Intrinsic optical biomarkers associated with the invasive potential of tumor cells in engineered tissue models

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Abstract: This report assesses the ability of intrinsic two-photon excited fluorescence (TPEF) and second harmonic generation (SHG) imaging to characterize features associated with the motility and invasive potential of epithelial tumor cells engineered in tissues. Distinct patterns of organization are found both within the cells and the matrix that depend on the adhesive properties of the cells as well as factors attributed to adjacent fibroblasts. TPEF images are analyzed using automated algorithms that reveal unique features in subcellular organization and cell spacing that correlate with the invasive potential. We expect that such features have significant diagnostic potential for basic in vitro studies that aim to improve our understanding of cancer development or response to treatments, and, ultimately can be applied in prognostic evaluation.

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OCIS codes: (100.2960) Image analysis; (190.1900) Diagnostic applications of non-linear optics.

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1. Introduction

The development of cancerous lesions that ultimately result in distant metastases is attributed to numerous genetic and environmental factors [1,2] that often lead to changes in cell morphology, organization, growth, and adhesion [3,4]. The transformation of cells from an epithelial to an invasive phenotype is one of the key steps in cancer metastasis. Therefore, improved identification of cancer cells at an early stage of their progression could result in important new methods for cancer diagnosis and treatment.

One of the critical early events in the development of epithelial cancer is the loss of E-cadherin, whose disruption leads to loss of cell-cell adhesion [5], individual cell migration [6], and elevated N-cadherin expression as cells undergo epithelial-to-mesenchymal transition (EMT) [7]. E-cadherin-mediated adhesion is essential for the assembly of cells into three-dimensional (3D) tissues and is required to maintain normalization of tissue architecture and homeostasis. In recent years, it has become clear that cancer is a disease of altered tissue architecture and that neoplastic progression is a consequence of abnormal interactions between tumor cells and their tissue microenvironment. The tissue microenvironment is defined by the complex network of intercellular interactions that are mediated by physical attachment, as in direct cell-cell or cell-extracellular matrix (ECM) interactions, and by biochemical signals, mediated by soluble molecules from surrounding cells, such as fibroblasts [8–10].

In light of this, it is essential to study the impact of the tissue microenvironment on cancer progression in tissue models that incorporate the 3D tissue context and architecture, so as to faithfully mimic their *in vivo* counterparts. Monolayer, two-dimensional (2D) culture systems do not generate spatially-organized, 3D structures that occur *in vivo* and have been of limited use in studying complex cellular responses. These inherent limitations have compelled cancer biologists to construct 3D tissue models of both normal and altered *in vivo*-like tissues that could provide novel experimental paradigms to study cancer progression. Due to the central role of tissue architecture in cancer progression, it is essential to study this process using techniques that preserve tissue structure, such as nondestructive imaging methods. For example, optical, high-resolution imaging approaches with depth-sectioning capabilities allow characterization of tissues non-invasively and dynamically over time without affecting tissue

architecture. Two-photon imaging approaches, such as two-photon excited fluorescence (TPEF) and second harmonic generation (SHG), are examples of such methods that yield images of cellular and extracellular tissue components with potential submicron level resolution and without requiring physical tissue sectioning [11,12]. In TPEF, image contrast originates from fluorescent dyes or endogenous fluorophores, such as NAD(P)H, flavins, keratin and lipofuscin, while in SHG the signal consists of light scattered in a non-linear fashion by molecules that lack centrosymmetry, such as collagen fibers [13]. Therefore, the combined acquisition of TPEF and SHG images has the potential to characterize key cellular and extracellular features in ways that reflect the dynamic manner in which they are remodeled and affect each other as invasion and metastasis occur.

This article reports on the ability of two-photon laser scanning microscopy (TPLSM) to identify changes in cell morphological and organizational features associated with distinct cell adhesion and migratory progenitors of epithelial tumor cells. We employ 3D tissue models, consisting of E-cadherin competent and E-cadherin deficient variants of squamous cell carcinoma (SCC) cells grown in collagen gels that mimic the tissue microenvironment in which these cancers progress. In addition, we examine tissue constructs that incorporate varying numbers of fibroblasts to assess the role of soluble factors that may affect cell phenotype and behavioral migration. We combine TPEF imaging with novel image-processing methods to non-invasively characterize SCC variants and assess the organization, adhesion, and migration of cells based on quantitative analysis of frequency domain patterns within autofluorescence images. We demonstrate that these optical techniques have the potential to monitor the morphological, structural, and microenvironmental changes that accompany the transition from clusters of E-cadherin-competent, adhesive cells to E-cadherin-deficient cells that migrate into the ECM. We anticipate that such tools could contribute significantly to advance the understanding of cancer progression and metastasis and ultimately the approaches used for its early detection, treatment, or prevention.

