processor, Waters 746 data module and Waters 464 pulsed electrochemical detector, equipped with a Hg-coated dual gold electrode. The separation was carried out using a Supelco LC-18 reversible phase column (7.5 cm x 4.6 mm, bead size 3 μm). To resolve glutathione from cysteine, a low pH mobile phase (pH = 2.0), consisting of 0.1 M phosphoric acid with 3.3 mM heptanesulfonic acid in water and 10% methanol was used, as described by Koch and Evans. Prior to separation, the mobile phase was purged with helium to displace dissolved O2 and reduce background current. Glutathione and cysteine concentrations were calculated by comparing the peak area of the samples with that of known standards. Consistency of measurement was maintained using external standards.
after every three samples. The volumes of the tissue sections used for HPLC measurements were obtained by the product of the section area, determined by digital microscopy of the parallel hematoxylin and eosin (H&E) stained section, and the section thickness.

2.3.4 Transmitted light microscopy

The H&E sections were imaged using a MicroComputer Image Device (MCID; Imaging Research Inc., St. Catharines, Ontario, Canada) linked to a Sony DXC-970 MD, 3CCD color video camera mounted on a Zeiss Axioskop microscope fitted with a Ludl Biopoint motorized stage. Using 10x 0.25 N.A. objective lens and an automated mini program, a microscopic field by field digitized tiled image of the entire tumor section was obtained. These images, showing the cellular morphology of the biopsies, were used as a guide for subsequent mercury orange fluorescence measurements.

2.3.5 Fluorescence microscopy and image acquisition

A second MCID image analysis system was used to tile the entire tumor section stained with mercury orange. This system has similar computer hardware and software to that used for transmitted light microscopy, but is linked to a Xillix MicroImager (Xillix, Vancouver, British Columbia, Canada) mounted on an Olympus BX50 reflected fluorescence microscope fitted with a Ludl Biopoint motorized stage. Using 10x 0.3 N.A. objective lens, tiled field images were obtained using an excitation filter centered at 540 nm which optimally excites mercury orange. Fluorescence emission was collected using a 585/40 nm band pass filter.
2.3.6 Image processing and analysis

Digitized mercury orange fluorescence and H&E images were saved as 24-bit TIFF files. Subsequently, the images were converted to 8-bit grayscale images using Adobe PhotoShop 5.0 software. Further image processing was performed using an application written in the Interactive Data Language (IDL 5.1, Research Systems Inc., Boulder, CO). The H&E images were inverted and subsequently thresholded at a brightness level that corresponded to three times the highest brightness value in the background, to exclude empty spaces in the tissue caused by freezing-thawing artifacts from the determination of total section area. The resulting binary image was used to calculate the total section area and volume. The Sony DXC-970 MD CCD used for transmitted light microscopy imaging has rectangular-shaped pixels, whereas the Xillix MicroImager CCD used for fluorescence imaging has square pixels; therefore, image areas rather than pixel numbers were used for comparisons of H&E and fluorescence images. The total positive area in the binary image was used as reference to find the appropriate threshold value for the mercury orange fluorescence image. After thresholding, the positive pixel addresses from the mercury orange binary image were used on the original grayscale image to collect the brightness values from corresponding pixels. The mean grayscale values were calculated by dividing the total sum of positive pixel brightness values by pixel number. Background was defined as the average pixel value in three randomly chosen image regions outside the tissue section and subtracted from the positive pixel brightness values.

To assess the mercury orange fluorescence levels in tumor cells and non-malignant cells within a tumor, areas predominantly populated with tumor cells were chosen on the H&E image and the corresponding areas of the mercury orange
fluorescence image manually outlined using the Adobe PhotoShop software. The mean brightness from 5-10 randomly chosen areas within one section was averaged and compared with areas containing predominantly non-malignant cells.

2.4 Results

2.4.1 NPSH measurements using HPLC

Peaks representing GSH and cysteine were readily resolved in all of the samples, with no additional peaks to indicate the presence of glutathione fragments, such as the dipeptides Cys-Gly or γ-Glu-Cys, in detectable amounts. The mean values for GSH and cysteine concentrations of all of the samples analyzed (n=34) were 2.86 mM (SEM 0.15) and 0.75 mM (SEM 0.06), respectively. Figure 12 and Table 2 show the mean values and standard deviations for the multiple biopsies obtained from individual patients. As seen, the intertumoral values of GSH ranged from 1.98 mM to 4.42 mM. The intratumoral variability was significantly smaller, since the pooled standard deviation for individual tumors was 0.39, compared to a SD of 0.86 for the total group of 34 samples (Anova, p<0.001). The intertumoral values of mean cysteine concentrations ranged from 0.34 mM to 1.40 mM. As with GSH, the pooled SD of 0.26 for individual tumors was smaller than that of the total group (SD 0.37, Anova, p<0.001).
<table>
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<th>Patient</th>
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<th>HPLC GSH</th>
<th>HPLC cysteine</th>
<th>Fluorescence</th>
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<tr>
<td></td>
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<td>Mean</td>
<td>SD</td>
<td>SEM</td>
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<tr>
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<td>10</td>
<td>3</td>
<td>1.98</td>
<td>0.27</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Table 2: NPSH content of cervical tumors
Figure 12: Glutathione and cysteine concentrations measured using the HPLC. 

GSH and cysteine were determined in frozen sections from multiple tumor biopsies. Symbols represent mean values of 2-4 sections from individual tumors, error bars are SEM.
Tiled images, composed of more than thirty individual 10x fields of views, of parallel tumor biopsy sections stained with H&E (top) and mercury orange (bottom).
2.4.2 NPSH measurements using image analysis

To compare NPSH levels in tumor cells and non-malignant tissues, and to assess the intracellular NPSH distribution, adjacent tumor tissue sections were stained with mercury orange and H&E. Representative tiled images covering the entire sample area, composed from more than thirty 10x objective viewing fields, are shown in Figure 13, panels A and B. The mean mercury orange fluorescence values, integrated across the entire tissue area, are shown in Figure 14 and Table 2. In Figure 15 these values are shown as bivariant plots versus HPLC-determined concentrations of GSH (top panel) and NPSH (GSH + cysteine; bottom panel). A close correlation between these two methods was observed for GSH ($r^2=0.92$) and for NPSH ($r^2=0.76$).

![Figure 14: NPSH content measured by quantification of mercury orange fluorescence](image)

Multiple (2-4) frozen tumor sections were stained with mercury orange and the fluorescence of the entire sections, excluding cavities, was averaged to generate the relative grayscale units. Bars represent mean values for individual tumors, error bars are SEM.
Figure 15: Correlation of NPSH measurements: HPLC vs. fluorescence quantification

Correlation of GSH concentrations and the integrated optical density (IOD) of mercury orange fluorescence \((r^2=0.92)\) is shown in the top panel. The correlation of NPSH (GSH + cysteine) concentrations and the grayscale of mercury orange fluorescence \((r^2=0.76)\) is shown in the bottom panel. Solid lines represent linear regressions. The linear regression intersect is not zero, reflecting the difference in sensitivities of HPLC and the GSH/NPSH fluorescence quantification.
Measurement of NPSH levels within individual sections was done by manually outlining areas containing predominantly tumor cells and areas of non-malignant tissue, using the H&E image as a guide. As can be seen in Figure 13, within areas that consisted substantially of viable tumor tissue the labeling intensity with mercury orange was heterogeneous, similar to our recent findings using human cervical cancer xenografts (Moreno-Merlo et al., 1999). The mercury orange fluorescence levels were higher by a factor of approximately two in areas containing predominantly tumor cells (mean 45.7 grayscale, SEM 2.2) compared to stroma (mean 24.6 grayscale, SEM 1.4). As shown in Figure 16, in individual tumor biopsies the NPSH contents of tumor and non-tumor tissue were closely correlated ($r^2=0.53$).

![Figure 16: NPSH content of tumor and non-malignant tissues](image)

The NPSH content (mercury orange fluorescence intensity) of areas populated predominantly with tumor cells is plotted against the NPSH content of areas of non-malignant tissue in the same section. Symbols represent mean values of individual tumor biopsies, error bars are SEM.
2.5 Discussion

In this study we have examined the heterogeneity of NPSH levels in multiple biopsies obtained from patients with cervical carcinomas who were entered into a study investigating the effects of hypoxia on the response to radical radiotherapy (Fyles et al., 1998). The major findings are that the intertumoral heterogeneity of the concentrations of GSH and cysteine exceeds the intratumoral heterogeneity, and that cysteine concentrations ≥1 mM were found in some samples, confirming an earlier report by Guichard et al. (1990). These levels of cysteine are much greater than those typically seen in tissue culture, suggesting that cysteine might exert a significant radioprotective effect in cervical carcinomas.

There is extensive literature showing that elevated cellular glutathione levels can produce drug resistance in experimental models, due to drug detoxification or to the antioxidant effects of GSH. In addition, radiation-induced DNA radicals can be repaired non-enzymatically by GSH and cysteine, indicating a potential role for NPSH in radiation resistance. Cysteine is the more effective radioprotector, but is usually present in lower concentrations than GSH. Whereas under fully aerobic conditions this radioprotective effect appears to be relatively minor, NPSH compete more effectively with oxygen for DNA radicals under the hypoxic conditions that exist in some solid tumors, hence might play a significant role in radiation resistance in the clinic.

Radiotherapy is a major treatment modality for cervical carcinomas. Recent randomized clinical trials (Rose et al., 1999) show that patient outcome is significantly improved when this is combined with cisplatin-based chemotherapy, and combined modality therapy is now widely used. It is important to establish the clinical relevance of
GSH and cysteine levels to drug and radiation resistance because of the potential to modulate these levels using agents such as buthionine sulfoximine; an irreversible inhibitor of γ-glutamylcysteine synthetase that can produce profound depletion of GSH in tumor and normal tissues (Griffith et al., 1979; Bailey et al., 1997). However, before the effects of such treatments can be fully evaluated, it is necessary to develop methods for accurate NPSH measurements in solid tumors.

Bulk NPSH levels have been determined in a wide range of tumor types using enzymatic assays that measure GSH and/or GSSG, and less frequently using HPLC methods that can also measure cysteine. Typically these studies have reported elevated tumor GSH relative to adjacent normal tissue, and intertumoral heterogeneity in GSH content. These findings are consistent with the idea that GSH could play a clinically significant role in drug resistance, although it should be noted that relatively few studies have the sample size and follow up duration necessary to detect a significant relation between tumor GSH content and response to chemotherapy, hence there are no consistent clinical data to support this idea.

Koch and Evans (1996) have shown that cysteine concentrations in established lines of rodent cells can be much greater when these are grown as tumors, compared to the in vitro values, suggesting that cysteine might play a more significant role in therapy resistance than previously considered. Relatively few studies have reported on cysteine levels in human cancers. However, an earlier HPLC-based study of cervical carcinomas by Guichard et al. (1990) reported cysteine concentrations greater than 1 mM in a significant number of cases. Compared to the present study, considerably greater intertumoral heterogeneity in cysteine and GSH was found by Guichard et al., possibly
due to differences in analytical technique, but the overall findings are similar. The fact that the variability in cysteine levels is greater than that for GSH suggests that these two thiols are regulated differently in tumors. Since cysteine is a more efficient DNA radioprotector on a molar basis than GSH (Bump et al., 1992), the finding of high cysteine levels may explain the failure of GSH depletion with BSO to result in significant radiosensitization of tumors in vivo (Guichard et al., 1986). Inhibition of γ-glutamylcysteine synthetase with BSO could result in elevated cellular levels of cysteine, since it is not being utilized for GSH de novo synthesis. In addition to its ability to repair radiation-induced DNA radicals, cysteine has the potential to detoxify cisplatin; a cytotoxic agent now routinely combined with radiotherapy to treat locally advanced cervical carcinomas.

A major advantage of enzymatic and HPLC assays is that they are quantitative; however, they do not provide information on the intratumoral variability of NPSH. In addition, biopsies from solid tumors can contain variable proportions of tumor and non-malignant cells, or viable and necrotic tissue, making the interpretation of such measurements even more complex. Sulfhydryl-reactive staining procedures with subsequent detection by flow cytometry or fluorescence microscopy can be used to assess cellular heterogeneity of NPSH. However, because of the potential for background labeling of protein sulphydryls and practical difficulties establishing and maintaining an accurate calibration, these methods are generally considered to be semi-quantitative.

