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3D organizational mapping of collagen fibers elucidates matrix remodeling in a hormonesensitive 3D breast tissue model

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Abstract

Hormones play an important role in normal and diseased breast tissue development. However, they can also disrupt cell-matrix interactions and their role in extracellular matrix reorganization during epithelial morphogenesis remains poorly understood, partly due to a lack of sensitive approaches for matrix characterization. Here, we assess the hormonal regulation of matrix reorganization in a three-dimensional (3D) breast tissue culture model using a novel metric, i.e., 3D directional variance, to characterize the 3D organization of collagen fibers visualized via high-resolution, second harmonic generation imaging. This metric enables resolving and quantifying patterns of spatial organization throughout the matrix surrounding epithelial structures treated with 17 β -estradiol (E2) alone, and E2 in combination with either promegestone, a progestogen, or prolactin. Addition of promegestone results in the most disorganized fibers, while the E2 alone treatment leads to the most organized ones. Location-dependent organization mapping indicates that only the prolactin treatment leads to significant heterogeneities in the regional organization of collagen fibers, with higher levels of alignment observed at the end of the elongated epithelial structures. The observed collagen organization patterns for all groups persist for tens of micrometers. In addition, a comparison between 3D directional variance and typical 2D analysis approaches reveals an improved sensitivity of the 3D metric to identify organizational heterogeneities and differences among treatment groups. These results demonstrate that 3D directional variance is

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Author contributions

Z.L., K.P.Q. and I.G. conceived and developed the analysis method. L.S. prepared tissue samples. Z.L. acquired the data. Z.L., C.A., D.P., Y.Z., E.S. and I.G. performed the analysis. C.S., A.M.S. and I.G. designed and planned the project. Z.L. and I.G. wrote the manuscript, which was reviewed and edited by all co-authors.

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Data availability

The authors declare that all data supporting the findings of this study are available within the paper and its Supplementary Information. Source data for the figures in this study are available from the authors upon request.

sensitive to subtle changes in the extracellular micro-environment and has the potential to elucidate reciprocal cellmatrix interactions in the context of numerous applications involving the study of normal and diseased tissue morphogenesis.

Keywords

Three-dimensional breast tissue model; Three-dimensional organization; Collagen fiber; Hormone treatment; Multi-photon microscopy

1. Introduction

Mammary gland morphogenesis is mediated by reciprocal cell-matrix interactions throughout life [1, 2]. Hormones such as estrogens, progesterone and prolactin affect such interactions, leading to changes in gene expression, cell proliferation, postnatal mammary gland development and disease progression [3–5]. As revealed by analysis of hormone receptor mutant mouse strains combined with tissue recombination techniques and proteomics, sequential and simultaneous activation of hormone signaling in the mammary epithelium is required for progression of normal morphogenesis [5, 6]. Although the establishment of hormone responsive breast epithelial cells in the 1970's using two-dimensional (2D) cultures provided valuable information regarding hormonal regulation of epithelial gene expression[7–9], three-dimensional (3D) organization is critical to understanding morphogenesis as well as cellular responses in the context of tissue organization [10–12]. Unlike monolayer cultures, mammary epithelial cells grown in 3D cultures recapitulate numerous features of the breast epithelium *in vivo*, including the formation of acini-like spheroids with a hollow lumen, apico-basal polarization of cells that make up these acini, the basal deposition of basement membrane components, and, in some cases, the production of milk proteins [10, 13–15]. Thus, 3D culture models adequately bridge the gap between complex whole animal models and simple 2D cultures [16–18].

Modified cell-matrix interactions have major effects upon key functional cellular features [19–21], and also upon the matrix biophysical and biochemical properties, including collagen fiber organization and mechanical properties [22]. Although the establishment of 3D breast tissue models provides a useful tool for the study of mammary gland morphogenesis, the role of matrix reorganization during this process and the associated biomechanical interactions remain poorly understood, partly due to a lack of sensitive detection and analysis approaches [17]. In this regard, second harmonic generation (SHG) microscopy has enabled non-invasive, label-free visualization of collagen fibers within 3D specimens with micron-level resolution [23–25]. However, the approaches used to characterize collagen fibrillar orientation and organization have focused almost exclusively on analysis of 2D images [26–31] with only a limited number of exceptions [32–35]. As these methods are either computationally expensive or lack the ability to provide organization readouts at the microenvironment scale, we recently developed a weighted vector summation algorithm which is able to define the voxel-wise orientation of collagen fibers in a 3D context [36]. Based on the orientation information, we further proposed a metric, 3D directional variance, to resolve and quantify the spatial organization of fiber-like

structures [37]. This newly-developed metric has shown potential to characterize organization of fiber-like structures in a broad range of applications, including assessment of a) collagen fiber differences between normal and cancerous tissues or among distinct cartilage regions, b) changes in the alignment of neuronal axons in response to traumatic brain injury, and c) dynamic alterations in the alignment of fibroblasts and collagen fibers in engineered lung tissue models [37].

In this article, we apply the 3D directional variance metric to the assessment of collagen fiber organization in a hormone-sensitive 3D breast tissue culture model. Human breast epithelial T47D cells which express the estrogen (ER), progesterone (PR), and prolactin (PrIR) receptors were used in this study because, among all established estrogen-sensitive cell lines tested, they formed the most representative mammary gland-like structures [38]. In fact, they responded to mammotropic hormones in a comparable fashion to that of the mammary gland *in situ* [38]. 3D cultures of T47D cells seeded in collagen type I matrix were treated with 17 β -estradiol (E2) alone, or E2 in combination with either promegestone or prolactin. Two-photon excited fluorescence (TPEF) and SHG images were acquired to resolve the epithelial morphology and collagen fiber organization. We used the 3D directional variance as a quantitative metric of the collagen fiber alignment to a) compare the organizational randomness of collagen fibers surrounding the epithelial structures in response to different hormone treatments, and b) to identify changes in fiber organization as a function of distance from the epithelial structure edges as well as along distinct regions of these structures. We evaluated correlations between spatial organization and collagen fiber density and demonstrated the sensitivity enhancements that were achieved when we used 3D as opposed to 2D-based analysis of the collagen fiber organization.