2. Materials and methods

Cell culture and retroviral infection: HaCaT-ras-II-4 (II-4) keratinocytes were grown in DMEM containing 5% fetal bovine serum (FBS). HaCaT-ras-II-4 cells were obtained from the laboratory of Dr. Norbert Fusenig (German Cancer Research Center, Heidelberg, Germany) in January 2007. The identity of these cells has been reconfirmed regularly and throughout the course of these experiments by testing for expression of the val-12 activated ras oncogene by Western blotting [14]. Human dermal fibroblasts were derived from newborn foreskins and grown in DMEM containing 10% FBS. These dermal fibroblasts are checked regularly for purity by immunohistochemical staining for the fibroblast marker Thy-1 (positive stain), for the keratinocyte marker keratin 10 (negative stain) and for the endothelial cell marker CD-31 (negative stain). All cells were grown at 37°C in 7.5% CO₂. In order to create cells with altered adhesion, II-4 cells were infected in the presence of 4µg/mL polybrene with 293 Phoenix producer cells transfected with either pBabe or pBabe-H-2K^d-Ecad plasmids by calcium phosphate methods [15]. Infected II-4 cells were maintained under puromycin selection (5µg/mL) starting 2 days post-infection.

Three-dimensional tissue constructs: 300,000 II-4 or H-2K^d-Ecad II-4 keratinocytes alone or with either 10,000 or 50,000 early-passage human dermal fibroblasts were added to neutralized bovine type I collagen concentrated at 3mg/mL (Organogenesis, Canton, MA). Three milliliters of this mixture was added to each 35-mm well insert of a deep-well six-well tray and incubated for 6-10 days in DMEM containing 10% FBS. While growing under submerged conditions, tissue culture medium was changed every 2-3 days. Tissues were imaged after 8-10 days. For tissue morphology, tissues were fixed in 10% formalin, embedded in paraffin, and hemotoxylin and eosin staining was performed on 6µm tissue sections. Images were captured with the Spot Advanced Program 4.5 using a Nikon Eclipse 80i microscope

(Nikon Instruments Inc., Melville, NY) equipped with a SPOT RT camera (Diagnostic Instruments, Sterling Heights, MI).

MPLSM data acquisition: TPEF and SHG images were acquired on a Leica TCS SP2 confocal microscope (Wetzlar, Germany) equipped with a Ti:sapphire laser (Spectra Physics, Mountain View, CA). Samples were placed on glass coverslips, excited with 740nm (TPEF) and 800nm (SHG) light and imaged using either a 63x/1.2 NA water immersion or a 20x/0.7 NA dry objective. The 63x/1.2 NA and the 20x/0.7 NA objective yielded 512 x 512 pixel images of 238 x 238 μm^2 and 750 x 750 μm^2 fields, respectively, in approximately 1 s. TPEF images were acquired by a non-descanned PMT with a filter cube containing a 700 nm short pass filter (Chroma SPC700bp) a dichroic mirror (Chroma 495dcxr), and an emitter bandpass filter centered at 455 nm (Chroma 455bp70). This excitation and emission filter combination allowed collection of fluorescence signal emanating from mitochondrial NADH [11,16–18]. SHG images were acquired in the forward direction through a bandpass filter centered at 400 nm (Chroma hq400/20m-2p). Four to ten fields from each tissue construct were examined for each experiment. Five separate experiments were conducted. Images were merged in ImageJ (v1.37) or rendered in OsiriX (v3.0.2). Analysis was performed with Matlab (Mathworks, Natick, MA).

Frequency-domain data analysis: Autofluorescence images were analyzed using a method described in detail previously [19]. Specifically, from the two-dimensional Fourier transform of 25 images from each group, we acquired angularly averaged power spectral density (PSD) spectra (the PSD corresponds to the squared amplitude of the fourier transform):

$$F(k_r) = Ak_r^{-\beta} + C \quad (1)$$

where $F(k_r)$ is the angularly-averaged radial PSD, k_r is the radial spatial frequency and the constant C corresponds to the high frequency noise floor of each image. The fits were restricted to the frequency range $0.14 < k_r < 1.0 \mu\text{m}^{-1}$, where inverse power law behavior of the PSD spectra was more evident (linear regime in log-log plots of PSD vs. frequency). Hurst parameters (H) were then individually derived from the power exponent β using the expression [20]:

$$2H = \beta - 2 \quad (2)$$

Circularity and area data analysis: Mean cross-sectional area and circularity were calculated for a random selection of tumor cells using ImageJ. Cell borders were identified manually by a user using the *Analyze Menu* in ImageJ. Images were not thresholded or pre-processed in any way prior to this selection. The perimeter and area of the cell were automatically calculated by ImageJ based on the length (in pixel units) and area (in square pixel units) of the user-defined border. Cells without apparent nuclei and fibroblasts were excluded from the analysis. Circularity was calculated from the measurements of area and perimeter with the equation: circularity = $4\pi(\text{area}/\text{perimeter}^2)$. A value of 1 indicates a perfect circle while a value that approaches zero indicates an elongated polygon. Cross-sectional area was converted from pixels to μm^2 by scaling appropriately for 63x magnification (238 μm /512pixels). More than 95 cells were randomly selected from each group.

