Multiscale Evaluation of Vascular Structure and Functional Oxygenation in Orthotopic and Heterotopic Head and Neck Cancer Xenografts

Liliana Berube, MS, Sai Inesh Nallamothu, Hannah Sondreal, BSc, David Murphy, PhD, Kwangok P Nickel, PhD, Mark Pagel, PhD and Randall J Kimple, MD, PhD, MBA

Department of Human Oncology, University of Wisconsin-Madison, Madison, WI

Department of Medical Physics

Department of Human Oncology

Introduction

- **The Pre-Clinical Gap:** Heterotopic (flank) models are the default, but they may not fully replicate the dynamic physiological and vascular niche of orthotopic head and neck cancers.
- **The Structural Paradox:** Traditional static histology often shows similar vessel densities between sites, which may mask key functional differences in blood perfusion and oxygen delivery.
- **Goal:** Establish a multi-parametric validation pipeline by combining OAI, ICG kinetics, and a multi-marker IHC panel to characterize functional hypoxia dynamics

Approach

Optoacoustic Imaging (OAI): Near-infrared laser pulses generate ultrasound waves via thermoelastic expansion, non-invasively mapping tissue chromophores.

- Oximetry (sO_2): Exploits distinct absorption spectra of oxyhemoglobin (HbO_2) and deoxyhemoglobin (Hb) to calculate tissue oxygen saturation in real time
- ICG Kinetics: Systemic Indocyanine Green (ICG) bolus maps microvascular perfusion

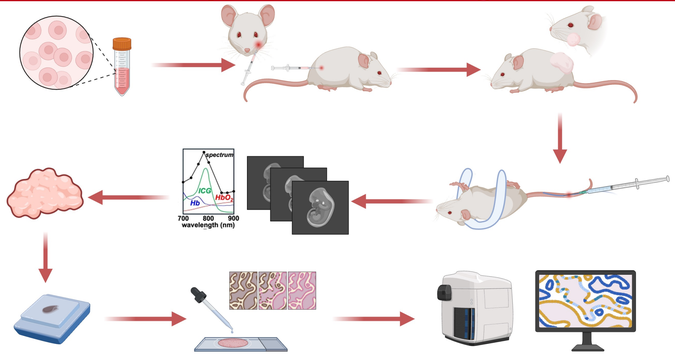
Dual-Staining (CD31/PDPN) for Structural Accuracy

- Lymphatic Noise: CD31 stains both blood and lymphatic vessels, artificially inflating vascular metrics in the lymphatic-dense buccal mucosa.
- The Solution: Dual-staining with PDPN (lymphatic-specific) mathematically excludes lymphatic vessels to isolate true, oxygen-carrying blood capillaries.

Comprehensive Angiogenic Profiling

- IL8: Pro-inflammatory endothelial cell migration and proliferation.
- VEGFA: Downstream endothelial cell survival, vasodilation, and vascular lumen patency.

Experimental Design



Xenograft Establishment: Heterotopic (flank) and orthotopic (buccal mucosa) tumor models of FaDu human head and neck squamous cell carcinoma

In Vivo Gas & Perfusion Challenge Protocol

- Normoxia: Baseline spectroscopic imaging during room-air breathing (21% O_2) to establish baseline hypoxia
- Hyperoxia: Switch to 100% O_2 breathing to measure vasoactive response and oxygenation change (ΔsO_2)
- ICG Infusion: Systemic ICG injection to resolve two-point kinetic evaluation (Wash-in Slope, MSE, etc.)