2. Materials and methods

2.1. Description of 3D directional variance algorithm

The development of the formulas to assess 3D orientation and 3D directional variance for axial (fiberlike) data has been described in detail recently [36, 37]. Briefly, the 3D orientation algorithm is based on the extension of a previously established 2D weighted vector summation technique [30]. The 3D directional variance for circular data (i.e. data for which 0° and 360° are equivalent), V_{3D} , is defined as [37]:

$$V_{3D} = 1 - \bar{R}_{3D}, \text{ where } \bar{R}_{3D} = \left(\bar{Z}_{3D}^c{}^2 + \bar{S}_{3D}^c{}^2 + \bar{C}_{3D}^c{}^2 \right)^{1/2}, \text{ and } \bar{C}_{3D}^c = (1/n) \sum_{j=1}^n \sin\varphi_j \cos\theta_j, \bar{S}_{3D}^c = (1/n) \sum_{j=1}^n \sin\varphi_j \sin\theta_j,$$

$\bar{Z}_{3D}^c = (1/n) \sum_{j=1}^n \cos\varphi_j$. The superscript c refers to the circular data, while θ and φ are the azimuthal and polar angles used to depict an orientation in 3D space, respectively. However, in the context of axial data (i.e. data for which 0° and 180° are equivalent), the definition of 3D directional variance needs to be modified. A general approach to transforming axial (fiber-like) data to circular data is to multiply angular values by 2. This strategy works for

transforming the azimuthal angle θ , but not for the polar angle φ . To address this problem, we consider additional angles β and γ (Fig. S1), which are azimuthal angles, like θ , and related to φ by:

$$\tan^2 \varphi = 1/\tan^2 \beta + 1/\tan^2 \gamma.$$

In this manner, the polar angle φ in the expressions for $\bar{C}_{3D}^a$, $\bar{S}_{3D}^a$ and $\bar{Z}_{3D}^a$ can be replaced by a function of azimuth angles β and γ . Specifically, we define $b_j = \sqrt{1/\tan^2(2\beta_j) + 1/\tan^2(2\gamma_j)}$, and the corresponding components suitable for axial data are expressed as:

$$\bar{C}_{3D}^a = (1/n) \sum_{j=1}^n (b_j / \sqrt{1+b_j^2}) \cos(2\theta_j),$$

$$\bar{S}_{3D}^a = (1/n) \sum_{j=1}^n (b_j / \sqrt{1+b_j^2}) \sin(2\theta_j), \text{ and } \bar{Z}_{3D}^a = (1/n) \sum_{j=1}^n SI / \sqrt{1+b_j^2}, \text{ where } SI = (-1) \cdot (\varphi - 90) / \left| \varphi - 90 \right|$$

when $\varphi = 90^\circ$, and $SI=1$ when $\varphi \neq 90^\circ$. The superscript a refers to the axial data. Using these modified components, we are able to acquire the 3D directional variance V_{3D} in the context of fiber-like data.

To calculate the 3D orientation of a certain voxel of the collagen fiber stack image, we first create a $n \times n \times n$ voxel window surrounding this voxel, and the intensity information within this window contributes to the determination of the orientation of this central voxel [36]. Based on our previous observations, a window of two to three times the fiber diameter leads to optimized orientation results [36]. Generally, to assess localized 3D directional variance, we use the same window size as for orientation analysis [37]. In this study, the diameter of collagen fibers is typically 1~1.5 μm , and we use $4.2 \times 4.2 \times 5 \mu\text{m}$ as the window size for both orientation and 3D directional variance analysis.

2.2. 3D breast tissue cultures

3D cultures of breast tissue were prepared using a previously described protocol [38]. Briefly, rat tail collagen type I (BD Biosciences) was used at a final concentration of 1 mg/mL [39], and neutralized with 1N NaOH according to the manufacturer's instructions. Human breast epithelial T47D cells were seeded at a density of 75,000 per gel. Cells were suspended in a final volume of 1.5 mL of collagen and then poured into 12-well plates (Becton Dickinson). The cell and collagen mixture congealed for 30 min at 37°C and 1.5 mL of the CD-FBS medium containing E2 (Calbiochem), E2 plus promegestone (R5020) (Perkin-Elmer), or E2 plus prolactin (Sigma) was added to each well. The hormone concentrations for each treatment were as follows: E2 alone, 10^{-10} M; promegestone addition, 10^{-10} M E2 plus 10^{-10} M promegestone; prolactin addition, 10^{-10} M E2 plus 10^{-7} M prolactin. Gels were detached as described by Dhimolea et al [17]. The 3D cultures were maintained for 2 weeks and the medium was changed every 2 days. Three replicates were prepared for each group. In order to visualize the epithelial structures the gels were whole-mounted and stained with Carmine Alum.