Collagen analysis: For collagen analysis the orientation index (OI) and entropy were calculated using the algorithms developed by Bayan et al. [21]. Briefly, the OI represents the percentage of fibrils within the image parallel to a dominant fiber direction and is calculated with Fourier-based methods. The entropy is a measure of the distribution of dominant lines in the image determined by the Hough transform. A lower entropy value correlates to more organized fibers.

Statistical analysis: Statistical testing for all quantitative comparisons was done in Matlab. The two-tailed student t-test was used to assess statistical difference with $p < 0.05$ between the mean values of the groups.

3. Results

3.1 II-4 cells and H-2K^d-Ecad II-4 cells exhibit different morphologic characteristics in 3D type I collagen gels

To assess the effects of distinct adhesion properties on the morphology and organization of cells within a type I collagen matrix, we acquired depth-resolved two-photon images of collagen gels populated with ras-transformed epithelial keratinocytes that are either E-cadherin competent (II-4 keratinocytes) or E-cadherin deficient (H-2K^d-E-Cad-II-4 cells). Figure 1 shows histological and optical sections of endogenous cellular NAD(P)H TPEF (green) and collagen SHG (red) of representative collagen gel constructs at 20x [Fig. 1(b) and 1(f)] and 63x [Fig. 1(c), 1(d), 1(g), and 1(h)] magnifications. As seen, TPLSM provides comparable 2D information to histological analysis, while allowing 3D image rendering [Fig. 1(d) and 1(h), [Media 1](#), and [Media 2](#)], offering superior tissue visualization. TPEF images display II-4 cells that maintain cell-cell contact and grow in dense, organized spherical clusters when cultured in a 3D collagen matrix, consistent with intact E-cadherin function [Fig. 1(a)–1(d)]. Under 20x magnification, smaller II-4 cell clusters appear more rounded, while the larger clusters are ovoid in nature [Fig. 1(b)]. In addition, II-4 cell clusters demonstrate well-delineated borders with the surrounding collagen matrix oriented and forming trabecular networks between adjacent clusters [Fig. 2(b)]. At 63x magnification, a well-defined band of collagen that surrounds the clusters of these cells without penetrating them is visible [Figs. 1(c) and 2(a)].

Fig. 1. TPEF shows E-cadherin deficiency causes individual tumor cell migration. II-4 and H-2K-E-Cad-II-4 cells imaged with 740nm and 800nm excitation light. Scale bar 100 μ m for 20x and 25 μ m for 63x. (a), (e) Histology of II-4 and H-2Kd-E-Cad-II-4 cells, respectively, show essential morphological characteristics of SCC variants. (*Inset*) Higher zoom images of SCC variants. (b), (f) 20x magnification of 2D optical sections of II-4 and H-2Kd-E-Cad-II-4 intrinsic NAD(P)H fluorescence (*green*) allows visualization of tumor morphology at a macroscopic scale similar to histology, without fixation or the use of dyes. Fluorescence images confirm H-2Kd-E-Cad-II-4 cells show limited capacity to adhere to one another, while II-4 cells demonstrate cell-cell contact and grow in dense, organized spherical clusters, suggestive of intact E-cadherin function. (c), (g) SHG images from collagen (*red*) overlaid with NAD(P)H fluorescence shows II-4 cell clusters have delineated borders that are distinct from surrounding collagen (63x magnification). (d), (h) Multiphoton imaging allows for straightforward generation of 3D rendered images, which show II-4 cells forming densely packed, round clusters as well as the diffuse, scattered organization characteristic of H-2Kd-E-Cad-II-4 cells.

These cellular and collagen organizational features can be contrasted to the appearance of the collagen gels populated with E-cadherin-deficient H-2K^d-E-Cad-II-4 cells [Fig. 1(e)–1(h)]. Specifically, individual cells are often seen streaming off the perimeter of loosely packed cell clusters towards unoccupied collagen matrix [Fig. 1(f)]. At 63x magnification, cells are seen

