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RECIPIENT: University of Rochester

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14. ABSTRACT In this project we will address the underlying mechanisms by which certain light-scattering properties of the tumor ECM predict metastasis. Understanding the mechanisms of this novel phenomenon may yield novel insights into metastatic processes, leading to new treatments for metastatic breast cancer. It may also uncover additional prognostic indicators, improving our predictive ability and further reducing overtreatment. The light scattering phenomenon in question is the direction that second harmonic generation light scatters from collagen fibers, the "F/B ratio". F/B is sensitive to the diameter of fibrils that are bundled into collagen fibers, as well as the spacing and disorder of their packing within the fiber, altogether known as a fiber's "microstructure". To begin to address why collagen microstructure, reported by F/B, predicts patients' metastatic outcome, we must first determine how that microstructure is defined by cells within the tumor, and the cues that influence those cells to do so.					
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1.INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Overtreatment of breast cancer is a pressing clinical problem as patients are subjected to the toxic side effects of chemotherapy even though they were not going to experience any post-surgical metastases. We recently discovered an innovative method to predict metastatic outcome in invasive ductal carcinoma (IDC) using light scattering from tumor collagen as an independent prognostic indicator. In this project we will address the underlying mechanisms by which these light-scattering properties predict metastasis. Understanding the mechanisms of this novel phenomenon may yield novel insights into metastatic processes, leading to new treatments for metastatic breast cancer. The light scattering phenomenon in question is the direction that second harmonic generation light scatters from collagen fibers, the “F/B” ratio. F/B is sensitive to the diameter of fibrils that are bundled into collagen fibers, as well as the spacing and disorder of their packing within the fiber, altogether known as a fiber’s “microstructure”. To begin to address why collagen microstructure, reported by F/B, predicts patients’ metastatic outcome, we must determine how that microstructure is defined by cells within the tumor, and the cues that influence those cells to do so, as well as how that microstructure affects tumor cells’ metastatic behavior. We believe that tumor cells and fibroblasts respond to presented matrix cues, including collagen subtypes, crosslink density, and the presented microstructure itself, to modify collagen microstructure (and hence F/B) by digestion of existing fibrillar collagen with MMPs, synthesis of fibrillar collagen, and LOX establishment of new crosslinks. We also believe that the microstructure of the tumor collagen in turn affects tumor cell metastatic ability at least in part by influencing tumor cell motility. This project will significantly impact the fight against breast cancer because understanding the mechanisms by which F/B predicts metastasis may uncover additional metastasis predictors, which will improve our ability to identify who should receive adjuvant chemotherapy. Improved predictive formulas can be incorporated into our ongoing development process and rapidly moved towards the clinic, thereby accelerating and enhancing the impact. Mechanistic understanding may also yield novel insights into metastatic processes. We are exploiting a novel way to study metastatic processes (by exploring the role of collagen microstructure) and thus newly discovered mechanisms affecting metastasis will likely themselves be novel. We will then be poised to explore druggable targets for antimetastatic therapies based upon these insights.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Metastasis, collagen, microenvironment, microscopy

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project? *List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or*

phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Major Task 1a. Evaluate F/B vs Col I:III and Col I:V ratios in IDC tumor samples. Major Task 1b. Evaluate F/B vs collagen crosslink density in IDC tumor samples.

Major Task 2a. Alter collagen ratios (I:III and I:V) in collagen gels and quantify resultant F/B modification by tumor cells and by fibroblasts. Major Task 2b: Alter crosslink density in collagen gels and quantify resultant F/B modification by tumor cells and by fibroblasts. Major Task 2c: Test mechanisms by repeating Major Tasks 2a,b after inhibiting formation of new LOX-induced crosslinks, collagen synthesis, and MMP activity. Major Task 2d: Repeat Major Tasks 2a-c in decellularized tumor tissue.

Major Task 3a: Add quantification of Col I:III and Col I:V ratio, and crosslink density, to our predictive formula, and assess its predictive strength.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

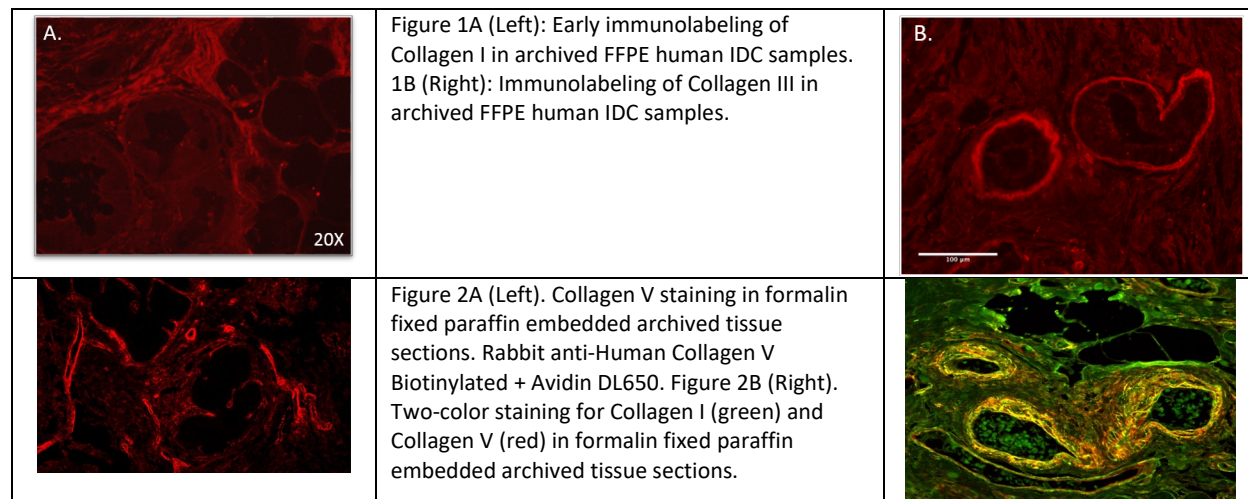
Please note this report focuses strictly on activities funded through this DOD award. Please also note that this report is cumulative: if a figure appears in a previous yearly report and does not appear here it is either because it has been reworked into a more complete figure (for example Figure 6a) or because it has been published and is now contained in an appended reference.

Introduction: This project began with the observation that a light-scattering phenomenon, specifically the directionality of second harmonic generation quantified as “F/B”, is prognostic of metastasis free survival time in untreated patients with invasive ductal carcinoma (IDC) that are estrogen receptor positive (ER+) and do not yet have significant visible signs of tumor cells in their lymph nodes (N0). The ultimate goal of the work is two-fold: 1) to propel that observation into a clinically useful tool for patients that will facilitate patient-tailored therapy by identifying who should, and should not, receive chemotherapy, and 2) to study the mechanisms underlying the relationship between F/B and metastasis in order to learn more about the metastatic process and hence develop anti-metastatic therapy. Tasks 1 + 2 of the grant address goal #2, while Task 3 addresses goal #1. To accomplish goal #1 broadly speaking we proposed to first confirm our results (that F/B predicts metastasis) in a US-based population with the modern distribution of neoadjuvant and adjuvant chemotherapy (the original discovery was made with a Dutch-based population who received neither adjuvant nor neoadjuvant chemotherapy), evaluate additional related predictors such as Col I/III content, then ask the more direct question if F/B plus related predictors can predict who will, and will not, respond to chemotherapy, then generate and validate the appropriate predictive model, then move to a multicenter trial to test that predictive model.

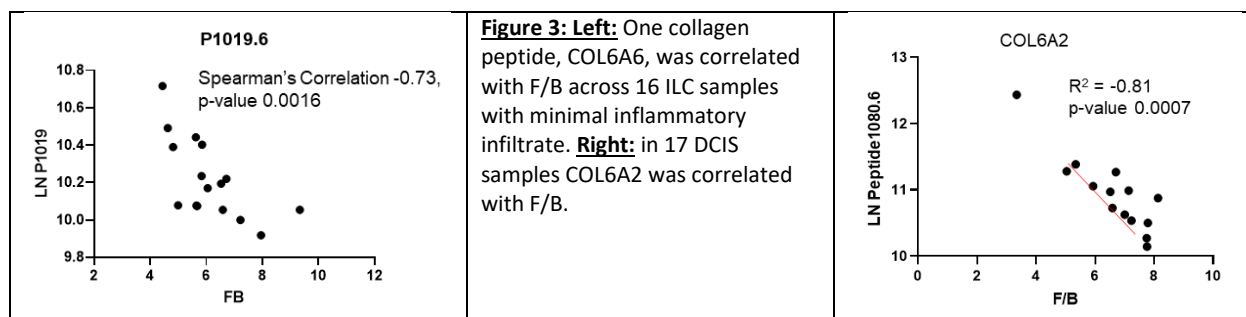
In the report that follows we state the Major Task title from the SOW in bold font, then report on our activities and products that are most relevant to that Task:

Major Task 1a. Evaluate F/B vs Col I:III and Col I:V ratios in IDC tumor samples. Major Task 1b. Evaluate F/B vs collagen crosslink density in IDC tumor samples.

Our original plan was to utilize immunolabeling to attempt to evaluate the relationships between collagen subtypes 1, 3, and 5 and F/B in breast tumor samples. Over the first year of the project we developed immunolabeling of collagen in our laboratory (Figure 1), including developing our ability to perform two-color staining (Figure 2).