2.3. Image acquisition

SHG images of collagen fibers and TPEF images of cells were obtained using a Leica TCS SP2 confocal microscope equipped with a tunable (710–920 nm) titanium-sapphire laser (Mai Tai; Spectra Physics; Mountain View, CA). Non-descanned photomultiplier tube (PMT) detectors collected light in the 400 ± 10 nm (SHG) and 525 ± 25 nm (TPEF) regions at an excitation wavelength of 800 nm. Circularly polarized light was generated by a quarter-wave plate to excite all orientations equally. The objective used in this study was a water-immersion 63x objective (NA 1.2; 220 μ m working distance). Images with a size of 512×512 pixels (238.1×238.1 μ m) were acquired at an interval of 1 μ m along the *z* axis. Generally there was only one epithelial structure acquired in a 3D stack image. Reconstruction of 3D images was performed by ImageJ (W. Rasband, National Institute of Health, USA), and image analysis was performed by custom code developed in MATLAB (available for download at <http://ase.tufts.edu/biomedical/research/Georgakoudi/researchNonDestructive.asp>).

2.4. Simulating fiber stacks to compare sensitivity of different analysis approaches

In order to compare the sensitivity of 3D directional variance algorithm versus typical 2D analysis approaches, we simulated fiber stacks with three organizational gradients in MATLAB. In order to generate stacks with a certain level of organization, first we simulated a number of fibers with similar θ (within (60°, 65°)) and ϕ (within (60°, 65°)) orientations, and then added randomly oriented (θ , ϕ ranged within (0°, 180°)) fibers in 3D space. Different gradients of fiber randomness were generated by adjusting the numbers of randomly oriented fibers, leading to fiber stacks with three distinct gradients corresponding to approximately 0.44, 0.52 and 0.60 in anticipated 3D directional variance. Five fiber stacks were generated for each gradient. Note that the intensity of each fiber was weighted based on its orientation and thickness so as to mimic the SHG signals which were sensitive to these characteristics [40–42].

2.5. Statistical analysis

In order to assess the presence of significant differences in either 3D or 2D directional variance, as well as fiber density among the three hormone treatments, an ANOVA with post-hoc Tukey HSD test was performed using JMP 12. A comparison in the error of angle θ , angle ϕ and overall error δ , as well as the percent error of 3D directional variance acquired from the uniform-intensity fiber stack or the fiber stack with orientation and size dependence was performed using a two-tailed t-test. A comparison of 2D directional variance determined using a frame-by-frame analysis approach was run using a nested model to avoid discarding any data information. Correlations between 3D directional variance and collagen fiber density were assessed, and significance was determined based on the null hypothesis that $R = 0$. Results were considered significant at $p < 0.05$. To assess the potential of fiber density and 3D directional variance as metrics that enable us to distinguish the characteristics of the tissues undergoing the three different hormonal treatments, we performed canonical linear discriminant analysis. Discrimination accuracies were calculated with the linear discriminant functions applied on either the entire data set or a leave-one-out cross validation scheme using SPSS.

3. Results

3.1. 3D directional variance quantifies fibrillar organization

The 3D directional variance metric was acquired based on the 3D orientation of fibers. To depict an orientation in 3D space (the blue line in Fig. 1A), an azimuth angle θ and a polar angle ϕ were determined (Fig. 1A). As references for visualizing orientation values in this study, the orientation maps of two typical planes are plotted in Figures 1B and 1C respectively, corresponding to the planes marked by magenta and green (Fig. 1A). Arrows in Figure 1A indicate the directions of increasing angle values. Note that throughout this study, optical sections were all parallel to the xy plane, and a ϕ orientation of 90° reveals that fibers are parallel to the optical sections (Fig. 1C). Once we defined the orientation of the fibers within a 3D stack of images, we used the recently developed 3D directional variance as a metric of 3D fiber alignment [37]. The value of this parameter ranges between 0 and 1, with 0 corresponding to completely aligned fibers within the analyzed volume, and 1 referring to fully random organization. To illustrate how 3D variance was used, we simulated a $400 \times 400 \times 400$ voxel stack of fibers with different orientations (Fig. 1D, left). The color-coded θ , ϕ orientations acquired by the weighted vector summation algorithm, as well as the 3D directional variance maps of two localized regions (with a size of $40 \times 40 \times 40$ voxels) were zoomed in (Fig. 1D, right). As can be seen from the θ , ϕ orientation maps, region 1 had more consistent hues both in θ and ϕ orientations than those in region 2. Within the corresponding 3D directional variance maps, there were bluer hues (corresponding to lower variance values) in region 1, in contrast to the redder hues in region 2. When we plotted the θ (black) and ϕ (blue) orientation distributions within these two regions, we found that both θ and ϕ distributions within region 1 (Fig. 1E, top) had a sharper peak than those within region 2 (Fig. 1E, bottom), consistent with observations from the color-coded orientation maps (Fig. 1D, right). As expected, the distribution of 3D directional variance of region 1 showed a peak at lower values (Fig. 1F), further supporting that the fibers are more organized (i.e. less random) in region 1.