either organized at the periphery of an empty lumen [Fig. 1(g)] or as part of linear arrays of cells that migrate randomly into the surrounding collagen without directionality or orientation [Fig. 1(h)]. Collagen fibers are detected interspersed between individual cells as expected [Figs. 1(h) and 2(d)], since loss of E-cadherin function leads to reduced cell-cell adhesion and subsequently augments cell motility and spacing. The images of NAD(P)H fluorescence (green) and collagen SHG (red) in Fig. 2(e) suggest that H-2K^d-E-Cad-II-4 cells do not organize the surrounding collagen matrix in a well-defined manner, yielding diffuse networks of fibers with no apparent orientation, in contrast to II-4 cells that orient the matrix between adjacent clusters of cells [Fig. 2(b)]. While the apparent orientation of collagen fibers based on SHG imaging using linearly polarized light can depend on the polarization direction of the incident light beam [22], our images indicate that the direction of the orientation of the collagen fibers in our specimens is dictated mostly by the location of the cell clusters [Fig. 2(b)]. In support of this observation, quantitative analysis of the collagen organization in the areas identified in Fig. 2(a) and 2(d), using approaches that have been described in detail previously [21], yields substantially higher orientation index (OI) and lower entropy values for the collagen immediately surrounding II-4 cells (OI = 33%, entropy = 3.78) than H-2K^d-E-Cad-II-4 cells (OI = 6.3%, entropy = 3.95).

Table 1. Quantitative effect of fibroblasts on tumor cells in collagen gel

	Circularity	Cross-sectional area (μm^2)	+ 10,000 fibroblasts		+ 50,000 fibroblasts	
			Circularity	Cross-sectional area (μm^2)	Circularity	Cross-sectional area (μm^2)
II-4	0.855 ± 0.021	443.5 ± 49.5	0.804 ± 0.018 ↓ 6%	519.5 ± 42.7 ↑ 20%	0.805 ± 0.021 ↓ 6%	628.3 ± 49.6 ↑ 41.6%
H-2K ^d -Ecad II-4	0.828 ± 0.019	451.4 ± 46.8	0.762 ± 0.019 ↓ 8%	837.5 ± 46.3 ↑ 85.5%	0.723 ± 0.022 ↓ 12.7%	748 ± 53.1 ↑ 65.7%

Percentages reported as compared to tumor cells alone.

Fig. 2. SHG images from collagen suggest adhesion capability and presence of fibroblasts affect local matrix structure. II-4 and H-2K-E-Cad-II-4 cells imaged with 740nm and 800nm excitation light. Scale bar 100 μm for 20x and 25 μm for 63x. (a), (d) At 63x magnification NAD(P)H fluorescence (green) and SHG from collagen (red) show collagen organization is unique for II-4 and H-2Kd-Ecad II-4 cells. Collagen organization is quantified for sub-images indicated with a white box as discussed in Results section. The respective entropy and orientation index (OI) values of these images are displayed in the upper right corner of the images demonstrating accurate collagen quantification is possible. 20x magnification of (b) II-4 cells, (c) II-4 cells with a high concentration of fibroblasts, (e) H-2Kd-E-Cad-II-4 cells, and (f) H-2Kd-E-Cad-II-4 cells with a high concentration of fibroblasts displays NAD(P)H fluorescence from cells (green) and SHG from collagen (red). II-4 cells organize collagen in a connected trabecular formation while H-2Kd-E-Cad-II-4 cells have diffuse, less structured fiber networks. Once fibroblasts are added, II-4 cell exhibit empty lumens (white arrow) and less structured collagen networks (c). Increased SHG signal is suggestive of increased fiber density.

To determine whether variations in matrix and cell organization were accompanied by changes in cell morphology, the cross-sectional area and circularity of the two cell types were quantified (Table 1) for random selections of cells (>95 in each group). We found that the average cell area was similar for II-4 and H-2K^d-E-Cad-II-4 cells, and was $443.5 \pm 49.5 \mu\text{m}^2$ and $451.4 \pm 46.8 \mu\text{m}^2$, respectively. Circularity was calculated for the same cells and shared a value closer to 1, which represents cells that are more rounded. We should note that the actual variation in circularity when considering the three-dimensional cell volumes may be smaller than the calculations we perform based on analysis of two-dimensional images, since we only consider cross-sections, which, especially for a highly elliptical cell can vary depending on the cell orientation. We found that II-4 cells have a mean circularity of 0.855 ± 0.02 while H-2K^d-E-Cad-II-4 cells have a significantly lower mean circularity of 0.828 ± 0.02 ($p < 0.05$).

Media 1 (a) 3D rendering of NAD(P)H fluorescence images from II-4 cells shows cells maintain adhesion and form rounded clusters. **Media 2** (b) 3D rendering of NAD(P)H fluorescence image from H-2K^d-Ecad II-4 cells shows individual migratory behavior.