Vessel Segmentation & H-Scoring

- True Blood Vessels: $CD31^+/PDPN^-$structures
- Lymphatic Vessels: $CD31^+/PDPN^+$or $CD31^-/PDPN^+$
- Semi-Quantitative H-Score: Cytokine expression across tumor sections calculated as:

$$\text{H-Score} = (\mathbf{1}\times\%\ \text{Weak}) + (\mathbf{2}\times\%\ \text{Moderate}) + (\mathbf{3}\times\%\ \text{Strong})$$

Orthotopic Niche Selectively Upregulates Localized VEGFA

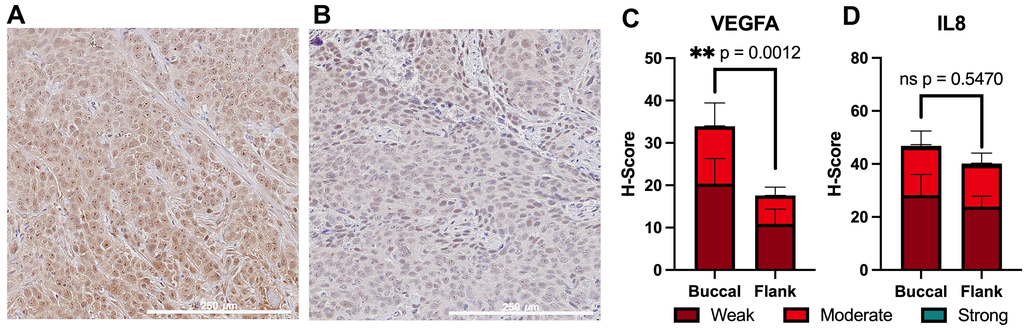


Figure 1. Localized VEGFA upregulation drives vessel patency.
(A) Representative high-magnification immunohistochemistry (IHC) tissue sections stained for VEGFA, showing localized brown chromogen deposition in buccal vs. flank tumors. **(B)** Semi-quantitative H-score analysis of VEGFA expression, demonstrating a statistically significant increase in orthotopic buccal tumors compared to the heterotopic flank ($p = 0.0012$). **(C)** H-score analysis of IL8 showing statistically equivalent expression between cohorts (p= ns), mirroring redundant upstream stroma-remodeling markers like HGFa (not shown). This preliminary comparison suggests that while both environments actively recruit vessels, they may diverge at the downstream step of VEGFA-mediated vessel maturation. Data presented as mean ± SD.