3.2. 3D directional variance is not highly susceptible to the fiber orientation and size dependence of SHG

SHG is sensitive to size and orientation of fibers with respect to the imaging plane [40, 43, 44]. In order to assess how this size and orientation dependence of SHG intensity affects the accuracy and sensitivity of 3D organization determination, we compared the error of 3D directional variance acquired from simulated 3D fiber stacks with or without the size and orientation dependence. The relationship between the SHG intensity and the polar angle ϕ orientation is shown in Figure 2A [40], and the fiber size dependence is based on data from Raub et al. [41, 42]. Both the θ and ϕ orientation was randomly defined within the range between 0° and 180° , and the fiber thickness varied from 3 to 7 voxels. Figure 2B shows a representative 3D image of the fiber stack where all the fibers have uniform intensity, whereas Figure 2C corresponds to the same stack, but with weighted intensity based on the angle and thickness of each fiber, as indicated by varied hues. Ten sets of fiber stacks were simulated to make a comparison. The 3D orientation algorithm was applied to acquire the θ and ϕ orientation of every voxel of each fiber, and the error of θ , ϕ orientation (θ and ϕ , respectively) or the overall angle error (δ , referring to the difference between the calculated

and the designated orientation in 3D space) were obtained. The overall angle error maps corresponding to a representative uniform or weighted-intensity fiber stack are shown in Figures 2D and E, respectively. A more detailed comparison can be achieved by zooming in a localized region, as shown in the insets, which correspond to the signal intensity, θ orientation, ϕ orientation and the overall angle error map. It is interesting to note that one of the fibers was almost invisible with a weighted intensity of 0.008 (as pointed by the arrow in the first inset), in contrast to the normalized maximum intensity of 1. However, it is still sufficient to acquire the θ and ϕ orientation with good accuracy, as demonstrated by the overall angle error (the fourth inset), although at certain regions the weighted case showed a higher error level (indicated by redder hues, as pointed by the arrow). A statistical analysis revealed that either the θ , ϕ error or the overall error was below or close to 6° in both cases, even though there was a significantly lower ϕ error and overall error in the uniform-intensity case.

Based on the orientation information, we further acquired the 3D directional variance for each stack, and calculated the percent error by comparing with the anticipated 3D directional variance. No significant difference in variance error was observed based on uniform- or weighted-intensity stack analysis, revealing that the 3D directional variance metric is even less susceptible to variations in signal intensity than that of the 3D orientation algorithm and that the effect of the orientation and size dependence of SHG could be minimum using this 3D directional variance assessment.

3.3. Different hormone treatments lead to differences in epithelial morphology and collagen organization

To resolve the cell morphology within the epithelial structures, TPEF images (excitation: 800 nm, emission: 525 ± 25 nm) of Carmine Alum stained T47D cells were acquired and reconstructed in 3D (Fig. 3A). Different patterns of epithelial organization in response to different hormone treatments were observed in 3D cultures, as reported previously [38]. In brief, in the presence of physiological concentrations of E2 alone, T47D cells formed rounded or slightly elongated (Fig. 3A) structures. In contrast, E2 in combination with the synthetic progestogen, promegestone, led to the formation of elongated structures with multiple cellular projections simulating side-branching [45](Fig. 3A). E2 in combination with prolactin resulted in the appearance of budding structures, which are spoon-like shapes at the tip of the ducts [8, 46](Fig. 3A). Collagen fibers surrounding cell clusters were visualized by SHG, and 3D reconstructions combining the cells and collagen fibers are shown in Figure 3B.

To acquire the voxel-wise 3D directional variance map, the orientations of collagen fibers were first calculated. All the calculations were done in a 3D context, and every voxel of the 3D stack was designated a θ and a ϕ orientation. Representative 2D images (Fig. 3C) within each 3D stack are shown along with corresponding distribution histograms (Fig. 3D) and color-coded θ and ϕ orientation maps (Fig. S2). The orientation distributions corresponded to the region within 23 μm from the cell structure edge. This range is comparable to previous studies based on 3D breast tissue culture models, where 2D analysis approaches were used to assess collagen fiber organization [28, 38]. When compared to the prolactin and

promegestone treatments, the E2 alone treatment showed a relatively sharper θ orientation distribution (black), with peaks close to 0° and 180° . This is consistent with the apparent directions of fibers visualized in the corresponding representative 2D images (Fig. 3C). We observed an even more dramatic difference in the ϕ distributions among these three groups. Collagen fibers in the E2 alone treatment yielded a single and sharp peak at $\sim 90^\circ$, which revealed that the majority of collagen fibers within this region are aligned in a primarily parallel direction to the optical sections. In contrast, relatively broader distributions for the θ and ϕ orientations in the prolactin and promegestone treatments revealed that collagen fibers are more randomly aligned in these cases. The corresponding 3D directional variance maps are shown in Figure 3E. E2 alone showed bluer hues than the other two treatments, and E2 plus promegestone led to even redder hues than E2 plus prolactin which indicated a higher variance level. However, as pointed by arrows in the E2 plus promegestone image, there were limited but clear blue hues at the tips of some branches, consistent with previous observations that collagen fibers were thickening and highly organized at the tip of the ducts [17, 28].

The distributions of 3D directional variance of collagen fibers within $23\ \mu\text{m}$ from these representative epithelial structures are shown in Figure 4A. The raw data (dots) were fitted by 8th order polynomial function (dotted line), which was determined as the optimal order fit [47]. The relative position of these distributions corresponds well to the hues observed from the 3D directional variance maps (Fig. 3E), and the peak location, which is the 3D directional variance value corresponding to the maximum of the polynomial fit, was then acquired for each 3D stack image. E2 alone treated samples show a significantly lower peak location than that of samples treated by E2 in combination with promegestone or prolactin (Fig. S3). It is worth mentioning that even though E2 in combination with promegestone or prolactin led to generally higher variance values than E2 alone, there are some localized regions in these samples, especially at tips of branches in E2 plus promegestone treatment (as pointed by arrows in Fig. 3E) or ends of buds in E2 plus prolactin treatment, showing bluer hues, which correspond to lower variance than neighboring regions. In order to provide a more quantitative assessment, we plotted the 3D directional variance distribution at these localized regions (marked by red or green contour in Fig. 4B). As expected, they show a peak at lower variance, in agreement with the highly aligned fiber organization at branch tips or bud ends where cells might exert strong pulling forces on the collagen fibers. These observations of differences in color-coded variance maps or variance distributions acquired by 3D analysis (Fig. 5A), were further validated by statistical analysis (Fig. 5D). The E2 treatment group had significantly lower mean directional variance than both the promegestone and prolactin groups ($p < 0.0001$ compared to promegestone, $p = 0.0011$ compared to prolactin), and the prolactin group showed a significantly lower directional variance relative to promegestone ($p = 0.0175$). It is important to note that there was no differential growth due to the different hormonal treatments that might affect the collagen fiber organization, as the total cell number was similar in 3D cultures under each treatment using the applied hormone concentrations [38]. Besides, we quantified the size of cell structures in each 3D image stack by counting the total voxel number corresponding to each cell structure, and no significant differences were observed (Fig. S4). These results confirm and expand our previous findings that hormones affect not only the morphology of epithelial