3.2 Co-cultures of dermal fibroblasts with II-4 cells and H-2K^d-E-Cad-II-4 cells in type I collagen gel affects tissue architecture

To investigate the influence that fibroblasts exert on the motility and organization of E-cadherin-competent and E-cadherin-deficient tumor cells 10,000 or 50,000 fibroblasts were mixed with either II-4 or H-2K^d-E-Cad-II-4 cells prior to their incorporation into the collagen gels. From the comparison of TPEF cross-sections of II-4 cells in the absence or presence of fibroblasts [Fig. 3(a)–3(c)], it is observed that the motility of II-4 cells is correlated with the amount of fibroblasts in the collagen gel. The migratory phenotype is apparent in SHG and TPEF images at 20x magnification [Fig. 2 (c)] where tissue constructs incorporating II-4 cells and fibroblasts show formation of clusters with empty central lumens. These structures are distinct from the H-2K^d-E-Cad-II-4 cell clusters [Figs. 1(g) and 3(g)], where a single-cell perimeter lines a central empty lumen. II-4 central lumen structures occur at depths of 3-5 cells, which may be suggestive of cell death at the center of the cluster [Fig. 2(c), arrow]. At 63x magnification [Fig. 3(b) and 3(c)], II-4 cells demonstrate enlarged cytoplasm and nuclei and are slightly elongated in their shape in the presence of fibroblasts, however not to the extent of H-2K^d-E-Cad-II-4 cells [Fig. 3(h) and 3(i)]. Quantification of these observations shows that II-4 cells in the presence of a low concentration of fibroblasts decrease in their circularity by 6% and do not significantly change in cross-sectional area ($p < 0.05$) as compared to II-4 cells alone (Table 1). The circularity value of the II-4 cells in the presence of a high concentration of fibroblasts remains the same as II-4 cells in the presence of a low

concentration of fibroblasts; however, a significant increase in the cell area is observed ($p < 0.05$) compared to II-4 cells alone or with a low concentration of fibroblasts (Table 1). Widened intracellular gaps accompany these morphological changes and they appear to be correlated with the amount of fibroblasts in the collagen gel [Fig. 3(b) and 3(c)].

From the TPEF cross-sections at 63x magnification of H-2K^d-E-Cad-II-4 cells in the absence or presence of fibroblasts [Fig. 3(g)–3(i)], it is clear that H-2K^d-E-Cad-II-4 cells organize and migrate in a similar way with and without fibroblasts. However, when fibroblasts are present, formation of cell structures with empty central lumens [Fig. 3(g) and 3(j)] is not observed. Instead, loose groups of one or a few cells surrounded by collagen are dispersed throughout the collagen matrix [Fig. 3(h), 3(i), 3(k) and 3(l)]. At 63x magnification, changes in cellular morphology of H-2K^d-E-Cad-II-4 cells in co-culture with fibroblasts are evident in these cells as seen by the appearance of enlarged cytoplasm and nuclei as well as elongated cell peripheries [Fig. 3(h) and 3(i)]. Changes in cell area and circularity show that H-2K^d-E-Cad-II-4 cells in the presence of a low concentration of fibroblasts exhibit an 8% decrease in circularity and double in their size when compared to these cells in the absence of fibroblasts (Table 1). When in co-culture with a high concentration of fibroblasts, H-2K^d-E-Cad-II-4 cells show 12.7% decrease in circularity while their cell area remains increased when compared to H-2K^d-E-Cad-II-4 cells alone (Table 1).

Fig. 3. Co-culture with fibroblasts influences low-grade invasive tumor cells to organize like high-grade invasive tumor cells. Scale bar 100µm for 20x and 25µm for 63x. 2D Optical sections of NAD(P)H autofluorescence (green) and SHG of collagen type I (red) in 3D gel [(a)–(c) and (g)–(i)] and Histology [(d)–(f) and (j)–(l)] of II-4 and H-2Kd-E-Cad-II-4 cells. (a), and (d) II-4 cells organize in tight bundles and do not migrate individually from origin cluster; (g), (j) H-2Kd-E-Cad-II-4 cells do not maintain cell-cell adhesion and migrate individually as lines or hollow spheres; (b), (c) II-4 cells cultured in the presence of (b) 10,000 and (c) 50,000 fibroblasts become enlarged and exhibit H-2Kd-E-Cad-like behavior; (h)–(i) H-2K-E-Cad-II-4 cells cultured in the presence of fibroblasts also become enlarged and maintain individual cell migratory behavior.

The presence of fibroblasts also affects the matrix organization by both E-cadherin-competent and E-cadherin-deficient cells. Figure 2 shows that when cultured with a high concentration of fibroblasts, both tumor cell lines are surrounded by a dense, yet disorganized collagen environment [Fig. 2(c) and 2(f)]. Furthermore, upon co-culturing with fibroblasts the intensity of SHG from the collagen increases, indicating that either more collagen deposition or more matrix remodeling occurs [Fig. 2(c) and 2(f)].