Perfusion Kinetics Trend Higher in Buccal Cohorts

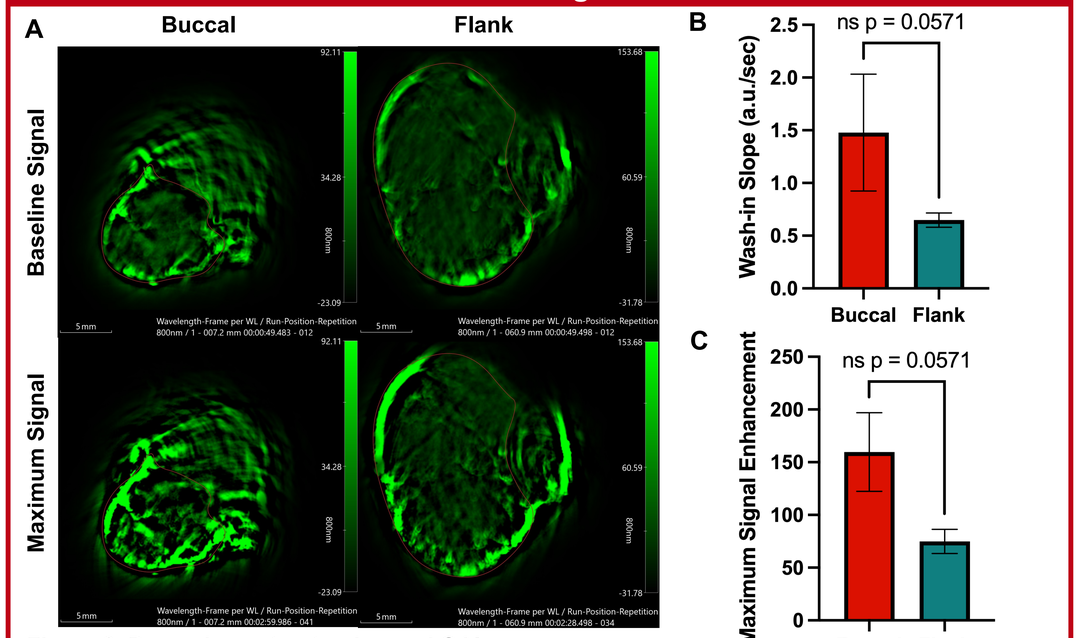


Figure 3. Dynamic contrast-enhanced OAI.
(A) In vivo perfusion maps at Baseline (T_0) and Time-to-peak (TTP) **(B)** Two-point kinetic estimation of the average Wash-in Slope, showing a trended increase in orthotopic buccal tumors compared to flank tumors (p **=** 0.0571).
(C) Peak tracer accumulation represented by Maximum Signal Enhancement (MSE), showing a trended doubling in the buccal cohort (p = 0.0571). Because the elapsed TTP (not shown) was comparable (p > 0.9999), these preliminary metrics suggest the buccal microenvironment supports a larger volume of functionally patent, perfusable vessels. Data presented as mean ± SD.

Dynamic Reoxygenation Capacity Restored Only in Buccal Tumors

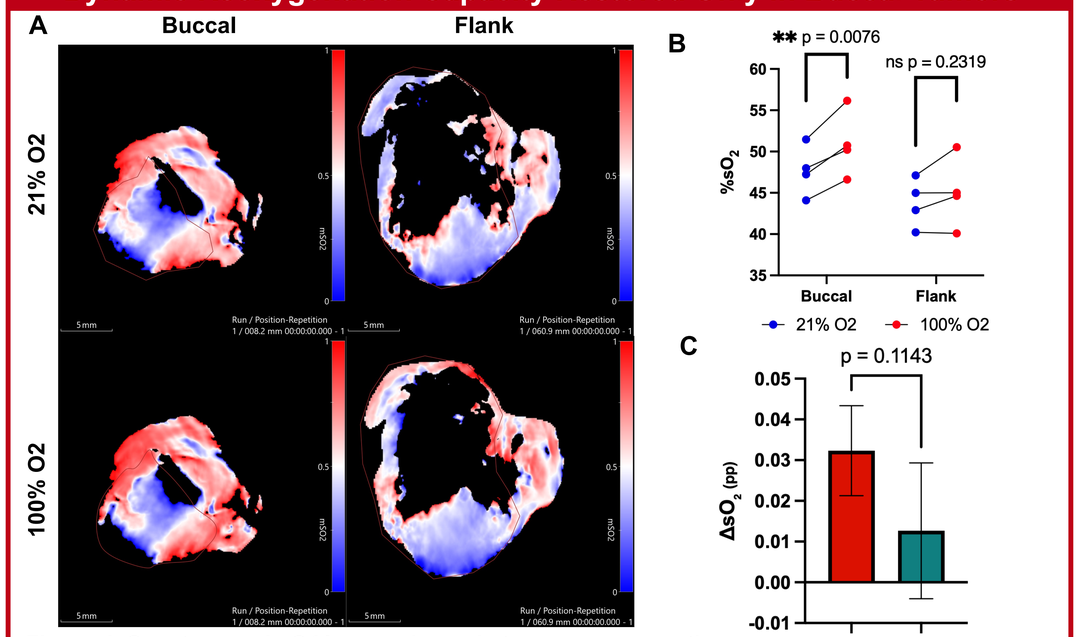


Figure 4. Spectroscopic OAI maps dynamic tumor reoxygenation.
(A) $\%sO_2$ maps showing a robust normoxia (21% O_2) to hyperoxia (100% O_2) shift in buccal tumors vs. a static, hypoxic flank. **(B)** Significant sO_2increase in buccal (p = 0.0076) vs. completely non-responsive flank tumors (p = 0.2319). **(C)** Trending threefold higher ΔsO_2change in buccal tumors (p = 0.1143). These preliminary kinetics suggest that heterotopic flank models may exhibit more limited dynamic reoxygenation capacities under these conditions, which could have implications for modeling fractionated radiotherapy. Data presented as mean ± SD.