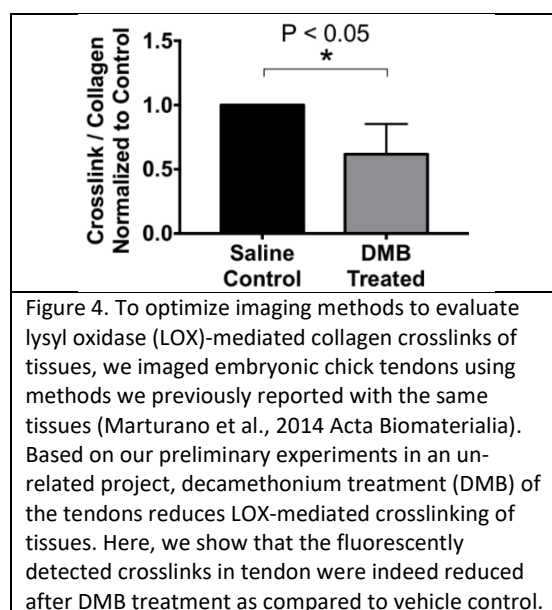


One acknowledged weakness of this plan was the issue of epitope availability, i.e. the fact that a collagen fiber has an inside and an outside, hence a particular collagen subtype may play a key role in defining its internal structure (and hence F/B) but may be poorly represented on the fiber surface, not bind many antibodies, and not contribute to an immunostain. At the 2019 SABCS meeting we initiated a collaboration with Dr. Peggi Angel of the Medical University of South Carolina, to use mass spectrometry to quantify the presence of 95 well characterized collagen peptides and compare them to F/B. This completely solves the problem of epitope availability and expands the range of collagen subtypes we can evaluate from 3 to 95, *and is in every way a better approach*. In a TMA of 60 invasive carcinomas and 20 invasive lobular carcinomas we found that higher stages revealed greater correlations, and F/B was correlated with COL1A2 ($R=0.752$) and COL4A1 ($R=0.737$) in stage III A/B invasive carcinoma. In stage IIA invasive lobular carcinoma F/B was correlated with COL6A2 ($R=0.730$) while in stage IIIA/B invasive lobular carcinoma F/B was correlated with COL3A1 ($R=-0.946$). One complexity was that, not surprisingly, there was variable amounts of tumor/stroma/inflammatory component in each disk in the TMA, perhaps adding to noise (although F/B did not correlate with the extent of these three categories). Invasive lobular carcinoma in particular has a minimal fibrous response, so we could eliminate 4 cores with inflammatory components above 10% and still retain 16 samples to study. In that reduced cohort a single collagen peptide, COL6A6 had a significant correlation with F/B across all ILC samples (Figure 3). As the samples were readily available on the TMA, we also evaluated the relationship between F/B and all 95 collagen peptides in 17 DCIS samples, and found a correlation between F/B and COL6A2 (Figure 3). This work is in its **final stages**, and was performed in the Brown lab, with an outside collaborator.



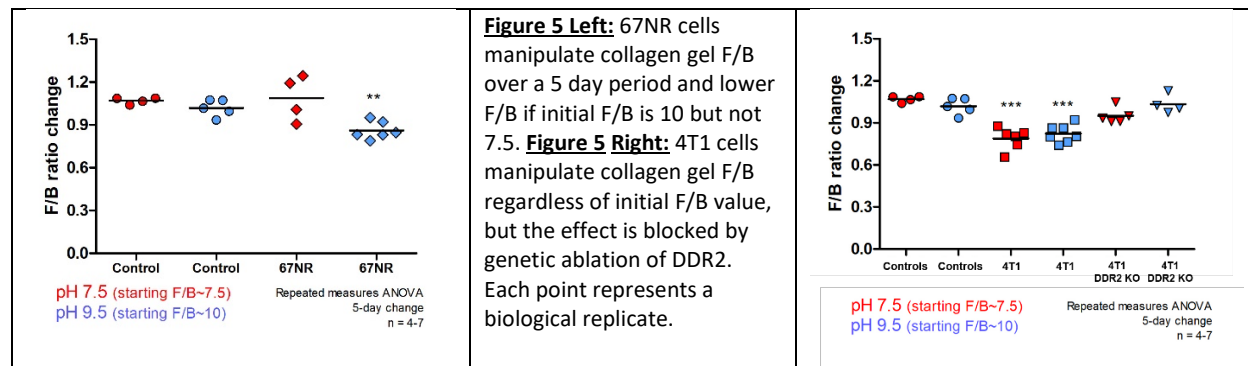
Major Task 2a. Alter collagen ratios (I:III and I:V) in collagen gels and quantify resultant F/B modification by tumor cells and by fibroblasts. Major Task 2b: Alter crosslink density in collagen gels and quantify resultant F/B modification by tumor cells and by fibroblasts.

Note that altering collagen ratios and crosslink densities, as stated in the title of these two subtasks, are ways of varying the fiber internal structure and hence SHG scattering directionality in collagen gels, which is the ultimate goal of these subtasks. In early work in this project we confirmed that we could use a novel autofluorescence-based assay developed by Dr. Kuo in her previous laboratory. To perform the work outlined in this proposal we first had to verify that we could successfully perform the technique in her new laboratory by reproducing a known result. In Figure 4 we see a confirmation of a previous result in the new laboratory, specifically that crosslink density of chick embryonic tendon (imaged with the autofluorescence assay) is correlated with elastic modulus (measure with AFM). Cryosectioned tendon samples were imaged with a 20X water-immersed lens at 800nm and 720nm excitation wavelengths for collagen and crosslink detection, respectively. Crosslink density per collagen content was determined by taking the average intensity ratio of crosslink to collagen signal.

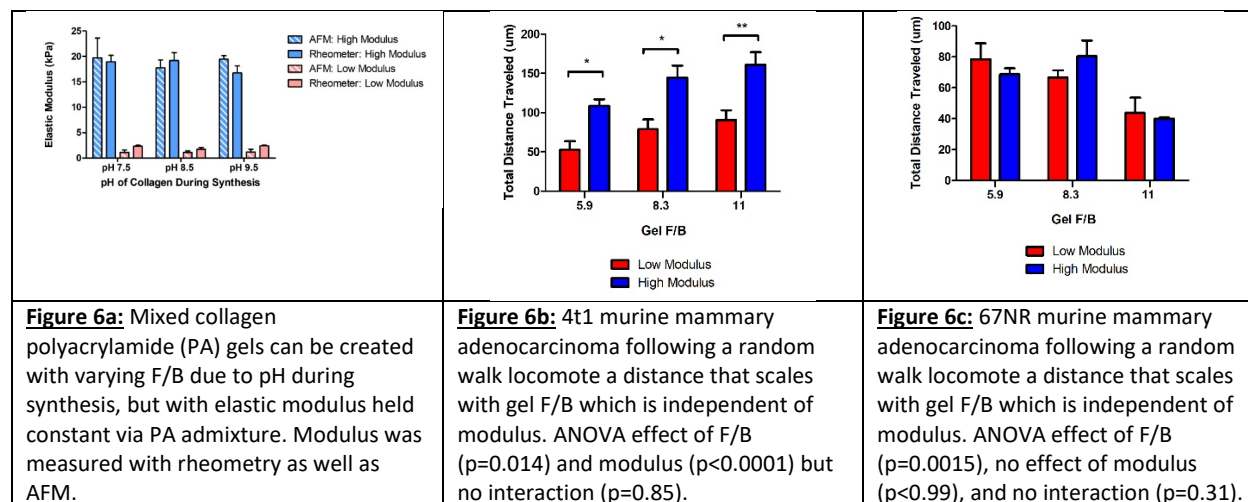


Another way to vary collagen fiber internal structure in gels is to vary pH during collagen synthesis, and as work progressed we found that we had better reproducibility with that method, so used that one instead. Consequently, we have determined the modifications in collagen F/B induced by tumor cell lines in collagen gels when presented with gels of different initial F/B, the goal of these subtasks, albeit with a different (and better) way of varying fiber internal structure of the gels than we first planned. We have found that the poorly metastatic 67NR murine mammary adenocarcinoma line reduces F/B in collagen gel when presented when an initial F/B of 9.5 but not when presented when an initial F/B of 7.5. Interestingly, the highly metastatic 4T1 line (a “sister” line to 67NR which arose from the same mouse) lowers F/B in collagen gels whether presented with an initial F/B of 9.5 or 7.5. This effect is blocked when Discoidin Domain Receptor 2 (DDR2), a collagen receptor, is knocked out of the

line, suggesting a possible mechanism for sensing collagen FIS (Figure 5). This work is in preparation for submission, and was performed in the Brown lab.