3.3 Co-culture of fibroblasts with II-4 cells as H-2K^d-E-Cad-II-4 cells in type I collagen show different frequency domain characteristics

To further characterize the two tumor cell lines, we used a Fourier-based analysis method we developed recently [19] to automatically process NAD(P)H autofluorescence images in order

to quantify the organization of mitochondria, which are the main sub-cellular structures yielding NAD(P)H emission [23]. The power spectral density (PSD), the squared amplitude of the Fourier transform, of these images [20] exhibits inverse power law behavior, which is indicative of self-affine fractal organization [24,25]. The exponent of this inverse power law is related to the Hurst parameter, which characterizes the fractal spatial organization of the mitochondrial network. For our data, the Hurst parameter was estimated in a frequency range that corresponds to features in the spatial domain of $1.0\mu\text{m}$ to $7.2\mu\text{m}$, which is reflective of features on the order of cell organelles and cell-cell spacing. This power-law model was applied to each group, with representative data and fits displayed in Fig. 4(a). Differences in the Hurst parameter, which is calculated from exponential fits of the PSD spectra [Fig. 4(a)], are found between II-4 cells in the absence and presence of fibroblasts as well as between II-4 cells and H-2K^d-Ecad II-4 cells in the absence of fibroblasts and presence of fibroblasts ($p < 0.05$) [Fig. 4(b)]. The mean slopes and Hurst parameter values from each group are shown with standard deviations in Table 2.

Fig. 4. Low-grade invasive tumor cells, high-grade invasive tumor cells, and fibroblasts in collagen type I gel have distinct frequency domain characteristics. (a) Representative Power Spectral Density (PSD) spectra with respective fits (colored dashed lines) of II-4 cells and H-2K^d-Ecad-II-4 cells as a function of fibroblast concentration calculated from NAD(P)H autofluorescence images. (b) Box and whisker plot displaying the median and range of Hurst parameters extracted from 25 fields of each cell group. The mean Hurst parameter values, shown with standard deviations in Table 2, are estimated from the slope (β) of the PSD spectra in a range that corresponds to features in the spatial domain of $1.0\mu\text{m}$ to $7.2\mu\text{m}$, which is reflective of fluorescent features on the order of cell organelles and cell-cell spacing. Differences in the Hurst parameter were found from the fits of the PSD spectra between II-4 cells alone and all other groups ($p < 0.05$) indicated by the black star (*), demonstrating the ability of this method to identify cells with a migratory/invasive phenotype.

The results obtained indicate that this type of rapid frequency domain processing can effectively distinguish between clusters with low-grade invasive and high-grade invasive potential. To investigate these trends in more detail, the distribution of Hurst parameters is represented with a box and whisker plot in Fig. 4(b). For all groups the average value of Hurst parameters falls within the range allowed by the self-affine model of $0 < H < 1$. II-4 cells alone exhibit a similar distribution of the interquartile range of Hurst parameters as the other groups, but the mean value is statistically lower ($p < 0.05$). The extent of the interquartile range of Hurst parameters observed in II-4 cells in the presence of a low concentration of fibroblasts is much larger than the other groups and is skewed to lower values. The low skew is in line with the finding that II-4 cells with a low concentration of fibroblasts are morphologically distinct from migratory groups (II-4 cells with a high concentration of fibroblasts, H-2K^d-Ecad II-4 cells alone, and H-2K^d-Ecad II-4 cells with fibroblasts). Despite the low skew, these Hurst values are not statistically different from the values of the other migratory groups ($p < 0.05$), indicating similar subcellular organization. Nevertheless, the low skew may suggest that II-4

cells in the presence of a low concentration of fibroblasts have dual sub-cellular character, including qualities of II-4 cells alone and those of migratory groups. The dual character may be the result of the need for a baseline amount of fibroblasts on a per-cell basis to catalyze sub-cellular changes. A skew to upper values is observed with H-2K^d-Ecad II-4 cells cultured alone, suggesting that this group may also possess a distinct sub-cellular organization, different from all other groups.

4. Discussion

The goal of this study was to identify the optical signatures associated with the invasive behavior of SCC cells in a 3D collagen matrix that mimics the *in vivo* microenvironment in which this cancer develops. We collected TPEF and SHG images to assess the organization, adhesion, and morphology of SCC engineered tissue variants upon E-cadherin abrogation and co-culture with fibroblasts. We took advantage of endogenous non-linear cellular fluorescence and matrix scattering properties to visualize and quantify tissues in a non-invasive manner. We demonstrate that non-invasive optical techniques can identify the following features of SCC progression in 3D tissues that mimic this disease in humans: a) abrogation of E-cadherin-mediated adhesion associated with individual cell migration, b) modulation of the migratory properties of SCC cells in response to contextual cues from fibroblasts in the surrounding microenvironment, and c) cell invasion associated with specific subcellular organization and collagen signatures that can be assessed in a rapid and automated way.