In the present study a strong correlation was found between the HPLC-determined GSH concentration and mercury orange fluorescence when these methods were applied to serial cryostat sections. This indicates that under carefully standardized conditions, the
mercury orange technique is able to give a quantitative estimate of tissue GSH levels. The correlation was less strong with respect to NPSH (GSH + cysteine) determinations, possibly due to greater solubility of the reaction product between mercury orange and cysteine. Because of this, and the relatively greater concentrations of GSH, we consider it unlikely that the mercury orange technique gives meaningful information about the intratumoral heterogeneity of cysteine.

GSH and cysteine levels varied by a factor of approximately 2 and 4, respectively; whereas mercury orange fluorescence levels varied by a factor of approximately 3. The observed discrepancies are may be explained by potential differences in sulphydryl binding kinetics and/or differences in quantum efficiencies for the GSH-mercury orange and cysteine-mercury orange reaction products. Another possibility is that a different mercury orange fluorescence image thresholding strategy, e.g., setting a constant instead of flexible brightness threshold level, would have resulted in different NPSH fluorescence quantification results. Constant brightness threshold setting would imply constant GSH diffusion and mercury orange reaction rates. The rationale for the approach taken in the course of this study is that flexible brightness threshold setting would more accurately account for slight variability induced by sample preparation and staining.

By comparing areas populated predominantly with tumor cells and areas of non-malignant cells within the same tumor section, we have found that mercury orange fluorescence intensity in tumor cells is approximately two times higher that in non-malignant cells. This is in agreement with our previous work in cervical tumor xenografts (Moreno-Merlo et al., 1999) and the observation that GSH levels in many tumor types are increased twofold above levels found in normal tissues (Ghazal-Aswad et al., 1996; Lee
et al., 1989; Honegger et al., 1988; Langemann et al., 1989; Cook et al., 1991; Parise et al., 1994; Jadhav et al., 1988; Berger et al., 1994; Hochwald et al., 1997; Chang et al., 1993; Murray et al., 1987; Wong et al., 1994; Perry et al., 1993; El-Sharabasy et al., 1993). The correlation seen between the NPSH contents of malignant and non-malignant tissues within the same tumor is relevant to the question of whether the heterogeneity of NPSH seen in human cancers is due to autonomous tumor factors such as expression of GSH-synthesizing enzymes, relative to systemic factors that determine the availability of GSH precursors.

By dual fluorescence staining for mercury orange and the hypoxia marker EF5, we have recently shown that NPSH levels are approximately 50% greater in hypoxic regions of ME180 and SiHa human cervical cancer xenografts, relative to better oxygenated tumor tissue (Moreno-Merlo et al., 1999). This finding suggests that tumor cells actively regulate NPSH levels in response to the tumor microenvironment. Because of the greater protection afforded by NPSH under hypoxic conditions, this response is likely to enhance the overall radioresistance of the tumors. In future experiments we plan to develop the wide field fluorescence image analysis technique in order address the underlying mechanisms of tumor GSH and cysteine regulation by examining the intratumoral relationships between hypoxia, mercury orange labeling intensities, and the expression of NPSH regulating enzymes such as γ-glutamylcysteine synthetase and γ-glutamyltranspeptidase. These experiments will be done using xenograft models, and biopsies obtained from cervix cancer patients being treated with EF5 as part of our research program investigating the mechanisms of hypoxia in human cancers.
CHAPTER 3: Effects of buthionine sulphoximine on glutathione levels in hypoxic and non-hypoxic cells in human cervical carcinoma xenografts

*This chapter is the modified text of a paper submitted for publication to British Journal of Cancer. The authors of the paper are Vojislav Vukovic, Trudey Nicklee and David W. Hedley. All experimental work was done by the author of the thesis or under his direct supervision.*
3.1 Abstract

The non-protein sulphydryl (NPSH) glutathione (GSH) is involved in mediating tumor chemoresistance and radioprotection under conditions of reduced oxygenation. Therefore, we have assessed the effects of buthionine sulfoximine (BSO) treatment on GSH levels in hypoxic and non-hypoxic areas of human cervical carcinoma xenografts ME180 and SiHa. GSH levels were determined using a sensitive HPLC assay in single frozen tumor sections. In BSO treated mice, GSH levels in ME180 and SiHa tumors were reduced by ~60% and ~50%, respectively. The 2-nitroimidazole derivative EF5 was administered i.v. to label tumor hypoxia. Distribution of NPSH and hypoxia in tissue was assessed using a double-staining fluorescence technique; hypoxic regions were visualized with an anti-EF5 mAb, and NPSH were stained with the thiol stain mercury orange. GSH levels in hypoxic regions were higher than in better-oxygenated areas in both ME180 and SiHa tumors. However, treatment with BSO produced a more profound GSH depletion in regions of hypoxia, resulting in similar post-treatment NPSH levels in hypoxic and non-hypoxic areas. This finding suggests that the likely mechanism for elevated GSH levels in hypoxic tumor areas is increased activity of \( \gamma \)-glutamylcysteine synthetase, the rate-limiting enzyme in GSH de novo synthesis.
3.2 Introduction

The non-protein sulphydryl (NPSH) glutathione has been shown to protect DNA from the effects of ionizing radiation in cell-free models and in cultured cells, and to contribute to resistance of solid tumors to radiation- and chemotherapy (Bump et al., 1992; Revesz et al., 1963; Biaglow et al., 1983a and 1983b; Hamilton et al., 1985; Vanhoefer et al., 1996; Siemann et al., 1993; Halperin et al., 1992; Kramer et al., 1989). The mechanisms that mediate this protection include scavenging of free radicals, chemical restitution of DNA radicals and provision of reducing equivalents to enzymes involved in the maintenance of protein sulphydryls in their reduced state. The protective effects of NPSH are potentiated under conditions of low oxygenation that are often found in human tumors (Hockel et al., 1991; Brizel et al., 1994), and therefore may contribute to increased resistance of hypoxic tumors to radiotherapy (Fyles et al., 1998). We have recently shown that NPSH levels are increased in hypoxic areas of ME180 and SiHa human cervical carcinoma xenografts (Moreno-Merlo et al., 1999). Although the mechanisms that underlie this observation are not fully elucidated, one possible explanation is that enzymes involved in GSH de novo synthesis and recycling, such as gamma-glutamylcysteine synthase (γ-GCS) and gamma-glutamyl transpeptidase (γ-GT) are overexpressed in hypoxic areas of solid tumors. O'Dwyer et al. (1994) have shown that hypoxia can upregulate the expression of γ-GCS, resulting in increased cellular GSH levels. It is also known that oxidative stress, often associated with perfusion-limited (intermittent) hypoxia, can upregulate the expression of γ-GT (Kugelman et al., 1994); an enzyme involved in the salvage of extracellular GSH and providing cells with precursors for GSH synthesis (Meister and Anderson, 1983).
Much effort has gone into attempts to develop strategies for modulation of tumor glutathione levels with the ultimate goal to increase the efficacy of cancer therapy. The $\gamma$-glutamyl amino acid analogue buthionine sulphoximine (BSO) is a specific inhibitor of $\gamma$-GCS, the rate-limiting enzyme in GSH synthesis (Griffith and Meister, 1979). BSO has been extensively used to reduce GSH levels in animal models and in the clinical setting (Yu and Brown, 1984; Minchinton et al., 1984; Bailey et al., 1994). In cultivated cells, BSO can cause profound depletion of GSH, to almost undetectable levels; with appropriate dose scheduling, BSO can reduce GSH levels \textit{in vivo} by $>90\%$ of those found in controls (Bailey et al., 1997). One important consideration, however, is the availability of BSO in poorly and/or intermittently perfused areas of the tumor. Hypoxic tumor cells are usually located beyond the oxygen diffusion distance in tissue ($>100-150~\mu\text{m}$ from the supplying blood vessel), and therefore may be exposed to smaller concentrations of BSO than better oxygenated cells, usually found in close proximity to blood vessels. In addition, in regions characterized by diffusion- and/or perfusion-limited hypoxia, tumor cells may show upregulation of enzymes that regulate GSH metabolism and GSH precursor availability, i.e., $\gamma$-GCS and $\gamma$-GT, thus rendering them more resistant to BSO-induced GSH depletion. Therefore, it is important to assess the effects of BSO on GSH levels selectively in hypoxic and non-hypoxic tumor cells.

In this study, we have investigated the effects of BSO treatment on GSH content of hypoxic and non-hypoxic regions of ME180 and SiHa human cervical cancer xenografts. Tumor-bearing mice were treated with the hypoxia marker EF5; upon bioreduction, this nitroimidazole derivative labels preferentially hypoxic cells (Lord et al., 1993; Evans et al., 2000). We have determined NPSH levels in frozen tumor tissue...
sections using a sensitive HPLC assay. Previously we have found excellent agreement between this assay and the mercury orange fluorescence quantification for measurement of NPSH (Vukovic et al., 2000). Using a dual-labeling fluorescence technique to detect NPSH and hypoxia in parallel cryostat sections, we have determined NPSH levels in hypoxic and non-hypoxic areas of ME180 and SiHa tumors. Our results indicate that treatment with BSO can abolish the existing GSH level gradient in hypoxic vs. non-hypoxic tumor areas, resulting in a relatively homogenous GSH distribution in BSO-treated tumors.

3.3 Materials and methods

3.3.1 Establishing of xenografts and treatment of mice

ME180 and SiHa squamous cell cervical carcinoma cell lines were originally obtained from the ATCC. Cells were maintained in growth medium (α-MEM), supplemented with 10% FBS and 0.1 mg/mL kanamycin (growth medium), at 37 °C in a humidified atmosphere containing 95% air plus 5% CO₂. Fifty microliters of a tumor cell suspension containing 5x10⁵ cells were injected into the gastrocnemius muscle of 20 female SCID mice for each cell line. After 9-12 days, when the tumors reached a size of 4-5 mm in diameter, the tumor-bearing mice were randomized and assigned to the control and treatment group, respectively. BSO (1mmol/kg in 200 μl) was given i.p. to the treatment group; the controls received an equal amount of distilled water. This treatment was repeated 20h later, 4 hours before tumor excision. After the second BSO treatment, EF5
was administered via a lateral tail vein to all animals, to give a total body concentration of 100 μM. Mice were sacrificed by cervical dislocation; the tumors were rapidly removed and snap frozen in liquid nitrogen.

3.3.2 Preparation of tissue sections

Frozen tumor blocks were cut to obtain groups of three consecutive sections. The first section was double-stained for the simultaneous detection of NPSH and hypoxia (Moreno-Merlo et al., 1999). Briefly, after first staining with mercury orange, the tumor section was stained with the Cy5 conjugated ELK3-51 monoclonal antibody, provided by Dr. Cameron Koch, University of Pennsylvania. The second section was fixed in 3.7% neutral buffered formalin for 10 minutes, rinsed and stained with hematoxylin and eosin (H&E); the third serial section was processed for HPLC measurements of NPSH.

3.3.3 HPLC measurement of NPSH

Tumor tissue extracts for NPSH determination were prepared as previously described (Chapter 2). Glutathione and cysteine levels were determined by HPLC-based electrochemical measurement, and the concentrations calculated by comparing the peak areas of the samples with those of known standards. The volumes of the tissue sections used for HPLC measurements were obtained by the product of the respective section areas, determined by digital microscopy of the parallel H&E section, and the section thickness. Results were expressed as nmol NPSH per mg tissue, assuming a tissue density of 1g per ml.
3.3.4 Transmitted light microscopy

The H&E sections were imaged using a MicroComputer Image Device (MCID; Imaging Research Inc., St. Catharines, Ontario, Canada) linked to a Sony DXC-970 MD, 3CCD color video camera mounted on a Zeiss Axioskop microscope fitted with a Ludl Biopoint motorized stage. Using 10x 0.25 N.A. objective lens and an automated mini program, a microscopic field by field digitized tiled image of the entire tumor section was obtained. These images, showing the cellular morphology of the biopsies, were used as a guide for subsequent mercury orange fluorescence measurements.

3.3.5 Fluorescence microscopy and image acquisition

A second MCID image analysis system was used to tile the entire tumor section stained with mercury orange. This system has similar computer hardware and software to that used for transmitted light microscopy, but is linked to a Xillix MicroImager (Xillix, Vancouver, British Columbia, Canada) mounted on an Olympus BX50 reflected fluorescence microscope fitted with a Ludl Biopoint motorized stage. Using 10x 0.3 N.A. objective lens, tiled field images were obtained using an excitation filter centered at 540 nm which optimally excites mercury orange. Fluorescence emission was collected using a 585/40 nm band pass filter. EF5 was excited using a 635/20 nm filter; the fluorescence emission was collected using a 685/50nm bandwidth filter.