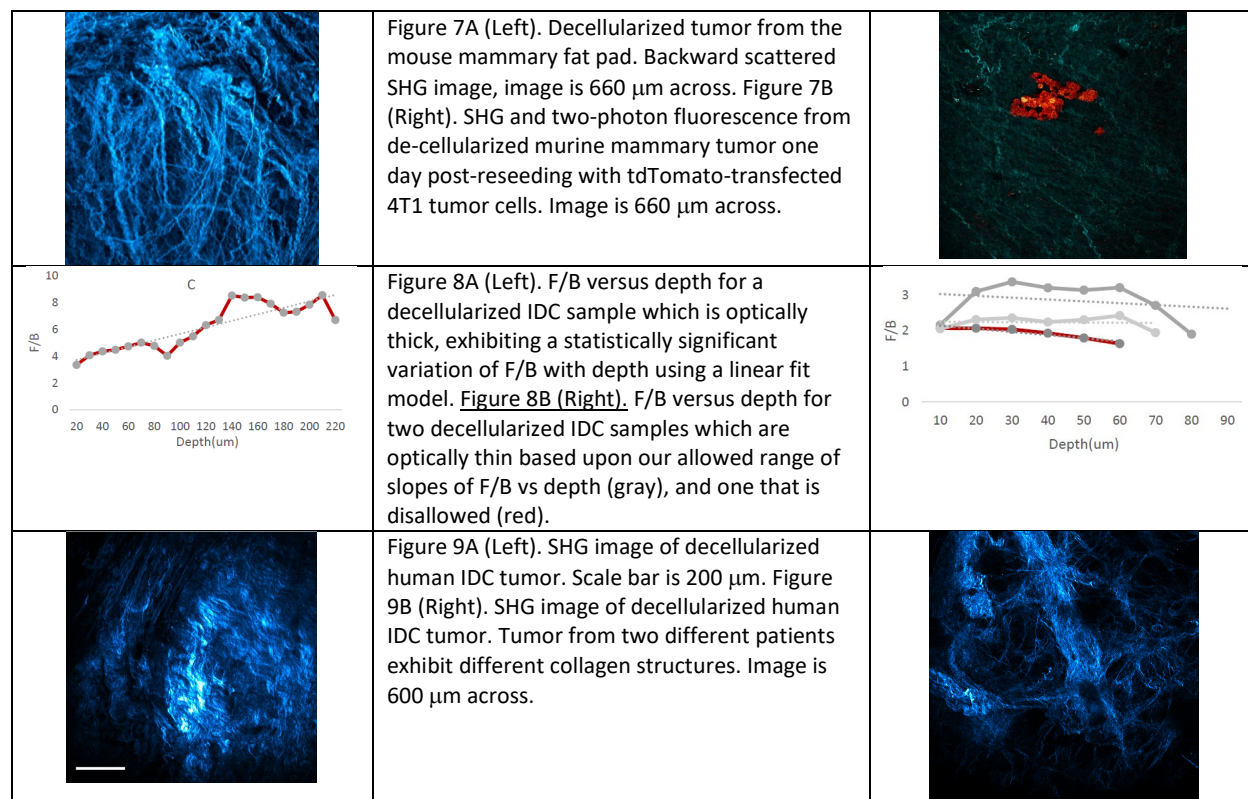
You will note that, while not explicitly stated in the Main Task *titles* above, one Subtask title is focused on matrix stiffness (“*Subtask 2: Verify stiffness is constant via AFM before cell application...*”): One possible model to explain why F/B is prognostic of metastasis is that collagen fibers form “roads” along which tumor cells crawl to escape the tumor. This was supported by an earlier paper of ours (Burke et al. 2015, Journal of Biomedical Optics 20(5):051024), that preceded this grant, and that showed that tumor cell motility varied with collagen gel fiber internal structure (FIS, the fiber physical properties which dictate F/B). The question remains, however, as to the possible mechanism by which tumor cells sense changes in FIS in order to respond to them with variations in behavior. One possible mechanism is by sensing changes in elastic modulus, i.e. stiffness, which may (or may not) accompany biologically relevant changes in FIS. Hence the subtask focusing on stiffness. To explore this possibility we generated mixed gels of varying FIS (as reported by F/B) but constant elastic modulus and introduced 4T1 and 67NR cells to those gels, tracking their random walk motility over a timescale of a few hours, short enough to avoid significant remodeling by the cells. Motility turned out to be a more rapid and higher throughput readout of tumor cell response to FIS than tumor cell F/B modification as first proposed. We found (Figure 6) that tumor cell motility varied with gel F/B even though elastic modulus was held constant in the 2-component mixed gel. This suggests that cells can sense changes in FIS independent of any possible concomitant changes in elastic modulus. Interestingly, the sign of the slopes of the two cell lines’ motility response to varying F/B in these pure collagen gels are different,



with 4T1 experiencing greater motility with increasing F/B, and 67NR experiencing less motility. *This is consistent with our observation in clinical samples that F/B has a positive correlation with metastasis in one breast tumor subtype, and negative correlation in another.* This work is **complete, submitted, and in revision** and is appended to this report, and was performed in the Brown and Kuo lab.

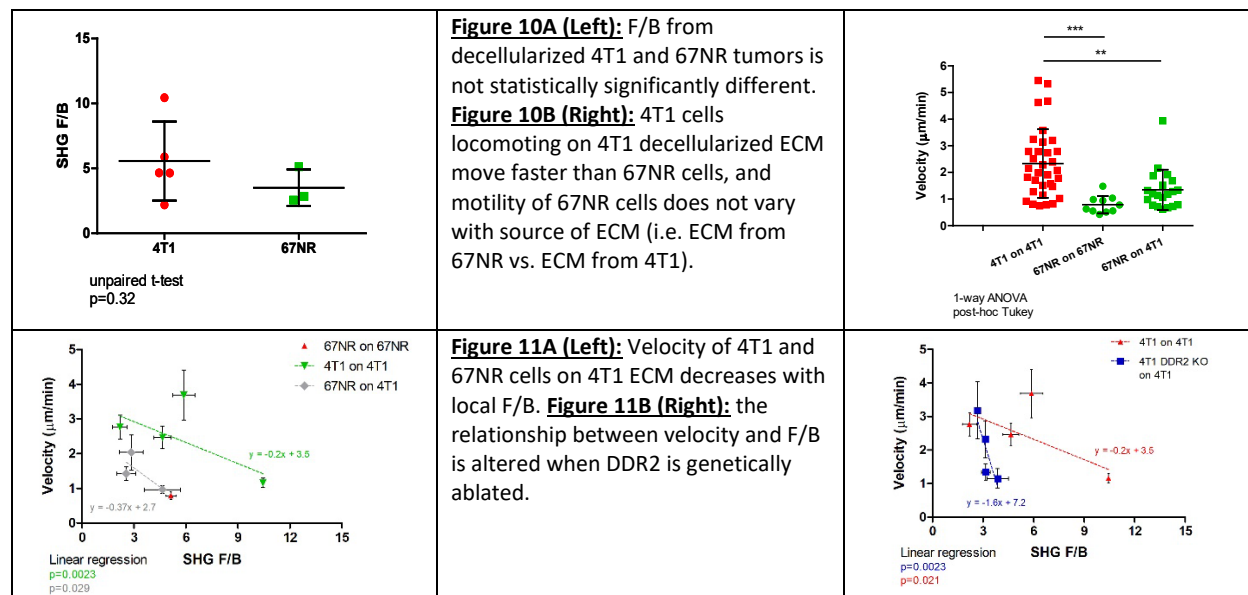
Major Task 2d: Repeat Major Tasks 2a-c in decellularized tumor tissue.

While pure collagen gels are useful tools, they do not reproduce all the molecular and cellular components of tumor collagen. However moving to a full *in vivo* model introduces a great deal of complexity and makes interpretation difficult. Therefore, we followed up on the results in the previous paragraph in decellularized tumor tissue, which has the molecular components of the tumor ECM but not the cellular components. This is the next logical step in a reductionist approach. When the project started we optimized our decellularization methods in murine tumors (Figure 7), developed our methodology to detect samples that were “optically thick (i.e. produced a measured F/B that varied with depth in the sample)” and created criteria to create samples that were “optically thin (i.e. with a measured F/B that did not significantly vary with depth)” (Figure 8), and applied them to human tumor samples (Figure 9). However, as described in previous yearly reports, our initial plan to study cell



response to F/B in decellularized human tumors was halted by a change in clinical procedure here at the University of Rochester Medical Center, which made those samples completely unobtainable. Therefore, we switched to decellularized mouse tumors. This proved to be an advantage as we could readily generate tumors from highly metastatic 4T1 tumors and nonmetastatic 67NR tumors. We found that tumors from the two different cell lines had the same F/B value on average (Figure 10). On average we found that the motility of 4T1 cells was higher than 67NR cells, and that the motility of 67NR cells did not vary with the source of the decellularized ECM (i.e. decellularized 67NR tumor vs. 4T1 tumor)(Figure

10). We have also found that the velocity of 67NR and 4T1 cells on decellularized ECM from 4T1 tumors decreases with F/B (Figure 11), in contrast with the results from the pure collagen gels described above, and suggesting that the “other” components of the ECM are playing a role in how tumor cells respond to changes in FIS. Finally, and most interestingly, the slope of the velocity-F/B relationship changes significantly when DDR2 is knocked out (Figure 11), again suggesting that this collagen receptor plays a role in cellular response to collagen FIS. This work is in its **final stages**, and was performed in the Brown lab.

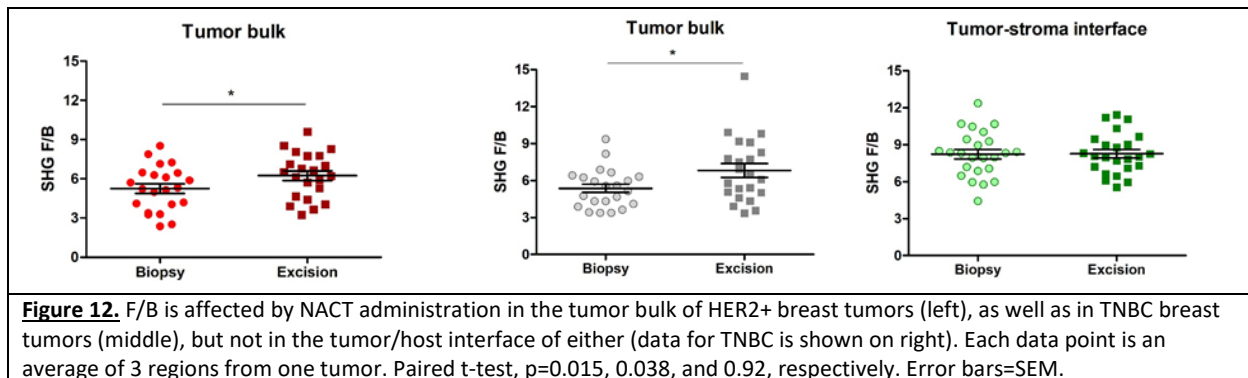


Major Task 3a: Add quantification of Col I:III and Col I:V ratio, and crosslink density, to our predictive formula, and assess its predictive strength.