Table 2. Migratory cell behavior is characterized by higher Hurst parameter values

	+ 10,000 fibroblast s				+ 50,000 fibroblasts	
	II-4	H-2K ^d -Ecad II-4	II-4	H-2K ^d -Ecad II-4	II-4	H-2K ^d -Ecad II-4
Slope (β)	2.20 $\pm$ 0.26	2.89 $\pm$ 0.59	2.61 $\pm$ 0.49	2.80 $\pm$ 0.39	2.81 $\pm$ 0.45	2.78 $\pm$ 0.49
Hurst = (β -d)/2	0.10 $\pm$ 0.01	0.45 $\pm$ 0.09	0.31 $\pm$ 0.06	0.40 $\pm$ 0.06	0.41 $\pm$ 0.07	0.39 $\pm$ 0.07

4.1 TPLSM probes cell adhesion in the context of tumor cell invasion

While it is well established that abrogation of E-cadherin is closely related to adhesion and migration of SCC cells [5,7,26–29], we describe its distinct effect on organization of SCC cells in 3D tissues. We study how optical techniques can be used to dynamically monitor the phenotype and behavior of E-cadherin-deficient and -competent SCC cells in collagen gels. Previously, non-invasive optical techniques in conjunction with fluorescence dyes have been used successfully to track cell migration upon epithelial-to-mesenchymal transition (EMT) in 3D tissues without chemical fixation and tissue sectioning [30,31]. In order to overcome the use of fluorescent dyes, endogenous fluorescence and scattering of tissues can be exploited with TPLSM. Other applications have successfully characterized 3D engineered tissues and tumor tissues using only endogenous sources of contrast [32–34]. While cellular features, such as the nuclear to cytoplasmic ratio, which is routinely used in histopathology, have been quantified from TPEF images [17,35], the characterization of subcellular organization for diagnostic purposes has remained largely unexplored, both in the context of histopathological and TPEF images. Furthermore, although TPEF spectral resolved tissue imaging has been utilized for the *ex vivo* evaluation of human skin [35,36] as well as the *in vivo* characterization of mouse skin [37], there is a lack of quantification of the dynamic changes that the local matrix as well as the cellular and subcellular tissue components undergo during normal and abnormal cellular processes.

In order to investigate the effect of E-cadherin abrogation on tumor cell morphology, we quantified the area and circularity of E-cadherin-competent and E-cadherin-deficient SCC cells, using endogenous NAD(P)H TPEF images. We report that the individual cell phenotype associated with loss of E-cadherin function is characteristically more elliptical than its low-grade counterpart. In agreement with this finding are reports that have demonstrated that

inhibiting E-cadherin function alters the morphology of normal kidney cells (MDCK) to a more invasive, elongated phenotype [38]. Furthermore, SCC-derived cells (UM-SCC-11B) exhibit a more elliptical phenotype that is consistent with decreases in E-cadherin expression, increases in N-cadherin expression, and an invasive phenotype [39]. We have previously used the 3D tissue model described in our current study to show that E-cadherin deficient SCC cells demonstrate a switch from expression of E-cadherin to N-cadherin in a 3D collagen gel [7]. Since N-cadherin expression, E-cadherin abrogation, and an elliptical phenotype are considered hallmarks of EMT, our findings that show cell elongation and migratory behavior, demonstrate the capacity of our imaging tools to identify SCC cells that underwent EMT [26,40,41].

To further characterize tumor cells and validate that their subcellular character is a reliable indicator of disease state, we used a Fourier-based analysis method, described in detail previously [19], to automatically process the NAD(P)H autofluorescence images and assess the fractal nature of mitochondrial organization. Several studies have shown that epithelial cells and tissues exhibit fractal morphology [42] and that the inverse power law behavior of these fractals can vary significantly between normal and diseased (dysplastic) specimens [42–45]. Indirect evidence for fractal microarchitecture of cells and tissues has also been shown in numerous elastic light scattering experiments, which have in turn revealed significant differences between normal and pre-cancerous or cancerous tissues [46]. In the current work, we find that the Hurst parameter, which is related to the slope of the inverse power law decay of the PSD and is used in this approach as a quantitative measure of self-affine organization, is significantly different for images of E-cadherin-efficient and E-cadherin-deficient cell clusters. E-cadherin-deficient cell clusters have Hurst values closer to 0.5, which suggests more random Brownian fluctuations in autofluorescence and translates specifically to a less organized assembly of mitochondrial structures within these cells. These changes may also be due to more significant variations in the spacing between E-cadherin deficient cells. An increase in the Hurst parameter value has also been detected from endogenous fluorescence of human papillomavirus (HPV) transfected epithelial cells, forming dysplastic 3D epithelia that lack differentiation when compared to their normal epithelial cell counterparts that organize in well-differentiated layers [19].