3.3.6 Image processing and analysis

Digitized mercury orange, and EF5 fluorescence images and H&E brightfield images were saved as 8- and 24-bit TIFF files, respectively. Initial image processing was
performed using the commercial software application Adobe PhotoShop, 5.0. The grayscale mercury orange images were thresholded slightly above the highest values in the background, to obtain images of the entire tissue section. Using the H&E image as visual guide, the resulting binary mercury orange image was manually modified to include morphologically viable tumor tissue only; the resulting tissue masks were further modified to delineate areas populated predominantly with tumor cells or the surrounding muscle, respectively. These binary images were used as masks to restrict analyses of NPSH and hypoxia staining specifically to the tumor and muscle tissues. The EF5 fluorescence images were thresholded at constant brightness values, corresponding to 2x the average intensity in non-malignant tissue and used as masks to outline hypoxic areas within the tissue. All subsequent image analysis was done using an application written in the Interactive Data Language (IDL 5.1, Research Systems Inc., Boulder, CO).

Upon staining with mercury orange, we have occasionally observed a focal clustering of the GSH-mercury orange reaction product, consistent with redistribution of GSH in the process of mercury orange staining. Therefore, such sections were discarded and only sections with unperturbed or minimally perturbed GSH localization, as judged by the mercury orange fluorescence distribution, were utilized for subsequent analysis. Using the tumor and muscle tissue masks, the mercury orange fluorescence was measured in the entire tissue section, as well as in the tumor and muscle tissues separately. Since EF5 and mercury orange images had identical dimensions and were optimally registered, the addresses of EF5-positive pixels were used directly as image array subscripts for determination of mercury orange fluorescence brightness values in EF5-positive areas. Addresses of the EF5-negative pixel set were consequently used to obtain the mercury
orange fluorescence brightness values in EF5-negative areas. The mean grayscale values were calculated by dividing the total sum of positive pixel brightness values by the pixel number. Background was defined as the average pixel value in three randomly chosen image regions outside the tissue section and subtracted from the positive pixel brightness values.

3.4 Results

3.4.1 HPLC measurement of NPSH

Shown in Figure 17 are the levels of GSH and cysteine in control and BSO-treated ME180 and SiHa tumor xenografts. GSH levels in control ME180 and SiHa tumors were similar (2.62 and 2.34 nmol/mg tissue, in ME180 and SiHa tumors, respectively). Treatment with BSO resulted in a significant decrease in GSH levels in both tumors types; in ME180 tumors, GSH levels decreased by approximately 60%, from 2.62 to 1.04 nmol/mg tissue (p<0.01). In SiHa tumors, the BSO-induced GSH depletion was similar to that observed in ME180 tumors, from 2.34 to 1.28 nmol/mg (p<0.01). Cysteine levels decreased significantly in both ME180 and SiHa tumors as a function of BSO treatment, by approximately 60% and 50%, respectively. One possible explanation for this observation is that BSO can compete with cystine for uptake into cells (Brodie and Reed, 1985) and subsequent reduction to cysteine.
Figure 17: NPSH levels in control and BSO-treated ME180 and SiHa tumors

NPSH levels in ME180 (white bars) and SiHa (black bars) xenografts were determined by HPLC in single frozen tumor sections from 8-12 control and BSO-treated tumors. Bars are mean values, error bars are SEM.
Figure 18: H&E, mercury orange and EF5 fluorescence images of a ME180 tumor

Wide-field fluorescence images of the entire cut surface of a ME180 tumor, composed of >40 individual fields of view at 10x magnification. For presentation, monochromatic fluorescence images were contrast enhanced and converted to indexed colour images. Brighter colours correspond to higher fluorescence intensity. MO - mercury orange, MO binary - separate masks (orange=tumor, green=muscle) allow for independent mercury orange fluorescence measurements in tumor and muscle tissue.
3.4.2 Hypoxia staining with EF5

The majority of analyzed ME180 and SiHa tumors stained positively for EF5 in 5-15% of the total tumor area. A frequently occurring motif of EF5 staining was the formation of ovaloid rims around vascular cords (Figure 18). Such patterns are consistent with labeling of chronic (diffusion-limited) hypoxia in tissue, since the diameters of these cords were usually 250-300 μm, the typical oxygen diffusion distance in tissue. In all of the analyzed cases the EF5 staining was confined to the tumor, since no specific EF5 staining was detected in adjacent muscle tissue.

3.4.3 NPSH measurements by image analysis

Wide-field microscopic fluorescence images, composed of individual fields of view and tiled into large images of the entire tumor cut surface, were analyzed to assess both the levels and the spatial distribution of NPSH in tumor and non-tumor tissues. In addition, by the combined analysis of images of mercury orange and EF5 fluorescence, the NPSH levels were determined specifically in hypoxic and non-hypoxic tumor areas. Since cysteine accounted for ≤ 10% of the total NPSH levels as assessed with the HPLC assay (0.29 and 0.13 nmol/mg in ME180 and SiHa tumors, respectively), mercury orange fluorescence was considered indicative of GSH levels in tissue.

Adjacent muscle tissues were stained more intensely with mercury orange than areas populated predominantly with tumor cells, indicative of higher muscular GSH levels. The mercury orange fluorescence intensities in muscle for control and BSO-treated mice are shown in Table 3. Upon treatment with BSO, muscular GSH levels decreased by approximately 35%, from 32 to 19 grayscale units.
Bars are SEM. Bars are mean values of 8-12 individual tumors, error.

Mercury orange fluorescence intensities, expressed asgray-scale units, in EF5-positive (white bars) and EF5-negative (black bars) control and BSO-treated MEF180 (top panel) and SHIa tumors (bottom panel). Bars are mean values of 8-12 individual tumors, error.

Figure 19: NPSH levels in control and BSO-treated MEF180 and SHIa tumors.

- tumor EF5
- tumor EF5+
Mean mercury orange fluorescence intensities of untreated controls were higher in ME180 than in SiHa tumors (19.8 vs. 16.2 grayscale units, p<0.05, in ME180 and SiHa, respectively), in agreement with the somewhat higher GSH levels in ME180 tumors determined by HPLC. The mercury orange fluorescence intensities varied usually by a factor of two within one section. Treatment with BSO reduced GSH levels in ME180 tumors by approximately 55%, from 19.2 to 8.8 grayscale units. In SiHa tumors, the BSO-treatment resulted in a similar depletion of GSH levels, from 16.2 to 8.2 grayscale units.

Using binary EF5 images as masks for segmentation of mercury orange fluorescence images, GSH levels were determined in hypoxic and non-hypoxic tumor areas. In both ME180 and SiHa xenografts, mercury orange fluorescence was higher in hypoxic than in better oxygenated areas (Figure 19). In ME180 tumors, mean mercury orange fluorescence intensities in hypoxic areas were 21.8 grayscale units, compared to 16.6 grayscale units in EF5 negative areas (p<0.05). In SiHa tumors, mean mercury orange fluorescence intensities were 18.2 and 15.1 grayscale units, for EF5 positive and negative areas, respectively (p<0.05, see Figure 19). Treatment with BSO resulted in a decrease of mean mercury orange fluorescence intensities in ME180 tumors from 21.8 to 8.4 grayscale units in hypoxic areas and from 16.6 to 8.7 grayscale units in non-hypoxic areas. Likewise, treatment with BSO resulted in a more pronounced relative loss of GSH in hypoxic areas of SiHa tumors; mean mercury orange fluorescence intensities were reduced from 18.2 to 9.6, and from 15.1 to 10.1 grayscale units in hypoxic and non-hypoxic areas, respectively (Figure 19). Therefore, treatment with BSO effectively abolished the difference in GSH levels between hypoxic and non-hypoxic tumor cells.
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<td>SEM</td>
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</table>

Table 3: Mercury orange fluorescence in ME180 and SiHa tumors

Mean values (±SEM) of mercury orange fluorescence in tumor and muscle tissue, and hypoxic and non-hypoxic regions of 8-12 individual control and BSO-treated ME180 and SiHa tumors.
3.5 Discussion

Increased levels of GSH and the presence of hypoxia have been independently associated with therapy-resistant tumor phenotypes (Vanhoefer et al., 1996; Hockelet al., 1993). These two factors have been shown to act synergistically in vitro to minimize the efficacy of radiation treatment; therefore, strategies designed to reduce tumor GSH levels are of potential clinical significance. Since it is known that hypoxia can induce overexpression of γ-GCS, the rate-limiting enzyme in GSH de novo synthesis, we have studied the effects of BSO, a specific γ-GCS inhibitor, on GSH levels in hypoxic and non-hypoxic areas of human cervical cancer xenografts. Our results indicate that hypoxic tumor cells are not more resistant to BSO-induced GSH depletion than non-hypoxic cells, consistent with the hypothesis that overexpression of γ-GCS is the likely mechanism responsible for increased GSH levels in hypoxic areas of ME180 and SiHa tumors.

In a preceding study, we have used mercury orange to stain NPSH and the 2-nitroimidazole derivative EF5 to label tissue hypoxia (Moreno-Merlo et al., 1999). This dual-labeling fluorescence technique allows for visualization of NPSH and hypoxia in the same frozen tissue section, thus permitting the analysis of their spatial correlation. Since fluorescence quantification is expressed in relative units, we were interested to compare the results obtained by image analysis to a biochemical assay. We have previously adapted a sensitive HPLC technique, based on electrochemical detection of NPSH, for measurements of GSH and cysteine in single frozen tissue sections. The results obtained by these two techniques are closely correlated (Chapter 2). In ME180 and SiHa tumors, GSH concentrations were in reasonable agreement with published data (Allalunis-Turner
et al., 1988). This finding is consistent with previous results in experimental (Koch and Evans, 1996) and patient tumors (Guichard et al., 1990). Treatment with BSO produced a ~50% reduction in GSH levels; this rate of decrease is typical for experimental tumors treated with similar BSO dose regimens (Minchinton et al., 1984; Yu and Brown, 1984).

Using the binary EF5 images to segment grayscale mercury orange fluorescence images, we have measured the relative content of GSH in hypoxic and non-hypoxic regions of ME180 and SiHa tumors. In controls, the GSH levels were higher in hypoxic areas, confirming our previous results obtained using a different image analysis technique (Moreno-Merlo et al., 1999). Treatment with BSO, however, effectively abolished the difference in GSH levels between hypoxic and non-hypoxic areas in both tumor types. Therefore, the relative decrease in GSH levels was more pronounced in hypoxic (by 62 and 47% in ME180 and SiHa tumors, respectively) than in non-hypoxic (by 48 and 33% in ME180 and SiHa tumors, respectively) tumor cells. This observation is consistent with (i) overexpression of γ-GCS being the likely mechanism responsible for increased GSH levels in hypoxic regions of ME180 and SiHa tumors, and (ii) faster GSH turnover in hypoxic areas and/or reduced ability of hypoxic cells to use alternative pathways to maintain their NPSH levels.

In the future we intend to characterize the tumor microenvironment using multiparameter fluorescence techniques with respect to spatial arrangements of the tumor vasculature, tissue perfusion and hypoxia, and to study the expression of enzymes involved in the maintenance of cellular NPSH levels.
CHAPTER 4: Multiparameter fluorescence mapping of non-protein sulfhydryl status in relation to blood vessels, perfusion and hypoxia in cervical carcinoma xenografts

\^This chapter is the modified text of a paper submitted for publication to Cytometry. The authors of the paper are Vojislav Vukovic, Trudey Nicklee and David W. Hedley. All experimental work was done by the author of the thesis or under his direct supervision.
4.1 Abstract

**Background:** Irregular vasculature architecture and temporal fluctuations in blood flow can result in chronic and intermittent tumor hypoxia. The aim of this study was to classify tumor hypoxia based on distance to blood vessels and tumor perfusion, and to characterize its biological significance by determining levels of non-protein sulfhydryls (NPSH) in regions of chronic and intermittent hypoxia.

**Methods:** A triple fluorescence method was developed for the spatial colocalization of the vasculature, perfusion, and hypoxia in frozen sections from SiHa cervical carcinoma xenografts. A parallel section was stained with the sulf hydryl stain mercury orange. Composite fluorescence images were generated by imaging and tiling individual fields of view into 2D image arrays. Image arithmetic techniques were combined with distance-based image segmentation to characterize expression of NPSH in the hypoxic tumor microenvironment.

**Results:** NPSH levels were higher in hypoxic areas of the SiHa xenografts (15.1 ±0.5 vs. 13.5 ±0.5 grayscale, p<0.03). When tumor hypoxia was classified by distance to the nearest blood vessel, significantly higher NPSH levels were found in hypoxic regions in proximity to blood vessels than in regions at a distance from blood vessels.