As summarized in the opening paragraph of this report, one of the two major goals of this project was to propel our previous observations into a clinically useful tool for patients that will facilitate patient-tailored therapy by identifying who should, and should not, receive chemotherapy. This tool is what we mean by “our predictive formula” in the Task description above. To accomplish this goal broadly speaking we proposed to first confirm our results (that F/B predicts metastasis) in a US-based population with the modern distribution of neoadjuvant and adjuvant chemotherapy (the original discovery was made with a Dutch-based population who received neither adjuvant nor neoadjuvant chemotherapy), evaluate additional related predictors (such as Col I/III/V content), then ask the more direct question if F/B plus related predictors can predict who will, and will not, respond to chemotherapy, then generate and validate the appropriate predictive model. Future work would then move to a multicenter trial to test that predictive model. While beginning the project, we were approached by a clinical collaborator with an exciting and highly relevant *pre-existing* data set: a set of formalin-fixed paraffin-embedded needle biopsy sections with the associated patient data denoting subsequent response to neoadjuvant chemotherapy recorded as the Residual Cancer Burden (RCB) score. This sample set allows us to rapidly gain insight into the question as to whether F/B, in addition to predicting metastasis, can also predict response to chemotherapy, albeit in the neoadjuvant setting. *This allows us to “skip a step”* and see if F/B predicts chemoresponse as well as metastatic outcome. This led

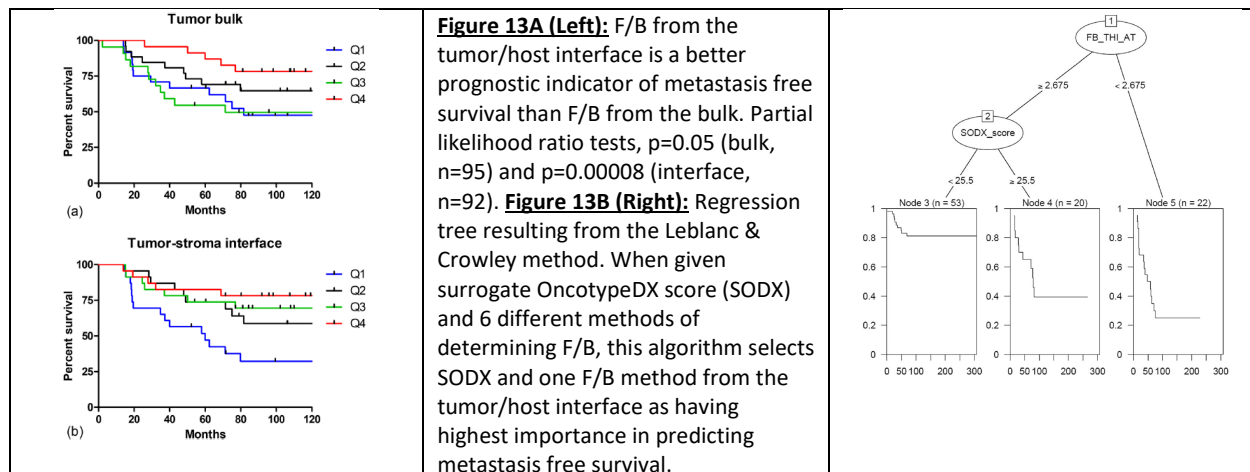
to the publication **Desa D, Bhanote M, Hill R, Majewski J, Buscaglia B, D'Aguiar M, Strawderman R, Hicks D, Turner B, Brown E. (2019) Second harmonic generation directionality is associated with neoadjuvant chemotherapy response in breast cancer core needle biopsies. Journal of Biomedical Optics. 24(8):1-9**, which is appended to this report. Highlights of the work include the observation that F/B was different between the tumor/host interface and the tumor bulk, that F/B from the tumor/host interface was correlated with response to neoadjuvant chemotherapy in HER2+ tumors (Fig. 3 in that paper), F/B from the tumor bulk was *not* correlated with response to neoadjuvant chemotherapy in HER2+ tumors (Fig. 3 in that paper), and that F/B was not correlated with response to neoadjuvant chemotherapy from either bulk or interface in TNBC tumors (Fig. 5 in that paper). This work was performed in the Brown lab.

Once we have “skipped a step” as described above, and advanced to the realization that F/B can predict response to chemotherapy, the question to naturally arise from the previous paper is whether chemotherapy *changes* collagen fiber internal structure, and hence F/B, in breast tumors. This was prompted by the observation that neoadjuvant chemotherapy can alter the characteristic “tissue microenvironment of metastasis”, or TMEMs, which are another optical predictor of metastatic outcome (Karagiannis et al Sci Trans Med 2017, *which came out after our initial grant submission*): as F/B is prognostic of metastasis, is it also altered by chemotherapy?? Using matched pre-and post-neoadjuvant chemotherapy samples from the same patients, we investigated the impact of neoadjuvant chemotherapy. Highlights of this project are that we have found that F/B in both HER2+ and TNBC tumors is altered by chemotherapy in the tumor bulk but not the tumor/host interface (Figure 12). This work is undergoing **final edits for submission** and is appended to this report. This work was performed in the Brown lab.



Based upon our results on needle biopsy samples described above, in which F/B was predictive of chemoresponse *if assessed in the tumor-host interface* but not if assessed in the tumor bulk, we felt it necessary to explore if there was also a location-specific effect on F/B’s ability to predict metastatic outcome. As described in **Desa D, Strawderman R, Wu W, Hill R, Smid M, Martens JWM, Turner B, Brown E. (2020) Intratumoral heterogeneity of second-harmonic generation scattering from tumor collagen and its effects on metastatic risk prediction. BMC Cancer. 20:1217**, which is appended to this report, we found that indeed there is such a location-specific effect on the prognostic ability of F/B. Highlights of this paper are that F/B is most prognostic of metastasis when determined in the tumor/host interface (Fig 4. in that paper), and most importantly, F/B combined with a surrogate for the

OncotypeDX genomic predictor *provide better prognostic value then genomic score alone* (Figure 13).
This work was performed in the Brown lab.



In summary, I feel that we have done a good job of meeting the goals of the grant, either exactly in the manner proposed or in a related system or with a related method. Quantifying success as measured by manuscripts, I think Task 3 was most successful, as it led to 2 publications and 1 manuscript in preparation. Task 2 was moderately successful, as it led to 1 paper in revision and 1 manuscript in preparation, while Task 1 was least successful, as it led to 1 paper in preparation.

project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to Report

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to Report.

- 4. IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

I feel that we have shown that F/B, measured from the tumor/host interface, is a clinically useful predictor of metastasis, and a significant improvement on current clinical practice using genomic methods alone. We have also provided mechanistic understanding of the prognostic ability of F/B by demonstrating that tumor cells respond to presented F/B by changing their behavior (matrix manipulation and motility) in ways that vary with cell type, and that several specific collagen subtypes underly the F/B of a tumor.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report.

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

A startup company, Harmonigenic Incorporated (investigators have no affiliation) was started just before this grant was funded, and the results herein (especially on the relationship between F/B, OncotypeDX, and metastatic outcome) have changed its overall strategies.

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report.

- 5. CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:

Changes in approach and reasons for change

As reported in previous yearly reports, what seemed to be a brief slowdown in our supply of fresh excess human breast cancer tissue became a permanent drought. We therefore switched to collagen gels and decellularized mouse tumor tissue.

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

See above.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume; year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Desa D, Bhanote M, Hill R, Majewski J, Buscaglia B, D’Aguiar M, Strawderman R, Hicks D, Turner B, Brown E. (2019) Second harmonic generation directionality is associated with neoadjuvant chemotherapy response in breast cancer core needle biopsies. *Journal of Biomedical Optics*. 24(8):1-9. Published, federal support acknowledged.

Schilling K, El Khatib M, Plunkett S, Xue J, Xia Y, Vinogradov S, Brown E*, Zhang X*. (2019) Electrospun Fiber Mesh for High-Resolution Measurements of Oxygen Tension in Cranial Bone Defect Repair. *ACS Applied Materials & Interfaces*. 11(37):33548-33558. Published, federal support acknowledged.

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Elias T, Desa D, Nyguen P, Goyal A, Benoit D, Buckley M, Kuo C.K., Brown E. Understanding the Role of Elastic Modulus in the Relationship Between Second Harmonic Generation Scattering and Tumor Cell Motility. In Review.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Desa D, Turner B, Buscaglia B, Hill R, Majeski J, Choe R, Strawderman R, Kuo C, Hicks D, Brown E. Using multiphoton laser-scanning microscopy to assess neoadjuvant therapy outcome in core needle biopsies: a novel methodology. San Antonio Breast Cancer Conference. December 2018*

Tresa Elias, Danielle Desa, Song Yuanhui, Danielle Benoit, Catherine Kuo, Bradley Turner, Edward Brown. Exploration of the Mechanisms Underlying the Relationship Between SHG Scattering and Metastasis. Annual Meeting of the Biomedical Engineering Society. October 2019. Philadelphia PA

DE Desa, M Bhanote, RL Hill, JB Majeski, B Buscaglia, M D'Aguiar, R Strawderman, DG Hicks, BM Turner, EB Brown. Second-harmonic generation scattering from breast cancer core needle biopsies is associated with neoadjuvant chemotherapy response. Photonics West Annual Meeting. February 2020, San Jose CA*

Nguyen PK, Deng F, Assi S, Paco P, Fink S, Stockwell C, Kuo CK, "Phenotype Stability and Expansion Potential of Embryonic Tendon Progenitor Cells In Vitro," Orthopaedic Research Society Annual Meeting, Phoenix, AZ, February 7-11, 2020. Podium Presentation.*

Desa, D. Wu, W., Hill, R., Martens, J., Strawderman, R., Turner, B., Brown, E. Intratumoral heterogeneity of second-harmonic generation scattering from tumor collagen and its effects on metastatic risk prediction. San Antonio Breast Cancer Society Meeting. Virtual. 2020.*

Wu W, Beilei X, Bernal E, Hill R, Desa D, Brown E. Breast cancer tissue sub-region classification from second-harmonic generation imagery via machine learning. IS&T Electronic Imaging. Jan. 2021.

Desa D, Wu W, Hill R, Martens J, Strawderman R, Turner B, Brown E. Intratumoral heterogeneity of second-harmonic generation scattering from tumor collagen and its effects on metastatic risk prediction. Oral presentation. SPIE Photonics West BIOS. Virtual. March 2021.*

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report.

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report.