In addition to altered subcellular organization, our data suggest that defects in cell-cell adhesion caused by E-cadherin abrogation are also reflected in matrix changes revealed by inherent optical signatures of collagen. For example, we observe a dense border of collagen that surrounds E-cadherin-efficient SCC cells, which may suggest that minimal breakdown of collagen is occurring near cell-matrix junctions. Consistently, data from other studies suggest that collagen fibers that are tangential to the epithelial cell border may apply physical restraint that limits cell migration into the adjacent matrix [47]. Beyond this collagen border, we find that collagen fibers appear significantly more aligned, forming trabecular networks between clusters of E-cadherin competent cells, while they organize only diffusely around E-cadherin deficient cells. This may suggest E-cadherin deficient cells are unable to create tensional forces on the matrix [15] resulting in poor collagen organization and observably diffuse, weakly SH-generating fibers. Furthermore, upon E-cadherin abrogation SCC cells have been found to up-regulate matrix metalloproteinases (MMPs), suggesting that E-cadherin-deficient SCC cells degrade the surrounding matrix at a greater degree than E-cadherin efficient SCC cells [15,48]. Thus, both weak collagen constriction and increased collagen breakdown or turnover maybe predictive signatures of the highly migratory phenotype we observed. In contrast, defined and aligned collagen fibers are reported to surround migratory tumor specimens from excised mammary tumors [47]. The migratory E-cadherin deficient model may not show defined fibers in aligned orientation because it is composed of solely type I collagen matrix, which makes it distinct from in vivo conditions.

4.2 TPLSM detects importance of local environmental cues in cancer progression

Numerous studies have established that interactions between cancer cells and surrounding fibroblasts play an important role in the progression of cancers [9,48]. In this study, we evaluate with non-invasive techniques the effects of fibroblasts in regulating cellular phenotype in order to link the migration and organization of low-grade invasive and high-grade invasive SCC cells in 3D tissues to distinctive structures of individual cell migration. We find that the presence of fibroblasts results in elimination of cell-cell adhesion and acquisition of migratory behavior. As a consequence, the empty lumen structures that we observe with E-cadherin deficient SCC cells alone [Figs. 1(g) and 4(g)] are no longer observed.

Since malignant transformation in tumors is often accompanied by changes in individual cell morphology [1], we assessed individual tumor cells for differences in cell cross-sectional area as a function of fibroblast co-culture. We find that addition of fibroblasts leads to an increase in the average size of SCC cells, regardless of E-cadherin function. This increase in average cell size upon fibroblast co-culture may reflect increased migratory behavior associated with the presence of growth factors such as hepatocyte growth factor (HGF) [8–10]. Furthermore, we find that E-cadherin-deficient cells are more sensitive to a lower concentration of fibroblasts as compared to E-cadherin efficient SCC cells, since they almost double in cross-sectional area (see Table 1). Thus, we conclude that maintenance of E-cadherin function plays a significant role in the magnitude of cell size increase upon co-culture with fibroblasts.

In addition, we find that fibroblast co-culture results in an elliptical or spindle-like morphology for the SCCs that is characteristic of invasive cells and reminiscent of EMT. In line with our observations, mesenchymal cells are characterized by the loss of cell polarity, which occurs because of loss of differentiated junctions and redistribution of mitochondria [49]. Upon co-culture with fibroblasts our analysis methods are sensitive to changes in SCC cell junctions and mitochondrial organization as reported by the Hurst parameter. Specifically, we find that TPEF images of all cells that exhibit a migratory behavior are characterized by a significantly higher Hurst parameter value, than the images of the non-migratory cells. Such increased Hurst parameter values have been previously associated with a lower differentiation state of keratinocytes [19].

5. Conclusion

We have established in the current study the presence of optical, non-invasive biomarkers associated with migratory tumor cell behavior induced by changes in the tumor cell microenvironment including the abrogation of E-cadherin-mediated cell adhesion and fibroblast-derived soluble factors. Specifically, we have used endogenous TPEF and SHG signals, emanating from cellular NAD(P)H and extracellular collagen, to visualize in high-resolution 3D images the organization and morphology of epithelial tumor cells embedded in a collagen matrix in the presence or absence of fibroblasts. These images demonstrate that abrogation of E-cadherin and/or co-culture with fibroblasts influence individual cell morphology, multi-cellular organization, and matrix structure. Our findings suggest that our image analysis can reveal changes in cell phenotype and behavior linked to altered cell-adhesion and motility and allows monitoring of the dynamic interactions found in the tumor cell-microenvironment that play an important role in the modulation of early invasion. The methods we have developed are designed to enable future in vitro evaluation of engineered tissues for studies to elucidate processes involved in cancer development or to assess the effects of novel drugs and therapeutic approaches. By harnessing the tremendous potential of 3D tissue models, we will help close the loop that exists between observations gleaned from rudimentary cell culture systems to those more linked to cancer progression in vivo. As we have demonstrated, the complex microenvironment present in 3D tissues offers new

opportunities to efficiently develop novel optical tools for cancer detection that will lead to their clinical application. Given advances in multiphoton microscopy that promise to allow endoscopic or deep-tissue evaluation via minimally invasive probes [50], we expect that the approaches that we present can be ultimately translated to in vivo studies to detect the behavior and phenotypic progenitors of cancer cells in real-time directly in cancerous tissues at various stages of their progression.

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