structures, but also the organization of the collagen fibers surrounding cell clusters [38]. This hormonal effect in fiber organization involves the cells, which in turn, organize the fibers [17].

3.4. A comparison between 3D and 2D analysis

In order to demonstrate the sensitivity enhancement achieved by employing a 3D image analysis approach for the acquired 3D stacks, we compared the achieved results with those of two possible methods that rely on 2D image analysis (Fig. 5) [28, 38, 48]. As shown in Figure 5B, the first 2D approach projected the entire 3D stack along the z dimension, followed by 2D directional variance analysis within the same region of 23 μm from the cell structure edge. As can be seen from the analysis results shown in Figure 5E, although there were similar trends in the mean directional variance as for the 3D analysis, the large standard deviation in the corresponding measurements led to non-significant differences among treatments. The latter approach is based on estimating the 2D directional variance frame-by-frame (Fig. 5C). The differences of the mean values among different treatments were subtle, and as in the previous case no statistical differences could be identified (Fig. 5F). We also validated the sensitivity improvement of 3D directional variance analysis over these two 2D analysis approaches using simulated fiber stacks with 3D directional variance values of 0.44, 0.52 and 0.60 (see Materials and methods). Applying the 3D variance algorithm to fiber stacks simulated to represent these three gradients of fiber organization (Fig. S5A), we found that the significant differences among different organizational gradients were faithfully maintained (Fig. S5B). However, the projection-based 2D analysis lost the significance in the 2D variance differences between Gradient 1 and 2 and yielded a higher p value for the variance differences between Gradient 2 and 3 (Fig. S5C). The frame-by-frame 2D analysis approach could not identify any significance in the variance of image stacks from any two organization gradient groups (Fig. S5D). The comparison from the actual 3D cultures, as well as that from the simulated fiber stacks, reveals that the 3D analysis is more sensitive in identifying organizational differences in 3D stacks of collagen fiber images than 2D image-based methods, which are only sensitive to changes in θ orientation.

3.5. Location dependence of 3D directional variance relative to cell structure

The results presented in Figures 3–5 demonstrate that differences in the configuration of epithelial structures are accompanied by differences in collagen fiber organization. Thus, we performed a more detailed analysis to assess the presence of differences in the 3D directional variance of the fibers depending on their localization along specific portions of the observed epithelial structures (Fig. 6). For this purpose, we examined both the epithelial morphology and the distance dependence of collagen fiber organization. First, we fitted an ellipse to the cell structure, then we selected the area extending 23 μm outside the fitted ellipse along the direction of each xy plane, and assessed the 3D directional variance within the body (region 2) and two end (region 1) regions separately (Fig. 6A). To segment these two regions, we followed the equation

$$MN = 0.3 \times MO,$$

where *MO* extends from the center of the ellipse to the furthest point along the major axis in each direction (Fig 6A). The mean and standard deviation of the 3D directional variance within each region were acquired and shown in Figure 6B. In the E2 and E2 plus promegestone groups, no obvious differences were observed between the localized region 1 and 2; however, both region 1 and 2 in the promegestone treated cultures had a significantly higher 3D directional variance than that of both regions of the E2 treated ones ($p < 0.0001$). On the other hand, significant differences existed between the end and body regions of the prolactin treated cultures ($p < 0.05$). Further, the end region of the prolactin group showed a significantly lower 3D directional variance than the promegestone group ($p < 0.05$), while the body region of the cultures exposed to prolactin showed a significantly higher variance than the E2 alone treatment group ($p < 0.0001$).

Besides the distribution of 3D directional variance surrounding the cell structure, we also assessed the directional variance metric as a function of distance away from the cell clusters. For every 3D stack, we created binary ring-masks outside the epithelial structure, each with a width of 11.6 μm , which was comparable to the diameter of individual cells, marked in different colors (Fig. 6C). Then the mean 3D directional variance within each ring-mask was acquired (Fig. 6D). We compared the directional variance among these three hormone treatments at each ring-mask, with the statistical results labeled on top of each data point. Generally, within a range of up to $\sim 60 \mu\text{m}$ away from the edge of the cell structure, the results remained statistically similar to those acquired for the regions immediately adjacent to the cells. At further distances only the differences between the E2 alone and E2 plus promegestone treated groups remained significant, at least up to a distance of $\sim 110 \mu\text{m}$. These results reveal that epithelial morphogenesis induces organizational alterations in collagen fibers, which are present not only next to the cells but also at a significant distance from the cells [49]. In this regard, it is known that cells use traction forces to organize collagen that mediate long scale cell-cell interactions [49].