**Conclusion:** The results of this study indicate differential expression of NPSH levels in regions of intermittent and chronic hypoxia in SiHa tumors.
4.2 Introduction

The presence of hypoxia has been demonstrated in a number of human malignancies, such as soft tissue sarcomas, squamous cell carcinomas of the head and neck, and of the uterine cervix, breast carcinomas, melanomas and prostate carcinomas (Vaupel et al., 1991; Lartigau et al., 1993; Fuller et al., 1994; Brizel et al., 1994; Movsas et al., 1999; Lyng et al., 1997; Lartigau et al., 1997). Hypoxic tumors, i.e., those with a significant proportion of low pO$_2$ values are less amenable to local tumor control by radiation therapy and are more likely to produce local and distant metastases (Vanselow et al., 2000; Brizel et al., 1996; Hockelet al., 1996; Fyles et al., 1998; Knocke et al., 1999; Molls et al., 1994; Rofstadt et al., 2000). These findings suggest that tumor hypoxia can contribute to the emergence of both therapy-resistant and biologically aggressive tumor phenotypes.

Thomlinson and Gray (1955) recognized that areas of necrosis were usually found in human cancers at distances from blood vessels that exceed the expected oxygen diffusion distance in tissue. This observation is consistent with the presence of oxygen gradients in tumor tissues; cells that are located further away from blood vessels are chronically exposed to reduced levels of oxygen. In addition, factors such as rheologic effects on erythrocyte deformability in the tumor vasculature, stasis of flow in tumor blood vessels, and longitudinal gradients of partial pressure of oxygen (pO$_2$) along the vascular tree (Dewhirst et al., 1999) have also been postulated to contribute to tumor hypoxia. Transient fluctuations in tumor circulation/perfusion can result in intermittent hypoxia and oxidative stress (Parkins et al., 1997). Intermittent episodes of hypoxia have been demonstrated in experimental tumors using electrode measurements of oxygenation.
(Dewhirst et al., 1998) and fluorescence labeling of perfusion (Durand and LePard, 1995). In those studies, transient flow was demonstrated in up to 50% of all tumor blood vessels. Similar observations were made in clinical tumors using laser Doppler flowmetry (Pigott et al., 1996). Therefore, the oxygenation status of tumors is characterized by the presence of chronic (diffusion-limited) and intermittent (perfusion-limited) hypoxia; however, current clinical diagnostic methods do not discriminate between these physiological entities.

The renewed interest in tumor hypoxia has resulted in a better understanding of molecular mechanisms involved in the response of tumor cells to the hypoxic microenvironment. A number of studies have investigated the adaptive responses of cells exposed to low oxygen conditions and oxidative stress; conditions that are likely spatially co-localized in solid tumors. The hypoxia-inducible factor 1 (HIF-1) is a transcription factor that is induced under conditions of reduced oxygenation and oxidative stress (Carmeliet et al., 1998; Forsythe et al., 1996; Iyer et al., 1998; Ryan et al., 1998; Mercurio and Manning, 1999). Oxidative stress also results in the upregulation of redox-sensitive transcription factors, e.g., Nuclear Factor kappa B (NFκB) (Wang and Semenza, 1993; Gius et al., 1999). Both HIF-1 and NFκB can mediate increased expression of enzymes involved in the maintenance of cellular glutathione (GSH) levels: γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthase. In addition, NFκB can also upregulate the expression of γ-glutamyl transpeptidase (γ-GT) (Kondo et al., 1999), an extracellular glutathionase involved in GSH recycling (Meister and Anderson, 1983).

Using a dual fluorescence labeling method, we have previously found elevated levels of non-protein sulfhydryls (NPSH) in hypoxic regions of human cervical
carcinoma xenografts (Moreno-Merlo et al., 1999). The aims of the present study were to characterize hypoxia in SiHa human cervical carcinoma xenografts with respect to tumor perfusion and distance to blood vessels, and to determine NPSH levels in areas of intermittent and chronic hypoxia. Our results suggest that (i) regions of both intermittent and chronic hypoxia may be present in SiHa xenografts and (ii) regions of presumably intermittent hypoxia have higher NPSH levels than oxic or chronically hypoxic tumor areas.

4.3 Materials and methods

4.3.1 Establishing of tumor xenografts

The SiHa cell line was obtained from ATCC and maintained in α-MEM, supplemented with 10% FBS and 0.1 mg/mL kanamycin. One million cells in a volume of 50 μl was injected into the left gastrocnemius muscle of ten 6-8 week old female SCID mice. After 11-12 days of growth when the tumors reached a size of 4-5 mm in diameter, mice were injected intravenously with 200 μl of a 10 mM 2-[2-nitro-1H-imidazol-1-yl]-N-(2,2,3,3-pentafluoropropyl) acetamide (EF5) stock solution, resulting in a total EF5 body concentration of 100 μM. The DNA minor groove-binding fluorescent dye Hoechst 33342 was used for visualization of tissue perfusion. Two hours and 40 minutes later, the animals were injected intravenously with 50 μl of a 3 mM Hoechst 33342 solution. Twenty minutes after application of the Hoechst 33342 dye, mice were sacrificed by
cervical dislocation, the tumors were quickly removed, placed into Tissue-Tek OCT embedding medium (Sakura Finetek, Torrance, CA) and snap frozen in liquid nitrogen.

4.3.2 Preparation of tumor sections and immunohistochemistry

Two groups of three 5 μm thick serial sections were cut from each frozen tumor using a HM 500 OM Microtome Cryostat (Microm Laborgeraete, Walldorf, Germany). The sections were adhered to 3-aminopropyl triethoxysilane (Sigma, St Louis, MO) treated glass microscope slides. The first section was fixed in 3.7% neutral buffered formalin for 10 minutes, rinsed and stained with hematoxylin and eosin (H&E). The second section was stained for NPSH with the sulphydryl-reactive dye mercury orange (Sigma, St Louis, MO). Mercury orange was first dissolved in acetone, then distilled water was added to produce a final concentration of 75 μM in 9:1 (v/v) acetone-water. In order to minimize the loss of reduced thiols through oxidation, the sections were cut and rapidly placed in the mercury orange solution and stained on ice for 5 minutes, followed by two rinses with 9:1 acetone-water. After rinsing with PBS, the slides were air dried. The third section was fixed in 3.7% neutral buffered formalin for 10 minutes, rinsed and stained with an anti-PECAM-1 (CD31) mAb (Pharmingen, San Diego, CA), using a 1:100 dilution, followed by incubation with a goat-anti-rat secondary antibody conjugated to the carbocyanine dye Cy3 (Jackson Immunoresearch, West Grove, PA). Tissue-bound EF5 was labeled with an anti-EF5 mAb (ELK3-51) conjugated directly to Cy5, supplied by Dr. Cameron Koch, University of Pennsylvania. All incubations were done for 1 hour at room temperature, and were followed by three rinses in PBS.
4.3.3 Fluorescence microscopy and image acquisition

The immunohistochemically labeled sections were imaged using a M5+ imaging system (Imaging Research Inc., St. Catharines, ON, Canada), linked to a Quantix cooled CCD video camera (Photometrix Inc., Tuscon, AZ). This system was integrated with a reflected fluorescence Olympus BX50 microscope fitted with a Ludl Biopoint computer controlled motorized stage. Using an UPlanFl 10x 0.3 N.A. objective (Olympus), individual fields of view were excited sequentially with UV (320-360nm), green (541-569nm) and red (630-650nm) light, using a filter wheel under computer control. A quadruple band pass cube was used to collect the emitted fluorescence at 450-470 nm, 590-610nm and 660-710nm. The resulting three 8-bit grayscale images were stored as 24-bit RGB files. Images of individual fields of view were tiled into a 2D array, to generate wide area images of the entire tumor cut surface (Figure 20). Cy5 and Cy3 spectral crosstalk was observed due to the technical limitation on setting fluorescence collection times individually for the respective channels. Therefore, Cy3 was used for labeling of blood vessels (CD31) and Cy5 for labeling of EF5; the rationale for this choice was that these two parameters are likely mutually exclusive in tissue, and the fluorescence spillover would be easier to remove by manual image editing. The maximal Cy5 crosstalk signal intensity was approximately 1/3 of the average Cy3 intensity, and in most cases was removed by simple brightness thresholding.
4.3.4 Image processing and analysis

After acquisition, the images were reduced in size by 2x2 pixel binning using the Adobe Photoshop v. 5.0 software, to facilitate subsequent processing and analysis. Mercury orange fluorescence grayscale images were converted from 12- to 8-bit. Using the M5+ software, the mercury orange fluorescence images were aligned with the triple stained RGB images, using image features as fiducial points. After image alignment, individual color channels from the 24-bit RGB triple-stained images were extracted using the Adobe Photoshop software and stored as separate 8-bit image files of the Hoechst 33342, Cy3 (anti-CD31) and Cy5 (anti-EF5) fluorescence. Fluorescence artifacts (dust, scratches) were manually edited where necessary. Subsequent image processing and analysis was done using an application developed in the Interactive Data Language (IDL, version 5.1, Research Systems Inc., Boulder, CO).

Figure 20: Triple fluorescence images of two SiHa xenograft tumors

Note the difference in EF5 staining area size and intensity (red color) and Hoechst 33342 perfusion (blue color) between the more hypoxic (panel A) and the less hypoxic tumor (panel B). Blood vessels are shown in green.
Creation of tumor distance masks

Tumor distance masks were created using blood vessels as anatomical landmarks. The distance masks were designed for mapping of the entire tumor section as a function of distance to the nearest blood vessel. First, CD31 images were binarized using a constant value to set the brightness threshold. Positive pixels in the binarized CD31 images were set as centers of square masks with 4 different side lengths: 100, 200, 300 and 400 μm. The CD31 images were then convolved with the masks in descending order; the pixels belonging to the respective distance classes were assigned different brightness values. Finally, the distance mask image was restricted to tumor boundaries using a binarized image of the mercury orange fluorescence as the tumor mask (Figure 21).

Figure 21: Distance to the nearest blood vessel map of a SiHa xenograft tumor

White pixels: location of the blood vessels. Areas in different shades of gray represent different distance classes (0-50 to >200 μm) from the nearest blood vessel.
Creation of perfusion masks

Hoechst 33342 binds to the DNA minor groove; therefore, only the part of the total cell area occupied by the nucleus is labeled. To normalize the nuclear staining areas to whole cell areas, three regions of high Hoechst 33342 binding per image were chosen and manually outlined in binarized Hoechst 33342 images. By analyzing the brightness value histograms of these regions, approximately one third of all pixels were found positive. Subsequently, binarized Hoechst 33342 fluorescence images were dilated (Figure 22), to extend the nuclear stained area to the total stained cell area. Image dilation was performed using the IDL library function MORPH_DILATE. Nuclear areas approximated the cellular areas when a 3x3 morphological operator was used for dilatation. The tumor perfusion masks were used for EF5-perfusion and NPSH-perfusion co-localization analyses.

Figure 22: Morphometric dilatation of a Hoechst 33342 image
A: Original Hoechst 33342 fluorescence image; B: Hoechst 33342 fluorescence image dilated using a 3x3 morphologic operator and restrained to the tumor area.
4.4 Results

4.4.1 Spatial distribution of hypoxia, perfusion and NPSH

Tumor hypoxia, perfusion and NPSH levels were determined as a function of distance to the nearest blood vessel. As shown in Figure 23, the spatial distribution of EF5 staining in SiHa tumors was heterogeneous. The percent of total tumor EF5 staining in the 0-50 μm distance class varied from 7.5 to 81.4%; therefore, a significant proportion of the EF5 staining was located well within the typical oxygen diffusion distance in tissue.

![Graph showing hypoxia as a function of distance from the nearest blood vessel](image)

Figure 23: Hypoxia as a function of distance from the nearest blood vessel

The curves represent EF5-to-blood vessel distance histograms for 10 individual tumors. Data points in the histogram were connected using a bicubic spline interpolation function.
Spatial EF5 staining patterns demonstrated significant intertumoral variability. In some tumors, >85% of the total EF5 staining was located within 100 μm from the nearest blood vessel. In a different tumor, however, ~70% of the total EF5 staining was located beyond 100 μm from the nearest blood vessel.

![Figure 24: Tumor perfusion as a function of distance from the nearest blood vessel](image)

The curves represent Hoechst 33342-to-blood vessel distance histograms for 10 individual tumors. Data points in the histogram were connected using a bicubic spline interpolation function.

Spatial patterns of perfusion in SiHa tumors are shown in Figure 24. In contrast to EF5 staining, Hoechst 33342 fluorescence decreased relatively uniformly as a function of
distance to blood vessels. In all cases, >80% of the Hoechst 33342 positive area was localized within 150 μm from the nearest blood vessel.