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Hill R, Brown E, Hicks D, Buscaglia B, Turner B, Desa D. (2019) A Method to Predict Response to Neoadjuvant Chemotherapy. Provisional Patent Application 62/795,828.

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Example:

Name: Mary Smith
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID): 1234567
Nearest person month worked: 5

Contribution to Project: Ms. Smith has performed work in the area of combined error-control and constrained coding.
Funding Support: The Ford Foundation (Complete only if the funding support is provided from other than this award.)

Name:	Edward Brown
Project Role:	co-PI
eraCommons:	EBBROWN
Nearest person month worked:	1
Contribution to project:	Dr. Brown provided general oversight in all aspects of data generation and analysis with particular focus on imaging as well as planning, writing, revising, and submitting IRB protocols.
Name:	Catherine Kuo
Project Role:	co-PI
eraCommons:	ckuo01
Nearest person month worked:	1
Contribution to project:	Dr. Kuo provided general oversight in all aspects of data generation and analysis with focus on mixed gel generation/analysis and crosslink density analysis.
Name:	Bradley Turner
Project Role:	co-Investigator
eraCommons	BMTMDMPH
Nearest person month worked:	1
Contribution to project:	Dr. Turner assisted with sample assessment, archived sample acquisition, and overall clinical relevance of the project.
Name:	Robert Strawderman
Project Role:	co-Investigator
eraCommons	STRAWDERMAN
Nearest person month worked:	1
Contribution to project:	Dr. Strawderman assisted with statistical analysis of data and project planning.
Name:	Danielle Desa
Project Role:	Graduate Student
eraCommons	N/A
Nearest person month worked:	12
Contribution to project:	Ms. Desa assisted with decellularization procedures, collagen gel creation, immunolabeling, imaging, and creation of a curated human samples dataset.
Name:	Tresa Elias
Project Role:	Undergraduate Student
eraCommons	N/A
Nearest person month worked:	2
Contribution to project:	Ms. Elias assisted with collagen gel creation and AFM analyses.
Funding Support:	Worked for class credit.

Name: Rachel Farkas
Project Role: co-Investigator
eraCommons N/A
Nearest person month worked: 1
Contribution to project: Dr. Farkas assisted with acquisition of human tumor samples and overall clinical relevance of the project.

Name: Phong Nguyen
Project Role: Graduate Student
eraCommons N/A
Nearest person month worked 9
Contribution to project: Mr. Nguyen optimized polyacrylamide gel formulations for specific stiffnesses, trained the Brown Lab on making polyacrylamide gels, and performed atomic force microscopy to evaluate gel stiffnesses. He also worked on IHC staining for collagens, but this was interrupted by the pandemic.

Name: Sabrina Pan
Project Role: Graduate Student
eraCommons N/A
Nearest person month worked 9
Contribution to project: Ms. Pan developed and optimized atomic force microscopy measurements and imaging techniques to evaluate tissue stiffness, collagen crosslinking, and collagen morphology of tissues.

Name: Jiewen Li
Project Role: Graduate Student
eraCommons N/A
Nearest person month worked 12
Contribution to project: Ms. Li has collaborated with Ms. Pan to collect sample tissues for protocol optimization, and has been developing gel fabrication protocols.

Name: Kelley Madden
Project Role: Co-Investigator
eraCommons Kelley_Madden
Nearest person month worked 2
Contribution to project: Dr. Madden assisted with immunolabeling and IRB protocol generation.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

One grant (NIH R21CA208921) that was providing other support to Dr. Brown has ended. Two grants (NIH P50HD103536, NIH 1 R01 DE029790-01) that provide other support to Dr. Brown have begun. None of these changes has impacted Dr. Brown’s effort on this project.

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner’s contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner’s facilities for project activities);*
- *Collaboration (e.g., partner’s staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner’s staff use each other’s facilities, work at each other’s site); and*
- *Other.*

University of South Carolina Medical Center
Charleston, South Carolina
Collaboration

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.

9. **APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Understanding the Role of Elastic Modulus in the Relationship Between Second Harmonic Generation Scattering and Tumor Cell Motility

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Abstract

Background: Second harmonic generation (SHG) is an intrinsic optical property of fibrillar collagen. SHG directionality is quantified by F/B, the ratio of forward- to backward-propagating signal, which is affected by collagen fiber internal structure, specifically the diameter, spacing, and disorder of fibrils within a collagen fiber. We have previously shown that F/B of primary invasive ductal carcinoma sections is prognostic of metastatic outcome. One possible cause of this relationship was revealed by our observation that tumor cells' motility on collagen I gels varied when collagen fiber internal structure in those gels was manipulated. The mechanism by which tumor cells sense changes in collagen fiber internal structure remains unknown: here we evaluate the role of elastic modulus in the relationship between collagen fiber internal structure (as reported by F/B) and tumor cell motility.

Methods: The 4T1 murine mammary adenocarcinoma, a model of metastatic triple negative breast cancer, (TNBC) and the 67NR line, a non-metastatic luminal phenotype, were introduced to a series of collagen-polyacrylamide mixed gels wherein collagen fiber internal structure and gel elastic modulus were independently controlled. F/B was measured with SHG, while elastic modulus was measured globally via rheometry and locally via atomic force microscopy (AFM). Tumor cell motility was quantified over three hours.

Results: The motility of both cell lines varied with F/B while elastic modulus was held constant, and did so at two physiological modulus values. Interestingly, 4T1 cell motility increased as F/B increased, while 67NR cell motility decreased.

Conclusions: Our results suggest that elastic modulus does not play a significant role in the observed relationship between collagen fiber internal structure (as reported by F/B) and tumor cell motility, for two cell lines that are models of TNBC and luminal-like breast cancer. Our observation that the two lines exhibit opposite motility trends as F/B increases is consistent with the trends in metastatic outcome versus F/B observed in our published clinical data for luminal versus basal patient cohorts, and suggests that a tumor's subtype may play a role in their response to collagen fiber internal structure.

Key words: Second Harmonic Generation, SHG, F/B ratio, collagen, metastasis

1 Background

Breast cancer is one of the most common cancers with approximately 12.8% of women being diagnosed within their lifetime (1). Between 2009 and 2015, women diagnosed with localized, or stage 1, breast cancer had a 98.8% 5-year survival rate (1). However, women diagnosed with distant metastases had a significantly lower 5-year survival rate of 27.4% (1). Metastasis to a secondary location is an onerous journey for a cancer cell. Once the primary tumor is established, cells begin to migrate within their microenvironment and may reach a blood or lymphatic vessel. Once a cell reaches the vessel it can intravasate into the lumen and, if it survives, reach a distant organ where it may lodge and/or extravasate and possibly develop into a metastasis (2).

One of the earliest steps in the metastatic process is migration through the tumor extracellular matrix (ECM). The ECM is made up of various proteins including fibrillar collagen type I, which can be imaged with an intrinsic optical signal caused by the scattering phenomenon known as second-harmonic generation (SHG)(3-5). SHG is observed when two incoming photons scatter off a noncentrosymmetric structure to produce one photon at twice the energy and half the wavelength of the individual incoming photons. In tumors the only significant source of SHG signal is fibrillar collagen (6). SHG imaging has revealed that tumor cells can move along collagen fibers, and that the orientation of collagen fibers with respect to the border between breast tumor and host tissue can predict patient outcomes (7). This suggests that collagen fibers may act as ‘roads’ facilitating tumor cell escape from the primary tumor. When an excitation laser is focused on one point along an individual collagen fiber, SHG photons can scatter in the “forwards” direction, i.e. the direction of propagation of the laser, or in the “backwards” direction, i.e. in the opposite direction. This directionality can be quantified by collecting photons in both directions using two detectors and calculating the forwards-to-backwards scattering ratio, or “F/B”. F/B is affected by properties of the internal structure of the collagen fiber including the diameter and spacing of the component fibrils that make up a fiber, as well as the order versus disorder in fibril packing (8-10). This internal structure is not the same as the direction of the fiber, but to extend the ‘roads’ analogy, F/B may provide information about road quality, as distinct from road direction.

Previously, we have shown that the F/B ratio of primary invasive ductal carcinoma (IDC) tumor biopsies is an independent prognostic indicator of patients’ metastasis-free survival time in 10-year follow-up data (11). This suggests that F/B may be an additional indicator to assist in identifying which patients will metastasize and therefore who requires adjuvant chemotherapy. Hence the predictive ability of the F/B ratio of primary tumors may be useful

in reducing the recognized clinical problem of “overtreatment” (12). To begin to understand why collagen fiber internal structure predicts metastatic outcome, in a previous study we determined the effect of varying collagen fiber internal structure on tumor cell motility in pure collagen gels (13). Collagen fiber internal structure was varied by varying gel polymerization conditions, and reported with F/B. We found that tumor cell motility was statistically significantly affected by varying collagen fiber internal structure (i.e. varying F/B) (13). This suggested that in patients, excised tumor F/B may be prognostic of metastatic outcome because the collagen fiber internal structure which dictates F/B also affects the efficiency with which cells escape the tumor. However, the underlying mechanisms of the relationship are still unidentified.

The elastic modulus of the extracellular matrix is known to have an impact on tumor cell behavior, including cell motility (14, 15). Therefore we hypothesized that cells could be reacting to modifications of collagen fiber internal structure because they are sensing matrix elastic modulus changes that may result from those modifications (15). To test this hypothesis, herein we characterized the motility of tumor cell lines in a series of collagen/polyacrylamide mixed gels in which we independently varied the collagen fiber internal structure and the elastic modulus (16-18). These gels provide an *in vitro* environment to study tumor cell motility and its relationship to ECM properties in the absence of other cellular actors, and their secreted factors, that may be present in an *in vivo* model.