3.6. Correlation between 3D directional variance and collagen fiber density

Collagen fiber density is another important characteristic of the matrix microenvironment that can be characterized in detail based on analysis of SHG images. We have previously found that a threshold of 25% of the maximum value within the stack identifies consistently the collagen containing regions [38]. Here, we also applied this thresholding method for collagen fiber identification within 23 μm of the cell clusters

in the acquired SHG stacks for each treatment. A significant difference in fiber density was observed only between the E2 and E2 plus promegestone treatments ($p = 0.0135$, Fig. 7A). This reveals that collagen fiber density is not as sensitive as 3D directional variance in identifying subtle alterations in response to different hormone treatments. Further, we observed that the correlation between collagen fiber density and 3D directional variance was not significant (Fig. 7B). This confirms that the organizational differences that we observed among different hormone treatments originated from the spatial alignment of the collagen fibers themselves and suggests that more generally these two metrics may provide complementary information regarding the matrix microenvironment. In this context, we did a classification analysis to distinguish the impact of different hormone treatments using a

combination of fiber density and 3D directional variance metrics (Fig. 7C). This multi-parametric analysis yielded an original classification accuracy of 81.5% and a cross-validated classification accuracy of 74.1%, which was higher than an accuracy of 40.7% or 66.7% achieved using the fiber density or the 3D directional variance alone, respectively (Fig. 7D). A higher classification accuracy based on the 3D variance than that based on the fiber density further supports the notion that the 3D directional variance is more powerful in resolving differences in the matrix microenvironment. It is interesting to note that fiber density and 3D directional variance showed a positive correlation in response to E2 treatment (Fig. 7C), but a negative correlation under treatments of E2 in combination with promegestone or prolactin (Fig. 7C). This, at least in part, explains the lack of significance in the correlation of these two metrics when considering all the data points (Fig. 7B).

4. Discussion

Our studies reveal that hormonal treatments have a significant impact not only on epithelial morphology, but also on the organization of the surrounding collagen fibers. Consistent with findings of hormone-induced mammary epithelial morphogenesis in animals, we observed lateral branching induced by promegestone in the presence of estrogens, and budding induced by prolactin in the presence of estrogens. Correspondingly, here we report that the E2 plus promegestone treatment led to the highest variability in orientation of collagen fibers, while the E2 plus prolactin and E2 alone treatments corresponded to increasingly smaller variations in fiber orientation. Thus, the more extensive deviations from the acinar epithelial organization that was typically encountered in the E2 plus promegestone treated cultures, were associated with progressively more randomly organized collagen fibers (Fig. 3). Previous studies found that areas of mammary gland requiring invasion and proliferation and low adhesion (terminal end buds, side branching) would have a unique ECM/integrin profile compared with areas that are dormant (such as primary ducts) [50]. Therefore, a higher level of heterogeneity in ECM/integrin profile from E2 plus promegestone treatment might be related to a complex alignment of collagen fibers and overall higher variability. Consistent with our observations of an overall higher but localized lower 3D directional variance under E2 plus promegestone treatment in contrast to the other two hormone treatments, Provenzano et al. also observed well aligned collagen fibers at the tips of branches in mammary gland, but highly wrapped fibers around the duct of the same gland [51]. The broadening of the polar angle ϕ distribution of the 3D fiber orientations appears to drive this increase in randomness (Fig. 3). This is one of the main reasons why the 3D directional variance metric is more sensitive in identifying differences in matrix organization than the corresponding 2D-based analysis metric, since the latter is only dependent on the fibers' orientation along the azimuthal angle. These differences in fiber organization were significant within at least 60 μm from the edge of the epithelial structures, with the overall trends persisting even beyond 100 μm (Fig. 6).

While we have previously demonstrated the high levels of sensitivity of the 3D variance as a metric of collagen fiber organization [37], we present here results from additional simulations which further validate this assertion. Specifically, we illustrate that SHG intensity dependence on fiber orientation and size does not affect the value of the 3D variance significantly, even if the error in assessing the exact angular orientation of the

collagen fibers increases to a small degree (Fig. 2). In addition, we use simulated stacks with fibers that represent distinct levels of alignment to demonstrate that while the 3D variance identifies these differences with high levels of significance, corresponding 2D-based analysis approaches either fail to detect differences or do so with reduced levels of significance (Fig. S5). Such detailed comparisons have not been presented for other methods of assessing the 3D orientations of collagen fibers [32–35]. As we have discussed previously [37], the 3D variance is over 30% more efficient than 3D Fourier-based analysis approaches [34], and completely independent of any reference to a specific coordinate system, as is the case for methods that rely on inertia moments, polarization, and diffusion tensor imaging [32, 33, 35]. Furthermore, an important merit of our 3D directional variance approach is that it can provide assessment of fiber organization at different scales by applying a different window size. In this study, its ability to provide localized organizational readouts within irregularly shaped volumes in a quantitative manner was essential for characterizing subtle changes in collagen remodeling in response to hormonal regulation.

While our results provide further support for the hormonal regulation of collagen remodeling, the underlying mechanism for this process is still under study. It was reported that these hormones may affect different proteins downstream of hormone-receptor interactions, all of them involved in cytoskeleton organization [52–54]. These proteins can mediate transmission of traction forces at localized regions surrounding cell structures and as a result remodel the collagen fibers. In order to understand this better, we have developed a mathematical model that will be used in our future work to find out how hormone treatment modifies cell-matrix interactions. Specifically, we use principles identified as fundamental for the elaboration of a theory of organisms, namely i) the default state of cell proliferation with variation and motility and ii) the principle of organization by closure of constraints. The success of this model suggests a step-wise approach whereby additional constraints imposed by the tissue and hormones could be rigorously tested by *in vitro* and *in vivo* experiments [55].

