Collagen fibers mediate water diffusion and anisotropy in breast tumors

Samata Kakkad[1,2], Jiangyang Zhang[1], Alireza Akhbardeh[1], Desmond Jacob[1], Meiyappan Solaiyappan[1], Michael A. Jacobs[1,3], Venu Raman[1,3], Dieter Leibfritz[2], Kristine Glunde[1,3], and Zaver M. Bhujwalla[1,3]

[1]*The Russell H. Morgan Department of Radiology and Radiological Science, The Johns Hopkins University School of Medicine, Baltimore, Maryland, United States,* [2]*Department of Chemistry and Biology, University of Bremen, Bremen, Germany,* [3]*Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University School of Medicine, Baltimore, Maryland, United States*

Introduction: Collagen 1 (Col1) fibers play an important role in molecular transport and cancer cell dissemination. Our goal in this study was to understand the influence of Col1 fibers on water diffusion, and to examine the potential of using noninvasive diffusion tensor imaging (DTI) to detect Col1 fibers in breast lesions. We previously observed in genetically engineered human MDA-MB-231 breast cancer xenograft tumors that fluoresce under hypoxia, relatively low amounts of collagen 1 fibers in fluorescent hypoxic regions [1]. This model was used to further investigate the relationship between Col1 fibers detected by second harmonic generation (SHG) microscopy, water diffusion and anisotropy, and hypoxia. We observed that water diffusion followed the Col1 fiber distribution, and that reduced Col1 fibers in hypoxic regions significantly decreased apparent diffusion coefficient (ADC) and fractional anisotropy (FA) values. We performed *in vivo* DTI measurements to confirm that DTI patterns in tumors observed *in vivo* spatially co-localized with DTI patterns observed *ex vivo* in the same tumors. These studies identify the potential use of noninvasive DTI as a surrogate marker to detect Col1 fiber density and further confirm the importance of Col1 fibers in molecular transport through the extracellular matrix (ECM).

Methods: Female severe combined immunodeficient mice were inoculated in the mammary fat pad with $2x10^6$ MDA-MB-231 cells stably expressing red florescence protein (RFP) under the control of a hypoxia response element (HRE) [2]. Once the tumor volumes were approximately 300-400 mm^3, a horizontal 11.7T Bruker system (Bruker Corp.) was used to acquire *in vivo* DTI with a 10 mm diameter solenoid coil. Mice were anesthetized with isoflurane and the respiration was monitored. DTI was acquired with 5 non-diffusion weighted and 30 diffusion directions, (b-value ~1500 s/mm^2, resolution = 105 x 105 μm^2 x 20 z-slices). Following *in vivo* DTI, the tumor was excised and fixed in 4% paraformaldehyde. A vertical 11.7 Tesla spectrometer (Bruker Corp.) was used to acquire *ex vivo* DTI. DTI of *ex vivo* samples was performed in three dimensions (3D) with two non-diffusion weighted images and eight diffusion-weighted images (b=1500 s/mm^2, resolution 60 x 60 x 60 μm^3). ADC and FA maps were calculated for both *in vivo* and *ex vivo* DTI. Following DTI acquisition, the tumor was sectioned at 1 mm slice thickness for optical imaging. Hypoxic regions were visualized by fluorescence microscopy using a 1× objective attached to a Nikon microscope. Tiled scan Col1 fibers distributions in 3D were acquired by SHG microscopy (incidence = 860 nm, emission = 410-470 nm) using a 25× lens on an Olympus FV1000 MPE multiphoton microscope (Olympus Corp.). Following SHG image acquisition, sections were paraffin-embed and sectioned at 5 µm thickness for immunohistochemistry. These sections were stained with hematoxylin and eosin (H&E). All analysis was done in MATLAB (Mathworks Inc.). Multimodality co-registration was performed by feature extraction to co-register first the *ex vivo* DTI data to *in vivo* DTI data, and then the optical images to the diffusion images using affine transformation. Metrics for registration were the dice similarity indices and set to a level > 0.85. Pearson's correlation coefficient was calculated for *in vivo* and *ex vivo* ADC and FA maps for four slices from each tumor. In addition, ADC, FA and Col1 fibers were quantified in normoxic *versus* hypoxic regions. Col1 fiber quantification analysis was done by our in-house program written in MATLAB (Mathworks Inc.) to quantify for inter-fiber distance and percent fiber volume [1]. H&E sections were co-registered to corresponding DTI images using affine transformation to detect necrotic regions, which were eliminated from quantification analysis.