When mercury orange fluorescence distribution was related to the distance to the nearest blood vessel there was a rapid decline from 0-150 μm from blood vessels with little staining beyond (see Figure 25), indicating that significant cellular NPSH levels cannot be maintained beyond the oxygen diffusion distance in tissue.

Figure 25: NPSH levels as a function of distance from the nearest blood vessel
The curves represent NPSH-to-blood vessel distance histograms for 10 individual tumors. Data points in the histogram were connected using a bicubic spline interpolation function.
4.4.2 Tumor NPSH levels

Tumor NPSH levels were determined in hypoxic vs. non-hypoxic, and in perfused vs. non-perfused tumor areas. Hypoxic areas were classified as intermittently or chronically hypoxic, based either on their distance to the nearest blood vessel or according to their perfusion status, as determined by co-localization with Hoechst 33342 fluorescence.

Using binary EF5 images for segmentation of the mercury orange fluorescence images, we have found higher NPSH levels in hypoxic than in non-hypoxic areas of SiHa tumors (15.1 ±0.5 and 13.5 ±0.5 grayscale, t-test p<0.03; Figure 26), thus confirming our previous findings (Moreno-Merlo et al., 1999).

![Figure 26: NPSH levels in hypoxic and non-hypoxic areas of SiHa xenografts](image)

Mean mercury orange fluorescence values in EF5-positive (black bars) and EF5-negative tumor areas. Error bars are SEM.
Classification of hypoxia based on distance to nearest blood vessel

Shown in Figure 27 are the NPSH levels in areas of intermittent and chronic hypoxia, classified as a function of distance to the nearest blood vessel. The distance classes were dichotomized as intermittent and chronic at distances of 50, 100 and 150 μm to the nearest blood vessel. In regions of intermittent hypoxia mean mercury orange fluorescence values remained fairly constant; in chronically hypoxic areas, however, mean mercury orange fluorescence values changed significantly as a function of split distance (Figure 27 and Table 4).

![Figure 27: NPSH levels in areas of intermittent and chronic hypoxia](image)

The distance classes were dichotomized as intermittent and chronic at distances of 50, 100 and 150 μm to the nearest blood vessel. Bars are mean values, error bars are SEM.
<table>
<thead>
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<th>Classification of hypoxia by the tumor perfusion status</th>
</tr>
</thead>
</table>

Tumor hypoxia was also classified as chronic and intermittent based on the perfusion status of EF5-positive regions. Using the binary Hoechst 33342 images as masks, binary images of EF5 fluorescence were segmented into two classes: perfused and non-perfused EF5-positive areas (Figure 28), followed by measurements of mercury orange fluorescence intensities in the respective classes. Quantification of hypoxic and perfused tumor areas revealed an inverse relationship with a strong correlation \((r^2=0.82)\) of these two parameters (Figure 29). This observation suggests that chronic (diffusion-limited) hypoxia may be a major component of the total tumor hypoxia in SiHa xenografts. However, EF5-positive areas were also partly co-localized with Hoechst 33342-positive areas, thus indicating the presence of intermittent (perfusion-limited) hypoxia in SiHa xenografts. The proportion of perfused EF5 areas varied widely in individual images, ranging from 0.5% to 19.9% of the total EF5-positive areas (see Table 5).
Figure 28: Intermittent and chronic hypoxia (classified by perfusion)
White areas represent non-perfused, gray areas represent perfused EF5-positive areas.

Figure 29: Association of tumor hypoxia and perfusion
Perfused regions in SiHa xenografts contained the majority of the total NPSH (Figure 30A), consistent with the analysis of spatial distribution of the Hoechst 33342 and mercury orange staining (Figures 24 & 25). Mean values of mercury orange fluorescence were almost identical in perfused and non-perfused areas (14.7 ± 1.4 and 14.4 ± 1.2 grayscale, p < 0.29, t-test; see Figure 30B).

Measurement of NPSH levels in perfused and non-perfused hypoxic areas showed that the mean mercury orange fluorescence intensities were slightly higher but not significantly different in perfused than in non-perfused EF5-positive areas; 17.7 ± 3.3 IOD and 16.4 ± 2.5 IOD (p < 0.17, t-test) in areas of intermittent and chronic hypoxia, respectively (see Figure 30C). Summarized, our results indicate that the NPSH levels in SiHa xenografts are similar in perfused and non-perfused areas, regardless of their oxygenation status.

<table>
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<tr>
<th>Image</th>
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<th>Perfused EF5 positive area [% of EF5 positive area]</th>
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Table 5: Colocalization of tumor hypoxia and perfusion
Figure 30: NPSH levels in relation to tumor perfusion status

The proportion of total mercury orange fluorescence in perfused vs. non-perfused areas is shown in panel A. Mean mercury orange fluorescence intensities in perfused and non-perfused areas are shown in panel B. Mean mercury orange fluorescence intensities in areas of intermittent and chronic hypoxia are shown in panel C. Bars are mean values, error bars are SEM.
4.5 Discussion

In this study we have applied quantitative multiparameter fluorescence microscopy and advanced image analysis techniques to characterize the relationship of hypoxia and NPSH levels in human cervical carcinoma xenografts. Using the distance to the nearest blood vessel as a criterion for classification of hypoxia, increased NPSH levels were found in areas of intermittent hypoxia, compared to non-hypoxic or chronically hypoxic regions.

The functional oxygenation status of tumors is often assessed by the use of hypoxia markers, such as the nitroimidazole derivative EF5 (Lord et al., 1993; Evans et al., 2000). In SiHa tumors, we have found the EF5 positive areas to comprise 0.5-11% of the total tumor area. Bussink et al. (1999) found very similar values for areas of hypoxic staining (3-10% of total tumor area) in laryngeal squamous cell carcinoma xenografts using the bioreductive compound 2-nitroimidazole-theophylline (NITP). It is of interest that tumors used in that study were approximately 8x larger in volume (R=0.6-0.8cm) than the tumors used in the present study (R=0.3-0.4cm).

Blood vessels are often utilized as anatomical and physiological landmarks for spatial domain analyses of the tumor phenotype (Rijken et al., 1995 and 2000; Bernsen et al., 1995; Haustermans et al., 2000; Fenton et al., 2000). Three monoclonal antibodies are routinely used for visualization of tumor blood vessels, the anti-von Willebrandt factor (factor VIII), anti-CD34 and anti-PECAM-1 (CD31) (Kuzu et al., 1992). We have chosen to use the anti-CD31 mAb since its cross-reactivity is limited to extravasated lymphocytes and macrophages. In our hands, the anti-CD31 mAb shows minimal cross-reactivity in cervical carcinoma xenografts using immunohistochemical staining in
combination with hematoxylin counterstaining (typically <1% of the total positive area). Furthermore, positively stained immunocompetent cells are readily discernible from the blood vessel staining based on differences in shape, size and distribution, and can be easily excluded from the analysis.

NPSH and in particular GSH have been traditionally associated with therapy-resistant tumor phenotypes (Meister, 1991); therefore, it is of importance to understand mechanisms that regulate the expression of NPSH in solid tumors. In a previous study we have addressed the issue of intra- and intertumoral heterogeneity of NPSH levels and found typically a twofold range in NPSH levels in human cervical carcinomas (Vukovic et al., 2000). One of the possible mechanisms responsible for the observed heterogeneity in NPSH levels is tumor hypoxia; expression of enzymes involved in the metabolism of NPSH is increased under conditions of reduced oxygenation and oxidative stress (Markey et al., 1998; Shi et al., 1994). Using a different image analysis technique, we have previously found increased levels of NPSH in hypoxic regions of SiHa and ME180 xenografts (Moreno-Merlo et al., 1999). In order to further characterize the relationship between NPSH and tumor hypoxia, EF5-positive areas were classified as areas of intermittent and chronic hypoxia based on two criteria: the distance to nearest blood vessel and tissue perfusion, as determined by Hoechst 33342.

One technical consideration in blood vessel/hypoxia colocalization studies was the issue of Cy3/Cy5 fluorescence ‘crosstalk’, due to non-specific Cy5 excitation with the Cy3 filter set. Since the Cy5 fluorescence intensity was only approximately 30% of the Cy3 fluorescence signal, the separation was done by brightness thresholding. This approach likely removed the majority of the non-specific Cy5 fluorescence (hypoxia)
from Cy3 images (blood vessels), thus minimizing the spectral ‘crosstalk’ and artificial spatial overlap of these parameters. Another technical limitation is that spatial relationships of hypoxia and blood vessels were assessed in individual frozen sections, i. e., two-dimensional approximations of three-dimensional tumor volumes, thus introducing a certain amount of error into the analysis.

The mean mercury orange fluorescence intensities in EF5-positive areas decreased as a function of distance from the nearest blood vessel, thus indicating that NPSH levels were higher in areas of intermittent than in areas of chronic hypoxia. Low NPSH levels in regions of chronic hypoxia are consistent with impaired cellular metabolic status. Further studies are needed to clarify whether the upregulation of NPSH levels in intermittently hypoxic regions is mediated by hypoxia- and/or oxidative stress-mediated stimuli.

The live cell DNA fluorescence stain Hoechst 33342 has been used extensively to characterize tumor perfusion, either as a single agent (Olive et al., 1985; Trotter et al., 1990) or in combination with carbocyanine dyes, i. e, DiOC7 (Trotter et al., 1989). In this study, we have used Hoechst 33342 to label perfused areas of the tumor rather than as a marker for perfused blood vessels (Durand and Raleigh, 1998). Colocalization analysis of EF5 and Hoechst 33342 fluorescence showed that 0.5 to 19.9% of the total EF5-positive areas were stained with Hoechst 33342, thus suggesting the presence of intermittent hypoxia in SiHa xenografts. It should be noted, however, that Hoechst 33342 was utilized to label perfusion over twenty minutes prior to tumor excision, whereas the EF5 staining indicates the ‘history’ of hypoxia over 2-3 hours prior to tumor excision. Therefore, the
colocalized area may not represent the true degree of intermittent perfusion in these tumors.

The analysis of distance profiles has indicated that >80% of the Hoechst 33342 staining was located within 100 μm from the nearest blood vessel, the typical oxygen diffusion distance in tissue (Tannock, 1968; Olive et al., 1992). Interestingly, the distance profiles showed a very similar distribution of NPSH; 78-95% of the total mercury orange fluorescence was located within 100 μm from the nearest blood vessel. This result is consistent with the analysis of spatial distribution of NPSH levels in hypoxic areas, and indicates that the maintenance of NPSH levels is dependent on the cellular metabolic status.

Intravenous application of Hoechst 33342 results in a transient reduction of the systemic blood pressure and reduced blood flow in tumors (Trotter et al., 1990); to maximize the proportion of stained vasculature and tissue, tumors should be removed 10-20 minutes after injection of the dye. At the doses used in this experiment, the half-life of Hoechst 33342 is ~2 minutes (Olive et al., 1985); therefore, large differences in staining intensity may occur between areas supplied by blood vessels that were open during and shortly after the injection and areas that were not perfused during that period. For this reason, Hoechst 33342 fluorescence images were binarized and no attempt was made to quantify the absolute amount of fluorescence in the images. Mean mercury orange fluorescence intensities were not significantly different in perfused vs. non-perfused EF5-positive regions, regardless of the brightness threshold value used for binarization of Hoechst 33342 fluorescence images. This contrasts with the results obtained using distance from blood vessels as a criterion for intermittent vs. chronic hypoxia.
Considering the uncertainties associated with labeling of tissue perfusion using Hoechst 33342 as the only parameter, these results suggest that quantification of perfusion may be required for adequate classification of tumor hypoxia. Irregular blood flow patterns can be detected by the sequential application of fluorescent perfusion markers Hoechst 33342 and DiOC₇ (Trotter et al., 1989). Intermittently perfused blood vessels can be identified by registration of staining mismatch, since oscillations in blood flow result in staining of blood vessels with only one perfusion marker. This step is followed by distance-based classification of tumor hypoxia selectively in continuously and intermittently perfused tumor blood vessels. The expectation is that EF5 staining would be located in the vicinity of intermittently perfused blood vessels and at and beyond the functional oxygen diffusion distance in areas surrounding continuously perfused blood vessels.

Another potentially useful combination of perfusion studies and distance-based measurements would be to determine the Hoechst 33342 diffusion distance for each individual blood vessel. This information could be used as an indicator of the potential oxygen diffusion distance in tissue. The expectation is that the majority of the EF5-positive staining would be detected at and beyond individual Hoechst 33342 diffusion distances.