Our previous clinical study revealed interesting subtype-specific behavior: in the cohort with ER⁺ IDC tumors (i.e. including the luminal subtypes) patients whose tumors had higher F/B experienced fewer distant metastases in 10-year followup data (11). Conversely, in the cohort with ER⁻ IDC tumors (i.e. including the TNBC and HER2 overexpressing subtypes) the relationship was inverted and patients whose tumors had lower F/B experienced fewer distant metastases. Therefore, in this study two cell lines were chosen: the metastatic 4T1 and nonmetastatic 67NR lines. The 4T1 murine mammary adenocarcinoma cells are a common model of basal-like TNBC and were used in our previous studies (19, 20). For the non-metastatic cell line, 67NR was chosen since it is a paired sister cell line to the 4T1 cells (i.e. derived from the same tumor) and has a contrasting luminal phenotype (21, 22).

2 Methods

2.1 Multiphoton Image Acquisition

Multiphoton laser scanning microscopy (MPLSM) was conducted using a Ti:Sapphire excitation laser controlled through a BX61WI upright microscope (Olympus, Shinjuku, Tokyo), with beam scanning and image acquisition controlled by an Olympus Fluoview FV300 scanning system. The excitation light was circularly polarized with 100 fs pulses at 80 MHz. Circular polarization at the sample was achieved by passing the excitation light through a Berek compensator (Model 5540, New Focus, Irvine, CA) before entering the scan box, and verified as circular entering the objective lens with a Thorlabs PAX5710 Polarimeter. The light was focused through an Olympus UMPLFL20XW water immersion lens (20x, 0.95 N.A.), which was also used to collect the backwards scattered SHG signal. A 670 nm dichroic mirror (670 DCSP-2P, Chroma) was used to separate the backscattered signal from the excitation light. The backscattered SHG signal was filtered by a 405 nm band-pass filter (HQ405/30m-2P, Chroma). Forward SHG signal was captured in the forward-scattered direction using an Olympus 0.9 N.A. optical condenser, reflected by a 565 nm long-pass dichroic mirror (565 DCSX, Chroma), and filtered by a 405 nm band-pass filter (HQ405/30m-2P, Chroma). The SHG signal was generated with an excitation wavelength of 810 nm. All signals were captured by Hamamatsu HC125-02 photomultiplier tubes.

2.2 *Cell Culture*

The 4T1 mouse mammary adenocarcinoma cell line (Caliper Life Sciences, Hopkinton, MA) was frozen after three initial passages. Cells were maintained in a media consisting of RPMI (Gibco, Invitrogen Inc., Carlsbad, CA), 10% fetal bovine serum, and 1% penicillin/streptomycin. 67NR cells were purchased from the Animal Model & Therapeutic Evaluation Core (AMTEC) Barbara Karmanos Cancer Institute, Wayne State University. The 67NR cell line is a sister cell population to the 4T1 cell line, both derived from a single spontaneous Balb/cF₃H. Cells were frozen after three initial passages. Cells were maintained in a media consisting of Dulbecco's Modified Eagle Medium (DMEM, Gibco 11965092), 10% fetal bovine serum, 1% 2mM L-glutamine (Cellgro 25-005-CI), and 0.1% 1mM Mixed Nonessential Amino Acids (Gibco 11140).

2.3 *Preparation of Collagen Polyacrylamide Mixed Gels*

Mattek petri dishes (Ashland, MA) with 14-mm diameter coverslip-bottomed wells were coated with 2.5M NaOH and allowed to air dry. 200 μ L of 97% 3-aminopropyltrimethoxy silane (APTES) was added to the coverslip bottom for 5 minutes and then rinsed three times with distilled water and gently dried with Kim Wipes. Each dish was flooded with 0.5% glutaraldehyde for 30 minutes and then rinsed three times with distilled water and allowed to air dry. Separate 12 mm glass coverslips were treated with RainX and allowed to air dry. Each coverslip was wiped to ensure an even coating and then sprayed again.

Collagen gels were made of human type I collagen solution at a concentration of 3 mg/mL (Advanced Biomatrix, San Diego, CA). With all solutions kept at 4°C to minimize gelation, 810 μ L of collagen type I was combined in a conical tube with 108 μ L of RPMI 1640 media, and 50 μ L of distilled water. The solution was vortexed at the lowest speed for 5 seconds to ensure the solution was evenly mixed. The pH of the solution was measured with a Mettler Toledo Micro Pro pH meter (Columbus, OH). The acceptable pH ranges for this solution is discussed below. In a petri dish, 6 droplets of 125 μ L each of the solution was pipetted, making sure the droplets did not touch or spread and that all droplets were equal in size to ensure equal gelation. The petri dish was placed in an incubator for 2 hours at 37°C and 5% CO₂.

Polyacrylamide is an easily manipulated polymer that can produce a wide range of elastic moduli, and polyacrylamide hydrogels do not significantly interact with the cell surface (16, 23, 24). When mixed with collagen the polyacrylamide can independently influence overall gel elastic modulus (16-18). Two polyacrylamide precursor solutions were made with different concentrations of acrylamide and bis-acrylamide. The first solution was 5% acrylamide: 0.1% bis-acrylamide and the second solution was 12% acrylamide: 0.25% bis-acrylamide; these combinations are known to produce polyacrylamide gels of significantly different elastic moduli (17). 375 μ L of the desired polyacrylamide precursor was added to a conical tube. Once the pure collagen gel droplets gelled, they were added to the polyacrylamide precursor solution. This solution was vortexed on medium speed for 20 seconds to break up the collagen gels and mix thoroughly with the polyacrylamide. Next, 0.5 μ L of N,N,N',N'-tetramethylethylenediamine was added to the solution and followed by 5 μ L of 10% w/v ammonium persulfate, for a final concentration of 0.5% and 5%, respectively; to minimize premature polymerization, this step was completed within 2 minutes. The solution was mixed by pipetting gently 10 times while avoiding bubbles. 100 μ L of the solution was pipetted onto the center of the treated square coverslip, and then coverslipped with the treated circular coverslip. The solution was left at room temperature for 30 minutes to fully polymerize. This produces a final mixed gel with a

uniform distribution of collagen fibers visible in SHG (Figure 1) embedded within a polyacrylamide matrix. The final mixed gels had a denser network of fibers than contained in the precursor pure collagen gels that were added to polyacrylamide precursor and vortexed.

The top coverslip was carefully removed with tweezers and the dish was washed with phosphate-buffered saline (PBS). The PBS was replaced with either RPMI or DMEM media, depending on which cells would be added to the gels. Gels were stored in the incubator overnight and subsequent experiments were performed within 24 hours.

2.4 *Determining the F/B Ratio of Collagen Polyacrylamide Mixed Gels*

During each imaging session, a standard fluorescein isothiocyanate (FITC) calibration sample was imaged with an excitation wavelength of 810 nm and 535 nm emission filters. Free FITC emits isotropically, which means the F/B ratio of a FITC calibration sample should be 1.0; any variation in the imaging system is reported by a variation in this measured ratio. To account for any day-to-day variation in the system, the background-subtracted SHG F/B ratios of the imaged samples on a given day (discussed below) are divided by the background-subtracted F/B ratio of the FITC calibration taken on that day. For each image pair, an F and B image was taken with the laser shutter closed and the average pixel counts for each image provided the background.

For SHG F/B ratio analysis of the gels, two xyz stacks of simultaneously collected forward and backwards images were taken at a given xy region on the gel; three different regions of each gel were imaged. The forward and backward image pairs were taken at 50 μm steps through the depth of the gel. Each image was 660 μm x 660 μm .

ImageJ was used for all image analysis (25). The forward and backward images were background subtracted with the average background value for the corresponding direction. For each matched image pair, two masks of both the forward and the backward-scattered SHG signals were created, in which all collagen pixels were set to 1 and background pixels were set to 0 by applying a predetermined threshold to each of the two images. One forward threshold and one backward threshold was used for each imaging session and applied to all images. These thresholds were determined by surveying random background-subtracted images and adjusting the image's look-up tables until only collagen fibers are highlighted, and all other pixels were rejected. The two resultant masks for each matched image pair were then multiplied together to create a single mask that identifies pixels with SHG emission above the threshold in both the forward and backwards direction. A single F/B ratio image was calculated from the F and B

image pair and then multiplied by the mask to set all non-fiber pixels to zero, thereby excluding these pixels from the analysis. The average pixel value of fiber pixels (i.e. non-zero pixels) for that region was then calculated for the masked F/B ratio image. This process was repeated in a for loop for each image pair in the stack for a single region. The average pixel value of each slice in the stack was then averaged to give a single F/B ratio for that region of the gel. The F/B ratio from all three regions were then averaged to produce a single F/B ratio to represent the entire gel.

2.5 *Manipulation of F/B Ratio*

The F/B ratio of the gels was altered by manipulating the pH of the collagen gel in the initial stage of making the mixed collagen-polyacrylamide gels as previously described (13). This is known to vary fibril diameter (26, 27), one element of collagen fiber internal structure known to alter F/B (13) (others being the less experimentally tractable fibril spacing and order versus disorder of fibril packing into fibers) (9, 10). The pH was altered by using increasing amounts of 2.5M NaOH in the collagen gel solution to bring the final pH to 7.5 - 7.7, 8.5 - 8.7, or 9.5 - 9.7, measured with a Mettler Toledo Micro Pro pH meter (Columbus, OH). The collagen gels were gelled and incorporated into the polyacrylamide as stated above.