Tumor Sites Exhibit Identical Vessel Density

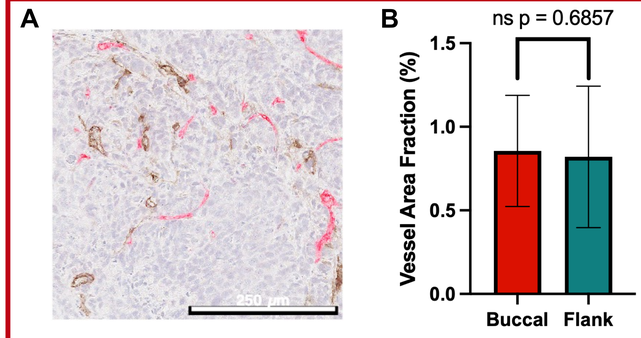


Figure 2. CD31/PDPN dual-IF isolates true blood vessels.
(A) Representative buccal tumor tissue segment overlaid with the filtered blood vessel mask ($CD31^+/PDPN^-$). This processing pipeline excludes overlapping lymphatics to isolate blood-perfusing capillaries. **(B)** Quantitative microvessel density (MVD) showing statistically equivalent blood vessel counts between the orthotopic buccal and heterotopic flank cohorts after lymphatic subtraction. These preliminary data suggest that static histology alone may not capture functional differences between these microenvironments. Data presented as mean ± SD.

Conclusions

Structural vs. Molecular Divergence: Despite exhibiting statistically equivalent microvessel densities in this pilot cohort, orthotopic buccal tumors showed a significant upregulation of downstream VEGFA ($p = 0.0012$) compared to heterotopic flank tumors.

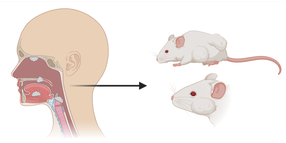
Preliminary Functional Differences: In vivo OAI demonstrated trends toward higher systemic tracer delivery (ICG wash-in/MSE) and a statistically significant dynamic reoxygenation response ($p = 0.0076$) in orthotopic buccal tumors.

Implications for Radiotherapy Modeling: heterotopic flank tumors did not demonstrate a significant reoxygenation response under hyperoxic gas challenge ($p = 0.2319$). This preliminary finding suggests that heterotopic models might underrepresent dynamic reoxygenation capacities relevant to fractionated radiotherapy.

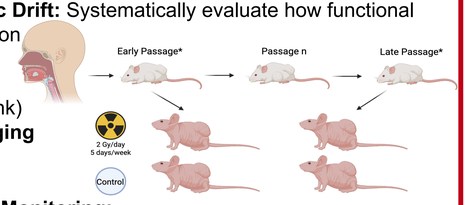
Feasibility of the Pipeline: This pilot study demonstrates the feasibility of combining non-invasive functional imaging with refined dual-marker histology, providing a methodological framework for characterizing tumor microenvironments in larger, upcoming PDX studies.

Future Directions

Standardizing PDX Heterogeneity: Scale this imaging pipeline to HNSCC Patient-Derived Xenografts (PDXs). By imaging tumors at both **early and l ate stages** of growth, we will calculate relative intra-tumor ratios (Late/Early) to normalize high inter-patient biological variability.



Characterizing Systemic Drift: Systematically evaluate how functional vascularity and oxygenation are affected by the **injection site** (orthotopic buccal vs. heterotopic flank) and across **serial passaging** generations.



Longitudinal Treatment Monitoring: Deploy sO_2 and DCE-OAI (Baseline to TTP) during therapy to track dynamic changes in tumor reoxygenation windows and vascular patency over the course of treatment.

Single-Cell Transcriptomic Validation: Perform single-cell RNA sequencing (scRNA-seq) on imaged tumors to determine if macroscopic functional perfusion and oxygenation trends correlate with specific endothelial and stromal transcriptomic signatures.

Acknowledgements

SciMed Graduate Research Scholars · NIH · Kimple Lab Precision Curative Medicine