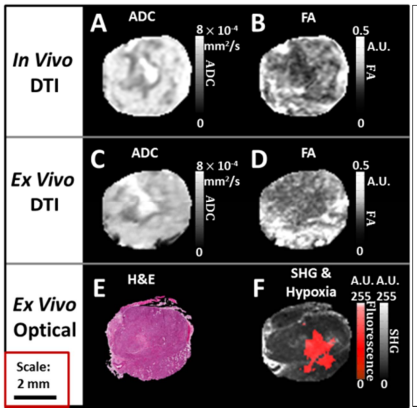

Figure 1: Apparent diffusion co-efficient (ADC) and fractional anisotropy (FA) map from *in vivo* DTI data (A-B); corresponding sections of ADC and FA map from *ex vivo* DTI (C-D); corresponding H&E section (E); and corresponding registered optical images, hypoxic regions in red overlaid with SHG image showing Col1 fiber distribution (F).

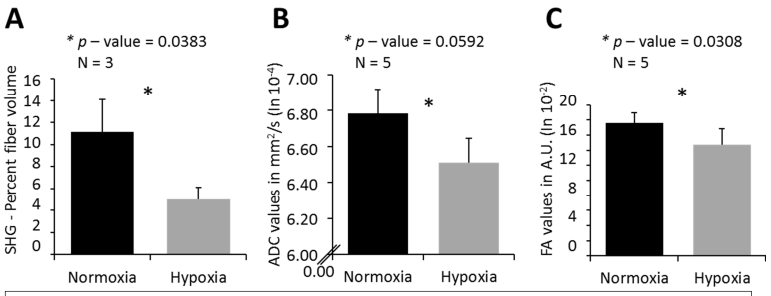

Figure 2: (A) Percent fiber volume was significantly lower in hypoxic regions as compared to normoxic regions (p-value = 0.0383, N = 3). Significantly lower (B) ADC and (C) FA values were observed in hypoxic regions as compared to normoxic regions (p-value = 0.0592 and p-value = 0.0308, respectively, N = 5).

Results and Discussion: Heterogeneous ADC and FA value distributions were identified in the DTI data acquired from both *in vivo* and *ex vivo* DTI (Figures 1A-D). The red fluorescent hypoxic regions were identified from the optical images (Figure 1F). Overall, hypoxic regions had lower Col1 fibers, and water diffusion (ADC) and diffusion anisotropy (FA) values than normoxic regions (Figure 2). A strong correlation was observed between spatially co-localized *in vivo* and *ex vivo* ADC maps as shown in Figure 3A and a trend towards good correlation between *in vivo* and *ex vivo* FA maps as shown in Figure 3B. Here we have shown that Col1 fibers can enhance water diffusion and increase diffusion anisotropy. Noninvasive DTI may be used as a surrogate marker to assess Col1 fiber density in breast cancers, which is important because high Col1 fiber density is associated with mammary tumor initiation, progression and metastasis [3]. The low ADC and FA observed in low Col1 fiber containing hypoxic regions indicate a functional role of these fibers in molecular transport. These results strongly support investigating the use of ADC and FA to noninvasively image Col1 fiber density as well as hypoxia.

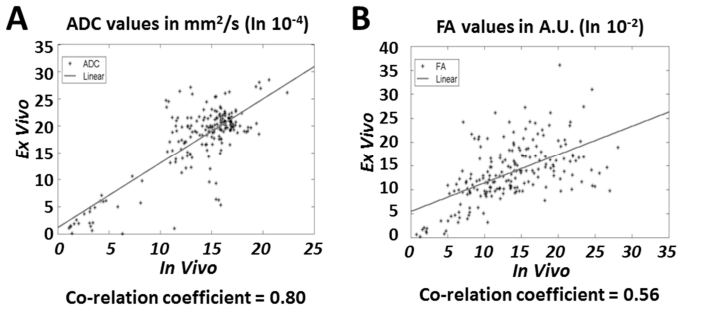

Figure 3: Correlation analysis between *in vivo* and *ex vivo* ADC values (A), showing a good correlation with co-relation coefficient of 0.80 and FA values (B), showing a trend towards good correlation with co-relation coefficient of 0.56.

References: [1] Kakkad et al., Neoplasia, 2010; [2] Krishnamachary et al., PLoS One, 2013; [3] Provenzano P. P. et al., BMC Med 2008.

Acknowledgement: This work was supported by NIH P50 CA103175 and P30 CA006973.