2.6 *Analysis of Tumor Cell Motility*

The following preparation and imaging were performed one gel at a time. An hour prior to imaging, all media was removed from the gel and 1×10^4 cells were resuspended in their respective media and added to the surface of the gel in a droplet. The gel was placed in the incubator for an hour (28) to allow the cells to adhere to the surface before all excess media was removed to minimize motion during the imaging session. In a preliminary experiment, representative gels were made with and without the addition of collagen, and we found that after the standard one hour adherence time, a gentle rinse washed away all cells in the collagen-free cases while the seeded cells remained adhered to the gel in the collagen-including cases (data not shown). This verifies that the cells were indeed interacting with the collagen versus the nonreactive polyacrylamide. Live cell images were taken on a Nikon Eclipse Ti microscope, using a Nikon MRH201201 air lens (20x, 0.45 N.A.). Cells were quickly transferred to a microscope stage incubator

(Pathology Devices, Westminster, MD), at 37°C, 5% CO₂, and 85% relative humidity. One phase contrast image was taken every 2 minutes for 3 hours and saved as a tiff file. To assess the extent of adhesion we quantified the aspect ratio of each cell on the first image of the three-hour timelapse (i.e. after the one-hour adhesion time) as well as the last image of the timelapse, and found no statistical differences within any group as well as no main effect of gel F/B or elastic modulus (2-way ANOVA with Bonferroni post-hoc tests, Supplemental Figure 1). This suggests that the initial adhesion time was sufficient for cells to fully adhere.

All image analysis was performed in ImageJ (25). For each gel, every cell that remained isolated in the time series (i.e. not touching another cell for more than 3 frames) was tracked using the “Manual Tracking” ImageJ plugin, which tabulates the XY location of each cell at each time point (25). From this data, the total distance traveled was calculated for each cell by summing the distance traveled between each time point. To confirm that the gels did not significantly move during the imaging session, a fiducial artifact on each gel was identified and tracked in the same manner; total distance traveled by fiducial artifacts did not exceed 4.37μm per imaging session and averaged 2.17μm ± 1.62μm, significantly less than the motion of all cells in all conditions. For comparison with previous literature (13), imaging sessions did not exceed three hours to ensure that there was no significant penetration of the cells into the gels and hence that the analysis remained two-dimensional, as well as to ensure that the cells themselves did not significantly manipulate F/B of the gel.

2.7 *Measuring Elastic Modulus with Atomic Force Microscopy*

A silicon nitride tip probe with a radius of 20 nm and spring constant of 0.06 N/m (Bruker, CA, USA) was used on an MFP-3D AFM (Asylum Research, CA, USA) to obtain a 64 x 64 two-dimensional force map over a 20 μm x 20 μm area in the center of gels that were 600-700 μm thick. Elastic modulus measurements at each point on the force map were calculated by fitting force displacements curves to the Hertzian model, and force maps were averaged to yield a single elastic modulus measurement for each gel, as we previously described(29-32).

2.8 *Measuring Elastic Modulus with Rheology*

Global elastic modulus measurements of 8 mm diameter gels were performed using a parallel plate torsional rheometer (Discovery HR-2, TA Instruments). To prevent slippage, the gap distance between the rheometer plate and base was

reduced in small steps ($\sim 10\ \mu\text{m}$) until the specimen was compressed with a constant axial force of $-0.3\ \text{N}$. After compression, dynamic torsional shear mechanical tests were performed at an angular frequency of $\omega=1\ \text{rad/s}$ across a range of strain amplitudes (0.01-1%). The shear storage modulus (G') was then computed at each strain level based on the measured time-dependent torque and angular displacement of the top plate. The value of G' for each gel was taken to be the average measurement of G' across all strain amplitudes. Assuming the gels were isotropic, the elastic modulus E was then determined according to $E=3G'$.

3 Results

3.1 *Development of Consistent Mixed Collagen/Polyacrylamide Gels that Maintain Optical Properties Necessary for MPLSM and Have Tunable Physical Properties*

A series of gels were produced over a range of F/B and elastic modulus values. Figure 1 shows representative forward-scattered SHG images of one image plane close to the surface of one gel from each condition, revealing no significant visible change in gel organization with F/B or elastic modulus.

To obtain a single representative F/B value for a gel, the sample being imaged must be optically thin, which means that the measured F/B must not be affected by the imaging depth into the sample. This is accomplished either with a sample that is not very scattering, is not very thick, or both. When plotting F/B versus imaging depth, non-optically thin samples would have a slope across the gel depth which was significantly different than zero. The methods described above produced small mixed collagen/polyacrylamide gels whose F/B value did not follow a significant trend as a function of depth within the gel (Figure 2). This was verified for each gel by plotting F/B as a function of imaging depth and performing a linear regression (Supplemental Figure 2), and in each gel tested the slope of the line of best fit was not significantly different from zero, thus ensuring that the measured F/B ratio was not significantly affected by imaging depth. To avoid including F/B of pixels outside the gel in that screening process, the slope of the middle 50% of the stack was analyzed.

3.2 *Manipulation of F/B Ratio and Confirmation that F/B was Not Affected by Addition of Polyacrylamide*

248

249 The F/B ratio of the gels was manipulated by altering the pH of the collagen gel during fibrillogenesis, and
250 elastic modulus was manipulated by altering polyacrylamide polymerization, each as described above. The pH of the
251 collagen was adjusted to either 7.5, 8.5 or 9.5. Figure 3 shows that F/B increases as pH of the collagen during synthesis
252 increases, which is consistent with our previous studies (13). A two-way ANOVA was performed to compare the F/B
253 of the high and low elastic modulus gels within a single pH category, and revealed no effect of elastic modulus
254 ($p=0.24$). A Bonferroni post-hoc test revealed that there was no statistically significant difference between the F/B of
255 the high and low modulus gels within each pH category. The range of F/B values that result from these manipulations
256 (~ 6 to ~ 11) is consistent with the range of values found in tumor tissue measured on the same system (11, 13).

257

258

259 3.3 *Evaluation of Elastic Modulus of Gels*

260

261 The elastic modulus of the gels was manipulated by altering the acrylamide and bis-acrylamide
262 concentrations in the polyacrylamide precursor solution. The ‘high modulus’ gels were composed of 12% acrylamide
263 and 0.25% bis-acrylamide and the ‘low modulus’ gels were composed of 5% acrylamide and 0.1% bis-acrylamide.
264 Figure 4 shows the elastic modulus of gels produced with these procedures, as measured by both AFM and rheology.
265 In each modulus category (i.e. low and high), a two-way ANOVA was performed with Bonferroni post-hoc test using
266 pH category and modulus measurement method as independent variables. No significant effect of pH category
267 ($p=0.79$) nor of method of modulus measurement was found ($p=0.66$), nor did Bonferroni testing find differences
268 between any measured values of modulus within each combination of pH/ modulus category. This reveals that the
269 tuning of F/B by varying pH during collagen gel formation does not affect gel modulus, and that the two methods of
270 measuring elastic modulus give the same results. Taken together this data demonstrates that the mixed gels had an
271 elastic modulus that could be tuned between two desired values (confirmed with both bulk rheology measurements as
272 well as local AFM measurements), that the average values of elastic modulus were 1.738 ± 0.168 kPa and $18.504 \pm$
273 0.685 kPa (values that are relevant to tumor physiology (33)), and that alteration of collagen gelation did not affect
274 the elastic modulus of the mixed gel.

275

3.4 4T1 and 67NR Cell Motility

Tumor cells moved about on the gels in typical fashion, with some cells appearing “stretched out” while others remained somewhat more rounded and moved about with a visibly “active” edge (See Supplemental Videos). Typical motility tracks are shown in Figure 5 and range from small motions in the general vicinity of the starting point to longer excursions. Overall, the histogram of distances traveled revealed a roughly Gaussian distribution without any marked secondary peaks indicative of a highly mobile or immobile fraction (Supplemental Figure 3).

Figures 6 and 7 shows the effect of varying collagen microstructure and elastic modulus on tumor cell motility of 4T1 cells and 67NR cells, while Table 1 provides the number of cells analyzed for each condition. For 4T1 cells (Figure 6), 2-way ANOVA reveals an effect of elastic modulus ($p < 0.0001$) and an effect of F/B ($p = 0.014$) on total distance traveled, but no interaction ($p = 0.85$). Bonferroni post-hoc testing reveals that the total distance traveled by the three low modulus groups are individually different from the three high modulus groups ($p < 0.05$, 0.05 , and 0.01 , respectively). This demonstrates that the motility of 4T1 cells can respond to variations in collagen fiber internal structure (as reported by F/B) independently of elastic modulus.

For 67NR cells (Figure 7), 2-way ANOVA reveals an effect of F/B ($p = 0.0015$) but no effect of elastic modulus ($p = 0.99$) on total distance traveled, and no interaction ($p = 0.31$). Bonferroni post-hoc testing reveals no differences between the total distance traveled by the low and high modulus groups at any of the three F/B conditions. Overall, this demonstrates that the motility of 67NR cells can respond to variations in collagen fiber internal structure (as reported by F/B) independently of elastic modulus. Interestingly, the trend for the most motile 67NR cells is negative (i.e. the slope of the relationship between distance traveled versus F/B is negative), whereas the trend is positive for the 4T1 cells.

To evaluate the role of elastic modulus in the relationship between collagen fiber internal structure, as reported by F/B, and tumor cell motility, we introduced cell lines to mixed collagen/polyacrylamide gels with three physiologically relevant F/B values and two physiologically relevant values of elastic modulus. First, we created a series of *in vitro* gels with multiple values of F/B and elastic modulus. To develop these gels in our laboratory, we incorporated polyacrylamide into our previous pure collagen gel protocols (13). After developing this protocol, we first verified that the visible morphology of the collagen fibrils did not vary when F/B and elastic modulus were tuned (Figure 1). Next, we verified that the measured F/B was constant throughout each gel and was not affected by the presence of the polyacrylamide nor by variation of the elastic modulus (Figure 3, Supplementary Figure 2). Next, we confirmed that varying collagen fiber internal structure (as reported by F/B) by varying collagen polymerization conditions did not affect the measured elastic modulus and that all three F/B categories had the same measured elastic modulus within each of two modulus categories (Figure 4).