This study illustrates the potential of quantitative multiparameter fluorescence microscopy and image analysis in characterizing the biological significance of tumor hypoxia. In future studies, we will analyze the expression patterns of the hypoxia- and redox-sensitive transcription factors HIF-1α and NFκB in the context of the hypoxic tumor microenvironment.
CHAPTER 5: Summary and future directions
5.1 Introduction

The non-protein sulphydryl glutathione has numerous functions in cells; perhaps the most significant one is the involvement in maintenance of the cellular redox status. The role of glutathione in tumor cells has been extensively studied, since elevated glutathione levels have been associated with resistance of tumors to chemo- and radiotherapy. At present, however, it is not clear whether increased cellular glutathione levels are the cause or merely the consequence of therapy-resistant tumor phenotypes.

Of particular interest is the association of glutathione with other determinants of the therapeutic resistance in tumors, e.g., hypoxia. Recent insights into molecular mechanisms that characterize the response of cells to conditions of reduced oxygenation and oxidative stress have identified HIF-1, NFκB, AP-1 and 2, ARE/EpRE and SP-1 binding motifs in the promoter regions of genes that code for the heavy and light subunits of γ-GCS. Increased levels of γ-GCS mRNA and glutathione levels have been observed in cultivated colon cancer cells exposed to hypoxia-reoxygenation; furthermore, increased levels of NPSH were found in hypoxic regions of human cervical carcinoma xenografts. Taken together, these observations are consistent with the upregulation of cellular NPSH levels in response to the hypoxic tumor microenvironment.
5.2 Summary and discussion of results

The clinical prospective study on heterogeneity of NPSH levels in cervical carcinomas is presented in Chapter 2. This study was conducted in patients enrolled into a trial studying the prognostic value of oxygenation status in cancers of the uterine cervix for radiation treatment outcome (Fyles et al., 1998). Published results on intra- and intertumoral heterogeneity of NPSH levels in solid tumors are conflicting. While some studies have reported on intratumoral variability in glutathione and cysteine levels up to a factor of 10x (Guichard et al., 1990), others have found only a 2-3x difference in glutathione levels in human tumors (Cook et al., 1991). One of the aims of this project was to assess the intra- and intertumoral heterogeneity of tumor NPSH levels; therefore, multiple punch biopsies were obtained from individual tumors. Glutathione and cysteine levels were determined in serial frozen tumor sections using a sensitive HPLC method with electrochemical detection and staining of a parallel section with the sulfhydryl dye mercury orange, followed by analysis of mercury orange fluorescence images. This approach allows for a close correlation of the HPLC and the relative fluorescence quantification method; the quantitative nature of the HPLC measurement is complemented by the ability to determine spatial variability of NPSH levels in tumor tissue at microscopic resolution.

Analysis of variance showed that the intratumoral variability (pooled SD = 0.39) of glutathione concentrations was smaller than the intertumoral variability (SD = 0.89, ANOVA, p<0.001). The same relationship of intra- and intertumoral variability was found for cysteine (pooled SD = 0.25; SD = 0.37, ANOVA, p<0.001).
The finding of high cysteine concentrations confirmed previous reports in experimental tumors (Koch and Evans, 1996) and human cervical carcinomas (Guichard et al., 1990). Cysteine is a more potent DNA radioprotector than glutathione (Bump et al., 1992). The proposed mechanism for the increased radioprotective properties of cysteine is its neutral charge at physiological pH values, and therefore, better accessibility to the negatively charged DNA phosphate backbone (Aguilera et al., 1992). The presence of cysteine in the milimolar concentration range has obvious clinical implications, since the majority of cervical carcinomas in advanced stages are treated with radiation therapy.

Using a computerized microscope stage, composite images were generated by tiling images of individual fields of view into 2D image arrays, thus allowing for imaging of the entire tumor cut section. Twofold higher levels of mercury orange fluorescence were found in areas populated predominantly with tumor cells compared to surrounding non-malignant tissue, consistent with studies on NPSH distribution in a number of human tumors (Honegger et al., 1988; Langemann et al., 1989; Cook et al., 1991; Hochwald et al., 1997; Chang et al., 1993; Murray et al., 1987; Wong et al., 1994; Perry et al., 1993; El-Sharabasy et al., 1993). The mean mercury orange fluorescence values integrated over entire tumor tissue sections varied by a factor of three (range: 26-79 IOD), similar to the variability observed using HPLC (~2.5x for glutathione, ~3x for cysteine), indicating a good correlation of these methods. Since our measurements were done in tissue sections, areas of necrosis, keratinization, hemorrhage and gross extravasation of immunocompetent cells were excluded from analysis. This discerning selection process has likely contributed to the relatively narrow range of NPSH levels, similar to the variability observed by Cook et al. (1991). These authors have used surgical excision
biopsies and therefore, could have selected macroscopically viable tumor tissue for their analyses. Most of the other studies were done on bulk NPSH measurements in core or punch biopsies, often without histological exclusion of necrosis or non-malignant tissue.

The finding of higher NPSH levels in hypoxic than in non-hypoxic tumor regions (Moreno-Merlo et al., 1999) suggests that tumor cells in hypoxic regions actively regulate their NPSH levels in response to the tumor microenvironment. Mechanisms that underlie increased NPSH levels in hypoxic regions may include increased glutathione synthesis and/or more efficient salvaging of extracellular glutathione. The rate-limiting enzyme for glutathione de novo synthesis is gamma-glutamylcysteine synthetase (γ-GCS); the key enzyme in recycling of extracellular glutathione is gamma-glutamyl transpeptidase (γ-GT). γ-GCS and γ-GT contain in their promoter regions binding sites targeted by transcription factors that are induced by oxidative stress (Mulcahy et al., 1997; Rahman et al., 1996a and 1996b; Haddad and Land, 2000; Moellering et al., 1999). O'Dwyer et al. (1994) observed increased γ-GCS and glutathione levels in HT29 colon cancer cells exposed to hypoxia-reoxygenation. Overexpression of γ-GCS and γ-GT has been associated with increased resistance of tumor cells to chemo- and radiotherapy (Richardson and Siemann, 1994; Bailey et al., 1992; Hanigan et al., 1999, Prezioso et al., 1994).

This observation led us to study the effects of buthionine sulfoximine (BSO), a specific inhibitor of γ-GCS (Griffith and Meister, 1979), on NPSH levels in hypoxic and non-hypoxic regions of ME180 and SiHa cervical cancer xenografts (Chapter 3). The hypothesis tested was that increased levels of γ-GCS significantly contribute to elevated NPSH levels in hypoxic regions of ME180 and SiHa xenografts. The purpose of this
study was to further characterize the association of hypoxia and NPSH by elucidating the mechanism(s) responsible for increased NPSH levels in hypoxic tumor regions. The rationale behind choosing γ-GCS as the primary candidate for the study was that although increased γ-GT activity has been associated with the maintenance of cellular glutathione levels, there are no published reports that γ-GT upregulation actually results in increased cellular glutathione levels.

To label hypoxia in tissue, all mice were treated with the nitroimidazole EF5 (2-[2-nitro-1H-imidazol-1-yl]-N-(2,2,3,3,3-pentafluoropropyl) acetamide). Upon bioreduction EF5 can bind to macromolecules in regions of reduced oxygenation and, therefore, selectively label hypoxic cells (Lord et al., 1993; Evans et al., 2000). As in the previous study, NPSH levels were assessed in parallel sections of frozen tumors by HPLC and by mercury orange fluorescence quantification; an additional serial section was stained with H&E and used as control of tissue architecture. Treatment with BSO resulted in a decrease of glutathione levels by 60% in ME180 and by 55% in SiHa cells. The observed decrease is in accordance with studies on glutathione depletion by BSO in experimental and human tumors (Lee et al., 1987; Minchinton et al., 1984; Yu and Brown, 1984; Bailey et al., 1994). The question might be raised why BSO treatment in vivo does not result in more profound glutathione depletion, as often seen in cultured cells (Biaglow et al., 1983a). Analyzing the pharmacokinetic and pharmacodynamic properties of BSO, Bailey et al. (1994) concluded that an optimized BSO-treatment protocol in patients should include a loading dose, followed by continuous BSO infusion, resulting in GSH levels <10% of baseline values.
Frozen tumor sections were double-stained with mercury orange and the anti-EF5 mAb ELK3-51. Mean mercury orange fluorescence intensities of untreated controls were higher in ME180 than in SiHa xenografts, consistent with slightly higher glutathione and higher cysteine levels in ME180 tumors determined by HPLC. Grayscale EF5 fluorescence images were converted to binary images and used as masks for segmentation of mercury orange fluorescence images. NPSH levels were higher in hypoxic than in non-hypoxic regions of ME180 and SiHa tumors, in agreement with previous results obtained using a different image analysis technique (Moreno-Merlo et al., 1999). Treatment with BSO resulted in a larger decrease of mercury orange fluorescence in hypoxic than in non-hypoxic areas of ME180 and SiHa tumors, therefore effectively abolishing the preexisting gradient in NPSH levels between hypoxic and non-hypoxic tumor regions. This observation is consistent with the hypothesis that increased NPSH levels are a consequence of γ-GCS overexpression in hypoxic tumor areas. Since upregulation of γ-GCS has been associated with oxidative stress rather than with hypoxia per se (Mulcahy et al., 1997; Rahman et al., 1996a and 1996b), it seems plausible that elevated NPSH levels occur in intermittently rather than in chronically hypoxic tumor regions.

The presence of tumor hypoxia has been found to correlate with poor patient outcome in a number of human malignancies; specifically with decreased rate of local tumor control by radiation therapy and decreased disease-free survival caused by regional and distant metastases (Hockelet al., 1996, Brizei et al., 1996, Fyles et al., 1998; Knocke et al., 1999; Molls et al., 1994; Rofstadt et al., 2000, Vanselow et al., 2000). Therefore,
hypoxia is associated with tumor phenotypes that are characterized by increased resistance to therapy and propensity towards metastasis.

Increasing evidence points to the existence of two distinct types of hypoxia in solid tumors: chronic (diffusion-limited) and intermittent (perfusion-limited). Tumor cells located beyond the oxygen diffusion distance in tissue are considered chronically hypoxic (Thomlinson and Gray, 1955; Tannock, 1968). Irregular spatial arrangement of tumor blood vessels can result in longitudinal pO₂ gradients along blood vessels; therefore, factors such as the cellular metabolic rate, blood flow rate and local oxygen concentration in blood vessels influence the effective oxygen diffusion distance in tumors (Dewhirst et al., 1999; Dewhirst, 1998). Rheologic effects on erythrocyte deformability in combination with transient stasis in flow are typical attributes of intermittent hypoxia in tumors (Kimura et al., 1996; Dewhirst et al., 1998). Temporal oscillations in significant portions of the vasculature have been observed in experimental (Durand and LePard, 1995) and patient tumors (Pigott et al., 1996); intermittent blood flow can result in multiple rounds of hypoxia and reoxygenation (Parkins et al., 1997). Tumor cells in regions of intermittent hypoxia are, therefore, likely characterized by adaptive responses to conditions of reduced oxygenation and oxidative stress, mediated by the ischemia-reperfusion injury. Tumor cells located in intermittently perfused areas belong to the metabolically active part of the tumor cell population. Since these cells are periodically subjected to microenvironmental stresses, it is conceivable that mutation and selection processes in this population occur at a higher rate than in better oxygenated tumor cells. Considering their distance to blood vessels, it is quite unlikely that tumor cells located in areas of chronic hypoxia have a high metabolic rate and undergo cell cycling and
division. One exemption may be the case of tumor reoxygenation in the course of fractionated radiotherapy (Denekamp and Joiner, 1982). Therefore, intermittently perfused tumor cells may actually be the major determinant of the hypoxic tumor phenotype.

The study described in Chapter 4 is an attempt at classification of tumor hypoxia as chronic and intermittent and the assessment of their respective effects on tumor NPSH levels. Tumor hypoxia was classified by two criteria: distance to the nearest blood vessel and spatial correlation of hypoxia and perfusion. Tumor blood vessels have been used as anatomical landmarks in a number of studies (Tannock, 1968; Rijken et al., 1995 and 2000; Bernsen et al., 1995; Haustermans et al., 2000; Fenton et al, 2000); the frequency or levels of different parameters of interest (e.g., cell proliferation, apoptosis) were expressed as a function of distance to blood vessels. Segmentation of an image by distance to blood vessels can be accomplished using several different approaches; in this study, we have created 5 distance classes (see Appendix, p. 132 for a detailed description) and classified the EF5-positive areas as regions of intermittent and chronic hypoxia, followed by quantification of mercury orange fluorescence. One potential consideration is the presence of longitudinal oxygen gradients in tumor blood vessels, resulting in potentially different functional oxygen diffusion distances for individual blood vessels. Furthermore, distance mapping in 2D images is only an approximation of the situation in solid tumors. Despite these limitations, NPSH levels were consistently higher in regions of intermittent hypoxia, regardless of the distance threshold used for classification of hypoxia. NPSH levels in regions classified as chronically hypoxic
decreased with increasing distance threshold values, indicating that maintenance of elevated NPSH levels is dependent on the metabolic status of tumor cells.