The localized 3D directional variance assessments uncovered additional differences in the manner cells interact with their surrounding environment (Fig. 6). Namely, only the budding structures, more frequently observed in the E2 plus prolactin treated cultures, were associated with a significant difference in the organization of the collagen fibers within the end and body regions of these structures. The significantly lower directional variance at the end than at the body regions of the epithelial structures suggests a likely reciprocal pulling interaction between the cells and the collagen fibers, consistent with previous studies [17, 28, 49, 56]. In contrast, the lack of a region-dependent fiber organization within the surrounding micro-environment of the epithelial structures treated with E2 plus promegestone suggests that there may be no preferred locations for initiation of side-branching. A schematic of the epithelial structures and surrounding collagen fibers in response to different hormone treatments are shown in Figure 8, which represents the cellular and ECM features resolved in this study, including the epithelial morphology, collagen fiber density, spatial orientation and organization.

Previous work focusing on 3D models of breast cancer and mammaryogenesis has suggested the reciprocal interactions between the organization of epithelial structures and the

alignment of collagen fibers [12, 17, 51, 57, 58]. Dhimolea et al. cultured 3D in vitro models using human breast epithelial MCF10A cells to study mammary epithelial morphogenesis, and found that MCF10A cells facilitated the organization of thick collagen fibers packed into large bundles at the tip of the ducts and parallel to the direction of ductal elongation. These collagen features were visualized using picrosirius red stained samples, which were imaged by polarized light microscopy [17]. Maller et al. studied the role of mammary stroma in the reduction of breast cancer risk attributed to early-age pregnancy [58]. Using SHG and atomic force microscopy, they found that the abundant collagen observed in the mammary glands of parous rats was more randomly organized and associated with a decrease in stromal stiffness compared with that of nulliparous rats, and further demonstrated that linearized (fibrillar) collagen I induced cellular phenotypes consistent with an invasive behavior in mammary tumor cells using 3D culture models [58]. Provenzano et al. used combined TPEF/SHG imaging of tumor explants cultured within 3D collagen gels to investigate collagen reorganization at the tumor-stromal interface, and observed that tumor cells reorganized a previously random matrix to a more aligned structure to facilitate local invasion [58]. A recent study in which a 3D culture model of MCF-7 cells was treated by a combination of E2 and prolactin also demonstrated that collagen fibers became more perpendicular to the cell surface [59], which was interpreted as a hallmark of cancer invasion, similar to the pulling interaction at the budding ends observed in the E2 plus prolactin treatment in our study. However, the majority of these studies were based on qualitative observations. Barnes et al. cultured 3D models of MCF10A cells to assess interactions between matrix and human breast cells during the process of mammary gland morphogenesis, and quantified the collagen fiber orientation and organization based on 2D analysis of matrix images acquired by reflectance confocal microscopy [28]. They found that elongated and branching ducts exhibited regions of fiber orientation that changed from being parallel to perpendicular to the cell surface. In addition, at the tip of branches there appeared thickening fibers, which were highly aligned, consistent with observations from the side-branching and budding formations in our present study [28]. Additionally, Montevil et al. developed a mathematical model to address the 3D model described by Barnes et al. The formation of acini and ducts could be explained by cells exerting forces that were transmitted to neighboring cells and collagen fibers, which in turn created constraints to cell movement and proliferation [55]. To the best of our knowledge, the T47D model is the first hormone-sensitive 3D culture model suitable to provide a systematic assessment of hormonal regulation of both epithelial and matrix organization [38].

The observations on the subtle changes in the extracellular micro-environment from this study open up opportunities to elucidate reciprocal cell-matrix interactions in the normal and diseased tissue morphogenesis. Given the local heterogeneities in collagen fiber organization already observed as a response to hormonal regulation, there is a potential to establish a correlation between cell morphological or functional characteristics, such as shape and size of the epithelial structure, presence of lumen, etc., with the alignment of the fibers surrounding the epithelial structures. Moreover, time-lapse microscopy has the potential to reveal the genesis of these heterogeneities and their relationship to the growth and shape of the epithelial structure [60]. Additionally, by combining SHG with some other imaging modalities, such as optical tweezers, the force distribution involved in these morphogenetic

events could be studied in a specific way which plays an important role during the evolution of the alignment between actin and collagen fibers [61, 62].

5. Conclusions

In summary, the 3D directional variance metric is a highly sensitive metric for assessing collagen fiber reorganization as a result of modified cell-matrix interactions induced by hormone treatment. This sensitivity cannot be achieved by previous 2D approaches. The combination of the label-free, noninvasive imaging capabilities of multiphoton acquisition and the performance of this 3D collagen fiber organization metric, has broad application potential for numerous studies that aim to elucidate cell-matrix interactions that drive normal and diseased breast tissue development and ultimately to improve diagnostics and therapies of breast diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. 3D directional variance is able to quantify spatial organization of fiber-like structures. (A) Definition of azimuth angle θ and polar angle ϕ used to depict an orientation in 3D space. Arrows indicate the directions of increasing corresponding angle values. (B) The θ orientation map of the plane marked in magenta as shown in (A). The polar angle ϕ is 90° on this plane. (C) The ϕ orientation map of the plane marked in green as shown in (A). The azimuth angle θ is 0° on this plane. (D) Validation of the 3D directional variance metric in characterizing spatial randomness of fibrillar structure. Left: a simulated $400 \times 400 \times 400$ voxel stack with differently oriented fiber components. Right: the θ orientation (top), ϕ orientation (middle) and 3D directional variance maps corresponding to two different regions ($40 \times 40 \times 40$ voxel) within the fiber stack, as marked in the left panel. The redder hues in 3D directional variance map in region 2 reveal higher spatial randomness. (E) θ (black) and ϕ (blue) orientation distributions of simulated fibers within region 1 (top) and region 2 (bottom). (F) Distribution of 3D directional variance corresponding to region 1 (green) and region 2 (blue), supporting that fibers within region 1 are more aligned than that in region 2.