The finding of higher NPSH levels in regions of intermittent hypoxia than in better oxygenated and chronically hypoxic regions is consistent with the presence of oxidative stress and its effects on enzymes involved in NPSH metabolism, e. g., γ-GCS and γ-GT. This observation complements the findings from studies described in Chapters 2 and 3, and emphasizes the relative significance of intermittent hypoxia in determining the tumor phenotype.
5.3 Conclusions

The aims of the work presented in this thesis were (i) to further characterize NPSH expression and the relationship between hypoxia and NPSH in cervical cancer, and (ii) to develop and implement analytical approaches and tools for multiparameter fluorescence image analysis. Summarized, the findings from the projects presented in Chapters 2-4 of the thesis are as follows:

- NPSH levels in areas populated predominantly with tumor cells are higher than in the stroma of squamous cell carcinomas of the uterine cervix.
- Intratumoral heterogeneity of NPSH levels is smaller than the intertumoral heterogeneity.
- Significant proportions of hypoxic regions are located within the typical oxygen diffusion distance in tissue, thus suggesting the presence of intermittent hypoxia in SiHa cervical carcinoma xenografts.
- NPSH levels in hypoxic regions are higher than in better oxygenated regions of cervical carcinoma xenografts. NPSH levels are higher in intermittently hypoxic regions than in non-hypoxic or chronically hypoxic regions, consistent with oxidative stress-mediated upregulation of \( \gamma \)-GCS and/or \( \gamma \)-GT.
- Treatment with BSO results in preferential depletion of glutathione in hypoxic regions, further supporting the hypothesis that oxidative stress-mediated upregulation of \( \gamma \)-GCS and/or \( \gamma \)-GT results in increased NPSH levels in hypoxic tumor regions.
5.4 Clinical relevance

The clinical study on microregional heterogeneity of NPSH levels in cervical carcinoma patients has found that levels of cysteine in some tumors may be up to 10x higher than typical cysteine levels in non-malignant tissue. The observed increase in cysteine levels is indicative of alterations in mechanisms that maintain the cellular redox state. On a molar basis, cysteine is a better DNA radioprotector than glutathione (Bump et al., 1992); therefore, elevated cysteine levels may confer increased radioresistance in these tumors.

The recently completed trial at the Princess Margaret Hospital on the prognostic value of oxygenation status in cervical carcinomas for radiation treatment outcome (Fyles et al., 1998) has identified a group of patients with large (diameter > 5 cm) hypoxic tumors (hypoxic fraction 5 mm Hg > 50%), to have a worse prognosis with respect to local tumor control. A retrospective clinical study in frozen tumor biopsies from the patients entered into this trial could directly test the hypothesis that increased levels of cysteine are associated with poor local tumor control.

Moreno-Merlo et al. (1999) reported higher NPSH levels in hypoxic than in better oxygenated regions of cervical carcinoma xenografts. The study on differential effects of BSO on glutathione levels in hypoxic and non-hypoxic tumor regions presented in Chapter 3 has found that administration of BSO can abolish this difference, indicating that increased expression of γ-GCS may be the likely mechanism responsible for increased NPSH levels in hypoxic tumor regions. This finding warrants a clinical study on γ-GCS expression levels in the frozen tumor biopsies; finding of increased levels of γ-
GCS in hypoxic tumor regions would support the addition of BSO as an adjuvant agent to the standard treatment protocol for cervical cancer patients.

The finding of higher NPSH levels in regions of intermittent than in regions of chronic hypoxia emphasizes the importance of further characterization of tumor oxygenation using multiparameter fluorescence imaging and analysis. A phase I clinical study on the feasibility of using EF5 as a marker of tumor hypoxia is currently in progress in the Princess Margaret Hospital. Multiple biopsies from prostate carcinomas, cervical carcinomas and soft tissue sarcomas are being collected and will be assessed using the methods and analytical procedures developed in the course of the preclinical study in human cervical carcinoma xenografts presented in Chapter 4. Classification of hypoxia as intermittent and chronic could result in a biologically more relevant assessment of the tumor oxygenation status. Furthermore, the biological significance of intermittent and chronic hypoxia with respect to established and novel markers of aggressive malignant disease could be directly assessed in human tumors.
5.5 Future directions

The studies presented in this thesis provide an insight into the complex relationship of the hypoxic tumor microenvironment and NPSH. The projects for future work, aimed at clarification and extension of the current studies, fall in three broad categories: preclinical studies, clinical studies and further development of methods and tools for advanced multiparameter image analysis.

5.5.1 Preclinical studies

Characterization of the tumor microenvironment provides a framework for the study of cellular adaptive mechanisms to conditions of hypoxia and oxidative stress. The obvious candidates for such studies are transcription factors that mediate cellular responses to hypoxia and oxidative stress (e.g., HIF-1 and NFKB) and proteins involved in the maintenance of the cellular redox state (e.g., thioredoxin, peroxiredoxins, glutaredoxins and Ref-1). The distribution and the expression levels of the aforementioned parameters can be studied in context of the spatial arrangement of the vasculature, tumor oxygenation and perfusion, by the combined use of multiparameter fluorescence imaging and advanced image analysis techniques. The choice of appropriate labeling techniques (sequential labeling and removal of antibodies) and fluorophore combinations should allow for the analysis of multiple parameters (4-6) in optimally registered images.

Another interesting area for future studies is the identification and validation of biological markers for intermittent and chronic hypoxia in xenograft models. In experiments similar to those described above, spatial expression of HIF-1 and NFKB can
be compared to EF5 staining patterns. If necessary, the EF5\HIF-1 colocalization experiments can be complemented by relating the observed expression patterns to tumor blood vessels using the image analysis methods described in Chapter 4. In addition, tumor circulation can be studied using a combination of perfusion markers, i.e., Hoechst 33342 and the carbocyanine dye DiOC$_7$ (Trotter et al., 1989b) for identification of intermittently perfused blood vessels. A combination of distance- and perfusion-based assessment of intermittent hypoxia is likely to produce more accurate results than the individual approaches. These studies can be extended to include other markers of oxidative stress, such as 8-oxoguanine DNA adducts (Reardon et al., 1997), or the detection of the reactive aldehyde 4-hydroxynonenal (Hammer et al., 1997). The ultimate goal of such studies would be the identification and validation of biological markers for tissue hypoxia that would enable large-scale retrospective studies in archive material. Ideally, such markers should allow for the differentiation of chronic and intermittent hypoxia in tumors.

5.5.2 Clinical studies

Further studies should address two clinically relevant issues: (i) the significance of elevated cysteine levels for cervical carcinoma patient outcome and (ii) characterization of the tumor oxygenation status using biological markers for hypoxia and oxidative stress.
As discussed previously, the finding of high cysteine levels in some cervical carcinomas raises the questions on mechanism(s) that underlie this observation and the clinical significance of cysteine as a radioprotector. One possible mechanism leading to increased cysteine levels in tumor cells is the overexpression of the glutathionase γ-GT. Prezioso et al. (1994) have found an association between increased γ-GT activity and radioresistance of B16 melanoma cells in vitro; the radioresistant phenotype was reversed by blocking γ-GT catalytic activity with the specific γ-GT inhibitor acivicin (Hanka and Dietz, 1973). The potential association of increased levels of cysteine and resistance to radiation therapy could be directly tested in a retrospective study in frozen tumor biopsies from patients entered into the trial on the prognostic value of oxygenation status in cervical carcinomas for radiation treatment outcome. Finding of significant correlates between elevated tumor cysteine levels and unfavorable patient outcomes and/or tumor oxygenation profiles would warrant the development of clinically feasible cysteine modulation studies.

Contingent on the successful identification and validation of biological markers of hypoxia in preclinical studies, the tumor oxygenation status could be determined in frozen tumor biopsies and compared to the tumor oxygenation profiles obtained by the Eppendorf pO2 histogram. In addition, the NPSH levels could be determined in hypoxic and non-hypoxic regions of human cervical carcinomas as an extension of previous studies in xenograft models (Chapter 2; Chapter 3). In parallel studies, the spatial expression profiles of γ-GCS and γ-GT should be analysed to further elucidate mechanisms that regulate NPSH levels in the context of tumor hypoxia.
5.5.3 Development of methods and tools for advanced multiparameter image analysis

Most of the image analysis tools utilized in the course of studies presented in this thesis are implementations of conventional image analysis routines based on combinations of mathematical and Boolean logic operators. These tools were developed in the Interactive Data Language (IDL, version 5.1), a structured high-level programming language optimized for the development of applications for standard image processing and analysis. Characterization of spatial relations of image features, however, requires the development of complex computer algorithms, e.g., pixel-based spatial operator functions. Being an interpreter-based programming language, IDL is dramatically slower (~100x) than standard programming languages (i.e., C and Fortran) in execution of programs that require intensive use of recursive logic and loops. The three-parameter fluorescence images generated in the course of the study described in Chapter 4 are typically 100-200 MB in size and show tumor areas of 3-5 mm in diameter. Punch biopsies from large cervical carcinomas sometimes have areas >1 cm²; the size of three-parameter fluorescence images of these tumors would be ~500 MB. For effective processing and analysis of images of that size, a number of improvements to the existing hardware as well as further development and optimization of analytical approaches and computer algorithms will be necessary.

Determination of NPSH levels as a function of distance from blood vessels (Chapter 4) is an example of a spatial mapping function. The choice of algorithms for this application was influenced by the above mentioned limitations of IDL. In order to optimize the execution of procedures for spatial mapping in large image files, specific
components of functions and individual routines should be developed in the C programming language and dynamically linked with IDL modules. The use of multiple CPUs and the redesign of software to make use of improved architecture characteristics should reduce the image processing times even further. Another approach to spatial mapping of images is the iterative 'growth' or image segmentation by morphologic characteristics of features of interest (see Appendix for detailed description). This method can be used for segmentation of the tumor area into vascular domains that adequately reflect blood vessel sizes. Subsequently, modeled vascular domains can be used for determination of NPSH levels or EF5-binding; in combination with perfusion studies, this analysis could provide information on the functional status of individual blood vessels and its effects on metabolism in tumor cells. Haustermans et al. (2000) have applied a global image segmentation approach to match modeled to observed tumor hypoxia by constructing tissue distance maps based on the tumor vasculature and relating the estimated non-perfused areas to tumor areas stained positively with pimonidazole. Therefore, spatial mapping techniques can be utilized to develop tools for complex image segmentation and sophisticated multiparameter modeling.

In routine image analysis only the information on the presence and the intensity of a parameter of interest is collected and analyzed. Such approaches discount the information about the 2 or 3D spatial arrangement of the parameter(s) of interest. Information on the spatial arrangement of parameters of interest (i.e., random distribution, clustering, and avoidance) adds another dimension to multiparameter analysis, and can contribute to the quality of correlation analyses. An example is the analysis of spatial arrangement patterns of the tumor vasculature. Using the appropriate
algorithms, the distance to the nearest neighboring blood vessel (nearest neighbor distance – NN distance) can be determined for each individual blood vessel in a tumor section.

Figure 31: Nearest neighbor distance profiles of blood vessels

Nearest neighbor distances were determined in 10 serial sections of a CaSki, ME180 and SiHa cervical carcinoma xenograft, respectively. For comparison, the nearest neighbor distance profile of a striated muscle from the SCID mouse is shown. Individual data points in the histograms were connected using a bicubic spline interpolation function.
A histogram of NNDs for three different cervical carcinoma xenografts is shown in Figure 31. Comparisons of the number of blood vessels in the respective distance classes indicate significant differences in the spatial arrangement of the vasculature in these tumors. Preliminary data from ongoing studies indicate that the degree of deviation from fractal (self-similar) arrangement of tumor blood vessels is correlated with the presence of tumor hypoxia, visualized by EF5 staining, in SiHa cervical carcinoma xenografts.

Expression patterns of biological markers in tumor tissue could be assessed using similar approaches; for example, the analysis of spatial distribution could indicate whether cell proliferation or apoptosis occur predominantly in one subpopulation or uniformly across the entire tumor cell population. Furthermore, these measurements can be combined with image segmentation techniques, to better outline heterogeneity of the tumor microenvironment.
APPENDIX: Procedures and algorithms for processing and analysis of images
Co-localization analysis

Characterization of complex biological processes often involves analysis of the spatial correlation of two or more parameters of interest. Multiparameter fluorescence images contain spatially correlated (registered) information recorded as individual grayscale images. The M5+ imaging system (Imaging Research, St. Catharines, ON) stores up to three individual 8-bit grayscale images as separate channels in RGB 24-bit image files.