Fig. 2. Assessment of the dependence of the fiber orientation and thickness on the determination of 3D directional variance based on SHG imaging.

(A) SHG intensity as a function of φ orientation. (B) A simulated 200×200×200 voxel fiber stack, where all the fibers have the same intensity regardless of the fiber orientation and thickness. (C) The same stack as that shown in (B), but with weighted intensity according to the angle and thickness dependent of SHG signal. Fibers are color coded according to the overall angle error (δ) within (D) a uniform and (E) a weighted intensity fiber stack. The insets shown in these two panels are corresponding to (from left to right): a localized

20×20×20 voxel intensity stack, the corresponding calculated θ map, the corresponding calculated φ map, and the corresponding overall angle error δ map. Note that in the weighted intensity stack case, one of the fibers is almost invisible (pointed by the arrow in the leftmost inset), with an intensity of 0.008 compared to the normalized maximum intensity of 1.

However, the θ and φ orientation can still be calculated with a good accuracy. **(F)**

Assessment of the error of the angle determination. $n = 10$ for either uniform- or weighted-intensity stacks. There is a significantly higher level of the φ error (φ , $p = 0.0087$) and the overall angle error (δ , $p = 0.0165$) in the weighted vs the uniform intensity stack. **(G)** No significant difference in percent error of 3D directional variance was observed between the uniform and the weighted intensity stacks.

Fig. 3. The 3D directional variance metric maps the spatial organization of collagen fibers surrounding the epithelial structure.

(A) 3D reconstructions of epithelial structures based on TPEF signals of Carmine Alum staining. (B) 3D reconstructions of combined TPEF epithelial structure (green) and SHG collagen fiber (magenta) images. (C) A representative 2D frame from the reconstructed 3D stack in each group. (D) θ (black) and ϕ (blue) orientation distributions of collagen fibers of the corresponding 2D frame within 23 μm from the edge of the epithelial structure. (E) Voxel-wise 3D directional variance maps of collagen fibers within the same 23 μm region. Arrows in E2+Promegestone image point to well-aligned fibers only at tips of branches. Scale bar: 50 μm .

Fig. 4. Distribution of 3D directional variance of collagen fibers at distinct regions.

(A) Distribution of 3D directional variance of fibers within 23 μm from the edge of the representative epithelial structures shown in Fig. 3. An 8th order polynomial function (dotted line) was fit to the raw data (dots). (B) Distribution of 3D directional variance at tips of branches in E2+promegestone treatment (top, marked by red line and arrow) or ends of buds in E2+prolactin treatment (bottom, marked by green line and arrow).

Fig. 5. A comparison between 3D directional variance and typical 2D analysis approaches. (A) Schematic of true 3D analysis based on the 3D directional variance metric. (B) Schematic of a typical 2D analysis approach, which converts the 3D stack into a 2D image by projecting the stack along the *z* direction, followed by 2D directional variance assessment. (C) Schematic of another typical 2D analysis approach which assesses the collagen fiber organization frame by frame via 2D directional variance. Mean and standard deviation of analysis results by (D) 3D directional variance, (E) *z*-projection 2D analysis approach and (F) frame-by-frame 2D analysis approach. There are three samples corresponding to each hormone treatment, and nine stack images per treatment (three per sample) are collected for 3D or 2D analysis. Scale bar: 50 μ m. *, $p < 0.05$; **, $p < 0.01$.

Fig. 6. Location-dependence of collagen fiber organization.

(A) Schematic of epithelial morphologybased fibrillar organization analysis. For each stack, the epithelial structure is fit with an ellipse, and the surrounding $23\ \mu\text{m}$ region is divided into two regions: the end region (region 1) and the body region (region 2), for collagen fiber organization assessment. (B) Mean and standard deviation of 3D directional variance within regions 1 and 2, corresponding to different hormone treatments. (C) Schematic showing the assessment of the distance-dependence of 3D directional variance away from the epithelial structure. Binary ring-masks are created outside the epithelial structure, each with a width of $11.6\ \mu\text{m}$. (D) Mean and standard deviation of 3D directional variance within each ring-mask in different hormone treatment groups. The color-coded solid triangles below the graphs

correspond to the ring-masks marked in (C). There are three samples corresponding to each hormone treatment, and nine stack images per treatment (three per sample) are collected for analysis. Scale bar: 50 μm . *, $p < 0.05$; **, $p < 0.0001$.

Fig. 7. Investigation of the relationship between 3D directional variance and collagen fiber density.

(A) Mean and standard deviation of fiber density within the surrounding 23 μm region of the epithelial structure. *, $p < 0.05$. (B) A correlation analysis between 3D directional variance and fiber density, with R and p values marked in the graph. $n = 3$ per treatment. (C) Classification of different treatments by combining both fiber density and 3D directional variance. Ellipsoids represent 90% of data coverage, while dotted lines and hourglass shades represent respectively linear fits and corresponding 95% confidence intervals for each

treatment group. **(D)** The original classification accuracy and the crossvalidated classification accuracy acquired from the usage of single metric (fiber density or 3D directional variance) or both of them.

Fig. 8. Schematic of epithelial structures and surrounding collagen fibers in response to different hormone treatments, summarizing the features including epithelial morphology, collagen fiber density, spatial orientation and organization.