Co-localization studies can provide answers to two questions: (i) whether two or more parameters are present at the same location and (ii) whether a relationship exists between the expression levels of the parameters. In the first case, co-localization studies are done in binary images; in the second case, the relative intensities from discrete locations are determined in grayscale images. Sample preparation for multiparameter fluorescence imaging involves a number of labeling, amplification and washing steps; often resulting in intra- and interexperimental variations of signal intensity. Therefore, co-localization studies are commonly done in binary images, since the ratios of specific to background staining should be relatively stable. A permutation of co-localization studies involves the use of a binary image for one parameter and grayscale image(s) for the other parameter(s). This method was used for analysis of non-protein sulfhydryl levels in hypoxic and non-hypoxic areas of cervical carcinoma xenografts (Chapters 3 and 4).

The first step in processing of images for co-localization studies usually involves the creation of a histogram of pixel brightness values, in order to choose an appropriate brightness threshold value and convert a grayscale into a binary image. In the ideal case,
the threshold value is a low point between the peaks that represent background and object brightness values (see Figure 32).

![Histogram of brightness values](image)

Figure 32: Histogram of brightness values
Lower brightness values represent background; higher values represent image features. Arrow shows the threshold level.

This ideal situation is rarely encountered; therefore, a number of mathematical approaches for the determination of optimal threshold values have been proposed (Huang and Wang, 1995; Sahoo et al., 1985). In most cases, however, different methods produce similar results, comparable to a simple mean brightness value threshold. In a situation of poor signal-to-noise ratio, elements in the background and regions of interest may contain pixels of the same brightness levels and therefore cannot be separated by a global threshold operation. For this reason, methods of increasing complexity have been proposed for regional gray-level segmentation, usually resulting in significant improvement in preservation of the original shape and size of the objects. For the purposes of the studies presented in the thesis, however, no assumptions are made about
shape or size of objects, and a simple gray-level segmentation with constant threshold values was done routinely. Threshold levels were determined as mean brightness of four background areas in the corners of the images.

For the assessment of spatial co-localization, the next operation is comparison of positive pixel addresses in images A and B and the counting of matches. The number of observed versus expected matches can be statistically analyzed and one of three possible outcomes determined: random distribution (no statistically significant positive or negative co-localization), positive-co-localization (more-than-random spatial overlap) and avoidance (less-than-random spatial overlap). The comparison of positive pixel addresses can be done in a number of ways; this particular implementation was done considering the advantages (fast array-based operations) and limitations (slow execution of loops and recursive logic) of IDL.

The co-localization algorithm consists of three modules:

1. The addresses of positive pixels in image A are found and stored as the variable address.
2. Image B is subscribed with address and the sum of brightness values of indexed pixels is calculated.
3. The overlap of parameters A and B is determined by calculating the ratio of observed over maximum sum of pixel brightness values.

The first module searches the image A for the addresses of positive pixels. Pixels are serially numbered, starting from 1; therefore, the pixel address is simply its serial number. Pixel serial numbers are used to subscribe image array B. The sum of brightness values of the indexed pixels in image B is the observed sum of brightness values ($S_{ob}$). In
a binary image, positive pixels have the brightness value of 255 and negative pixels are 0. If all subscribed pixels were positive, i.e., the overlap of A to B was 100%, the sum $S_{\text{max}}$ for the total pixel number $N$ would be:

$$S_{\text{max}} = N \times 255$$

The number of overlapping pixels is determined by first finding the number of co-localized pixels (total brightness sum divided by 255), and subtracting this value from the total number of pixels for parameter A ($N_a$).

$$\text{Number of overlapping pixels} = N_a - (S_{\text{obs}}/255)$$

The random overlap is calculated from frequencies of occurrence of both parameters:

$$\% \text{ random overlap} = \frac{N_a \cdot N_b}{T^2}$$

$N_a$ and $N_b$ are the respective numbers of positive pixels for parameters A and B, and $T$ is the total number of pixels in one image (the images are of identical size).

Measurements of the levels of parameter A in regions that are parameter B-positive or negative use a grayscale and a binary image. The implementation of this method is straightforward and similar to the previously described algorithm.

Scheme of the quantitative co-localization algorithm:

1. Addresses of positive and negative pixels in image B are found and stored as variables *positive address* and *negative address*.

2. Image A is subscribed with the variables *positive address* and *negative address*, and the sum of brightness values in positive and negative areas is calculated.
Feature-based image analysis

Image processing and analysis procedures are usually done on a global basis; e.g., global contrast enhancement and setting of threshold are applied to and take effect on the entire image. An example of global image analysis is the assessment of tumor hypoxia as percent of EF5-positively stained area in the entire tissue section. To address the issue of spatial distribution of hypoxia in tumors, EF5-positive area can be analyzed in relation to another parameter of interest, i.e., tumor vasculature. This approach is termed feature-based image analysis. Analysis of the spatial relationship of blood vessels to tumor hypoxia involves three steps: (1) the identification of blood vessels, (2) definition of their spatial domains and (3) segmentation of the EF5 fluorescence image on the basis of blood vessel spatial domains.

Creating binary images of the tumor vasculature

After visual inspection, the grayscale CD31 fluorescence images are converted to binary images by brightness thresholding. Due to the non-uniform distribution of endothelial cells along the perimeter of the blood vessel, brightness thresholding often produces discontinuities in the outline of larger blood vessels (Figure 33, panels A and B). Object fragmentation artifacts can be minimized by the use of mathematical morphology methods; image dilatation and erosion are used for processing of binary images on the basis of object shape (Russ JC, 1998). Image dilation involves the processing of image objects by a structuring element, and therefore is similar to convolution. The origin of the structuring element is overlaid over each pixel in the image. If the image pixel is nonzero,
each pixel of the structuring element is added to the result using the Boolean logic "or" operator.

Figure 33: Processing of a CD31 fluorescence image
A: original grayscale image; B: binarized version of A; C: dilated and eroded version of B (dilation was done using a 7x7, erosion was done using a 5x5 structuring element).

Dilation of an image results in an actual increase of the number of positive pixels; to preserve the original object size, dilation is followed by image erosion. Analogous to dilation, the origin of the structuring element is overlaid over each pixel in the image. If any of the image pixels contained in the structuring element is zero, the origin pixel is set to zero. The results of a dilation-erosion operation (also referred to as image closing) are shown in Figure 33, panel C. Closing of the CD31 fluorescence image removes most of the blood vessel fragmentation. If the features in the image C were counted, the resulting number would be closer to the original (image A) than the number determined by feature count in image B.
Spatial image mapping

Often it is of interest to analyze the spatial relationship of multiple parameters. The approach utilized for classification of hypoxia as a function of distance from blood vessels (Chapter 4) involves the creation of distance maps on a nominal scale, or distance class maps. The areas surrounding blood vessels are assigned to distinct distance classes. This method is computationally efficient, since the number of required iterations is usually very small (3-6). The implementation is fairly straightforward and involves the creation of a series of 2D matrices (kernels) of increasing size. These matrices are sequentially convolved with either all or with the pixels that define the perimeter of the blood vessel (Figure 36). The shape of the matrices is determined by performance and quality requirements; compared to a square, a circular matrix produces more accurate distance maps at the expense of slower performance.

Scheme of the distance class maps algorithm:

1. Addresses of pixels contained in all blood vessels (all pixels or pixels in the perimeter) are registered and stored in the variable *address*.
2. Each element of the variable *address* is convolved with a series of matrices of decreasing size. Each matrix labels its pixels with a different distance code.
3. The resulting image is restrained to the original tissue size.
Panel A: Fluorescence image of the vasculature marker CD31. Panel B: The distance class map (0 - >120μm) for a SiHa xenograft was generated using a square matrix with 30 μm increments. Blood vessels are shown in white, the distance classes are shown as different shades of gray.
Further applications

The previously presented methods for image analysis were utilized in the studies presented in this thesis; concurrently, a number of alternative methods and algorithms were designed and implemented for use in future projects.

Definition of vascular domains

Individual afferent blood vessels supply discrete tissue regions with oxygen and nutrients; these regions are termed vascular domains (Kayar et al., 1982). The modeling of vascular domains starts either from the centroid (point of gravity) or from the contours (perimeter) of the respective blood vessel. The advantage of the centroid model is simpler processing; the obvious disadvantage is that this approach does not consider the differences in blood vessel sizes. Modeling of vascular domains from the contours of the object solves this problem. The accuracy of the modeling is affected by the fact that the shape of blood vessels is changed in frozen or fixed tissue. Irrespective of the starting point, vascular domains are modeled either as continuous areas or as distance classes. A process similar to image dilation is used for modeling of continuous vascular domains.

Scheme of the continuous vascular domain modeling algorithm:

1. Addresses of pixels contained in all original objects (centroids or perimeters of the blood vessels) are registered and stored.

2. The first set of addresses is passed on to a function that analyses the neighborhood of each pixel and tests whether: (i) the neighborhood pixels belong to the tumor (ii) the neighborhood pixels are 'free' (not 'claimed' by another blood vessel). Pixels that
meet both conditions are assigned to the vascular domain of the current blood vessel. Addresses of the newly associated pixels are stored, and control returns to the calling function.

3. The next set of addresses (for the next blood vessel) is passed to the neighborhood test function (step 2).

4. Upon completion of one iteration for all blood vessels, the next iteration starts with the first blood vessel that has returned newly associated addresses in the previous iteration, and a new ‘layer’ of pixels is added to the vascular domain.

5. The iterations are continued until all available space is assigned to vascular domains of individual blood vessels (see Figure 34). Alternatively, the number of iterations can be limited (e.g., to the estimated oxygen diffusion distance).

Figure 34: Image of modeled continuous vascular domains

Panel A: Fluorescence image of the vasculature marker CD31. Panel B: Areas shown in different shades of gray represent individual vascular domains in a SiHa xenograft with the blood vessel superimposed.
In small images (~1MB), the IDL implementation of this algorithm on the PC platform is executed at reasonable speed (~10 minutes). The speed of execution, however, decreases exponentially with image size; CD31 images from cervical carcinoma biopsies are typically 10-30 MB in size. Modeling of continuous vascular domains in IDL implementation requires 4-6 hours (dependent on the tumor vascular density) for a 10 MB image on a Pentium III 500 MHz PC. The advantages of continuous modeling of vascular domains are that (i) further assessments can be done in individual blood vessels (ii) distance is mapped continuously; therefore, functions of distance are expressed on a interval scale.

Figure 35: Image of a continuous distance map
Panel A: Fluorescence image of the vasculature marker CD31. Panel B: Distance map of the image in panel A. The distance from blood vessels in presented in shades of gray (darker areas – greater distance from blood vessel), white pixels represent the blood vessels.
A variation of the continuous vascular domain modeling is the creation of continuous distance maps. The algorithm is essentially a simplified, global version of the continuous vascular domain modeling algorithm; the difference is that blood vessels are not treated as individual entities, and modeling is done simultaneously on all blood vessels (Figure 35). Continuous vascular domain modeling requires finding and labeling of individual blood vessels, resulting in numerous searches through the image array \(N = \text{total number of blood vessels}\). In continuous distance maps modeling, the finding and labeling of blood vessels requires only one pass through the image array, thus resulting in a significant reduction in initial execution time.

**Nearest neighbor distance profiles**

One of the parameters that characterize the expression of features of interest is their 2D or 3D spatial arrangement. The nearest neighbor (NN, or closest individual, CI) approach to test for non-random dispersion has been used in ecological surveys (Cottam et al., 1953). Kayar et al. (1982) have adopted this approach to study distribution patterns of capillaries. Essentially, the probability of encountering an individual within any given distance from an arbitrary location is determined by using a set of random points in the image and determining the distance from each point to the nearest capillary. This method has also been applied to the analysis of biomedical images, i.e., the spatial analysis of apoptosis in tumors (Barbini et al., 1996).
The original implementation of the NN algorithm was modified to determine the NN distances between all blood vessels in the image.

Scheme of the IDL implementation of the NN distance algorithm:

1. All blood vessels in the area of interest are counted and labeled.

2. The centroids for every blood vessel are determined and their X, Y coordinates are stored.

3. For every centroid, the nearest neighbor (nearest blood vessel) is determined by calculating distances to all blood vessels and finding the smallest distance at subpixel accuracy.

4. A histogram of distance classes is generated.

The analysis of histograms generated from different cervical carcinoma xenografts and skeletal muscle (Figure 31, p. 113) indicates significant intertumoral differences in the spatial arrangement of the vasculature.
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