As demonstrated in Figure 6, in mixed collagen-polyacrylamide gels the motility of 4T1 cells increases as F/B of their microenvironment increases. These results confirm our previous study where we observed 4T1 motility increasing as F/B increased in pure collagen gels whose elastic modulus was not controlled (13). Furthermore, this specific trend of tumor cell motility increasing with F/B is consistent with the trend of metastasis-free survival time decreasing with F/B observed in patient tumor samples in the analogous (ER⁻, containing basal TNBC) cohort of human patients. This suggests that F/B may predict metastatic outcome in patient samples because changing collagen fiber internal structure (which affects F/B) alters the outflow of cells from a tumor. Importantly, 4T1 motility increased with F/B independently of elastic modulus. This suggests that the 4T1 model of TNBC can respond to changes in internal collagen structure through some other mechanism. In the 67NR model of luminal breast cancer we again observed that tumor cell motility varies with F/B and does so independently of elastic modulus (Figure 7). This suggests that the 67NR model of luminal breast cancer can also respond to changes in internal collagen structure through some other mechanism. Interestingly, we observe the opposite trend between motility and F/B in the 67NR cells versus 4T1 cells, where 67NR tumor cell motility *decreases* as F/B of the microenvironment increases. This is again consistent with the data from patient samples where metastasis-free survival time increases with increasing F/B in the ER⁺ patient cohort, which contains the luminal subtypes (11). This again suggests that F/B may predict metastatic outcome in patient samples because changing collagen fiber internal structure (which affects F/B) alters the

outflow of cells from a tumor. However it appears that the response of luminal-type breast tumor cells to alterations in collagen internal structure is opposite to the response of basal-type TNBC breast tumor cells (Figures 6 and 7), although in both cases the response is independent of elastic modulus, but directly to changes in the collagen fiber internal structure.

The mechanisms by which these two cell lines respond to tuning of collagen fiber internal structure independently of elastic modulus remain unknown. There are myriad differences between the two lines, just as there are between patient breast tumors of luminal and basal subtypes, including in their metabolism, overall gene expression patterns, etc. (34, 35). While ER, PR, and HER2 status are useful markers to distinguish these subtypes we do not have any evidence that those particular receptors are involved. One attractive candidate for sensing the variation in fibril diameter that is induced by varying pH during collagen gel synthesis (hence tuning F/B) is the discoidin domain receptor (DDR) family, which are collagen sensors known to be sensitive to the morphological state of collagen fibers (36), and whose activity is critical for breast cancer metastasis and tumor collagen organization (37).

This work also confirms observations in the literature that the motility of tumor cells varies when elastic modulus of their substrate is varied, and that different cell lines respond to changes in elastic modulus in different ways. At equivalent values of elastic modulus and F/B we observed that 4T1 cells were more motile than 67NR cells (Figure 6 and 7). This is consistent with literature observations of their relative migration (38) and with the fact that unlike the highly metastatic 4T1 cells, 67NR cells very rarely metastasize to secondary locations and are typically unable to leave the primary site (21). At constant values of F/B the motility of 4T1 cells increase with elastic modulus while the motility of 67NR cells do not change (Figure 6 and 7). This is consistent with literature observations of MDA-MB-231 (a model of basal TNBC cancer, analogous to 4T1s) and MCF7 (a model of luminal breast cancer, analogous to 67NRs) which exhibited the exact same behavior (i.e. increasing motility versus no change in motility) on substrates of similar elastic modulus (39).

There are several limitations to this work. While we do not observe any significant variations in visible gel morphology when F/B and elastic modulus are tuned (Figure 1), there may be some gel property that we are unexpectedly varying that is not visible in our images. Also, while AFM offers a well-established approach to test the mechanical properties of tissues at the cellular length scale (32, 40), the pressing of a nanoscale AFM tip on a collagen fibril embedded in a network of collagen and polyacrylamide is a somewhat different interaction than the tugging of a nanoscale cell filopodia on that fibril. Hence, while the AFM and rheometer measurements suggest that the nanoscale

and macroscale elastic modulus of the gel network is constant as F/B is varied, there is a possibility that the cells can sense mechanical differences in the fibrils not apparent to nanoscale AFM tips, as encompassed by the difference in AFM/fibril versus cell/fibril interactions.

5 Conclusions

To summarize, using a collagen/polyacrylamide gel system capable of independently varying collagen fiber internal structure and elastic modulus, we evaluated tumor cell motility under the influence of these two parameters. Our results suggest that variation in collagen fiber internal structure (as reported by F/B) can induce variation in tumor cell motility directly, independently of elastic modulus, in either a model of basal-like metastatic TNBC or a model of luminal-like non-metastatic breast cancer. Furthermore, we found that in the 67NR model of luminal breast cancer cell motility decreases as F/B increases, while in the 4T1 model of basal TNBC cell motility increases with F/B. This is consistent with observations of patient samples wherein luminal subtypes exhibited longer metastasis free survival (MFS) with increasing F/B while basal subtypes exhibited shorter MFS with increasing F/B (22). Taken together these results suggest that variations in collagen fiber internal structure do not modify tumor cell motility via any induced changes in elastic modulus. Furthermore, these results suggest that luminal and basal-type breast cancer reacts to changes in collagen fiber internal structure in different ways. In future work we hope to continue to explore the mechanism by which tumor cells sense changes in collagen fiber internal structure. Our observation that these cells are not sensing changes in collagen fiber internal structure through elastic modulus suggests that there may be cell surface receptors that are directly sensitive to variations in this property. Furthermore, these fiber internal structure sensors may be different on basal versus luminal subtypes or may be linked to different signaling pathways. This increased understanding of the mechanisms relating F/B, collagen fiber internal structure, and metastasis, may improve our understanding of the metastatic process, possibly leading to improved prognostic tools or antimetastatic therapies.

Abbreviations

SHG: Second Harmonic Generation, F/B: Forward-to-backwards ratio, IDC: Invasive Ductal Carcinoma, ER+: Estrogen Receptor positive, ER-: Estrogen Receptor negative, ECM: Extracellular Matrix, MPLSM: Multiphoton

386 Laser Scanning Microscopy, PBS: Phosphate Buffer Saline, FITC: Fluorescein Isothiocyanate, AFM: Atomic Force
387 Microscopy, MFS: Metastasis-free Survival

388

389 **Declarations**

390 **Ethics approval and consent to participate**

391 Not applicable

392 **Consent for publication**

393 Not applicable

394 **Availability of data and materials**

395 The datasets used and/or analyzed during the current study are available from the corresponding author on
396 reasonable request

397 **Competing interests**

398 The authors declare that they have no competing interests

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404 study, data collection, data analysis, data interpretation of data, nor in writing the manuscript.

405 **Authors' Contributions**

406 TE designed the protocol for collagen-polyacrylamide gels in consultation with PN and CKK, and prepared all gels
407 for experiments, imaged and analyzed F/B data from all gels, performed cell motility experiments, and completed
408 statistical analyses. TE is responsible for writing the majority of the manuscript. DD contributed to the design of the

409 experiment as well as data interpretation and troubleshooting. AG performed all rheometer measurements, as well as
410 analyzed elastic modulus data. PN performed all AFM elastic modulus measurements and contributed to AFM
411 analysis. CK provided consultation on making collagen-PA gels, access to AFM, as well as expertise and advice
412 involving AFM and motility results. DB provided access to live cell imaging microscope, cell culture hoods, and
413 incubators. MB provided access to rheometer as well as expertise in analyzing and comparing elastic modulus
414 results. EB contributed to the design of the experiments, troubleshooting, data interpretation, and writing the
415 manuscript.

416 All authors read and approved the final manuscript.

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418 Not Applicable

419 **Authors' Information**

420 Not Applicable

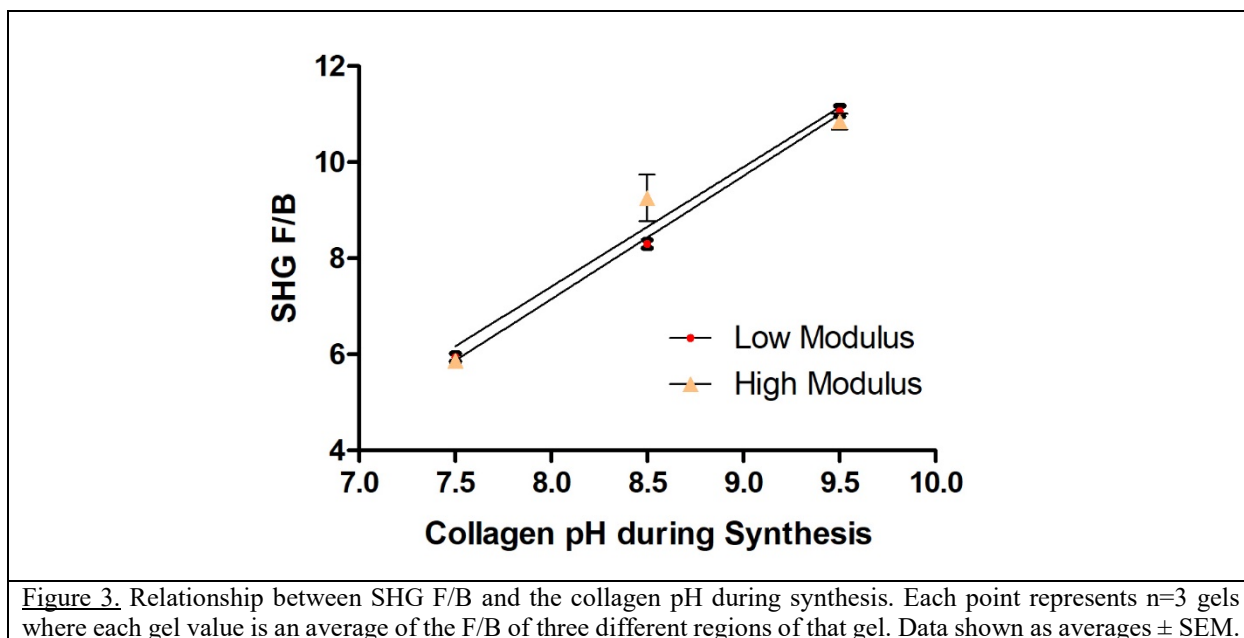
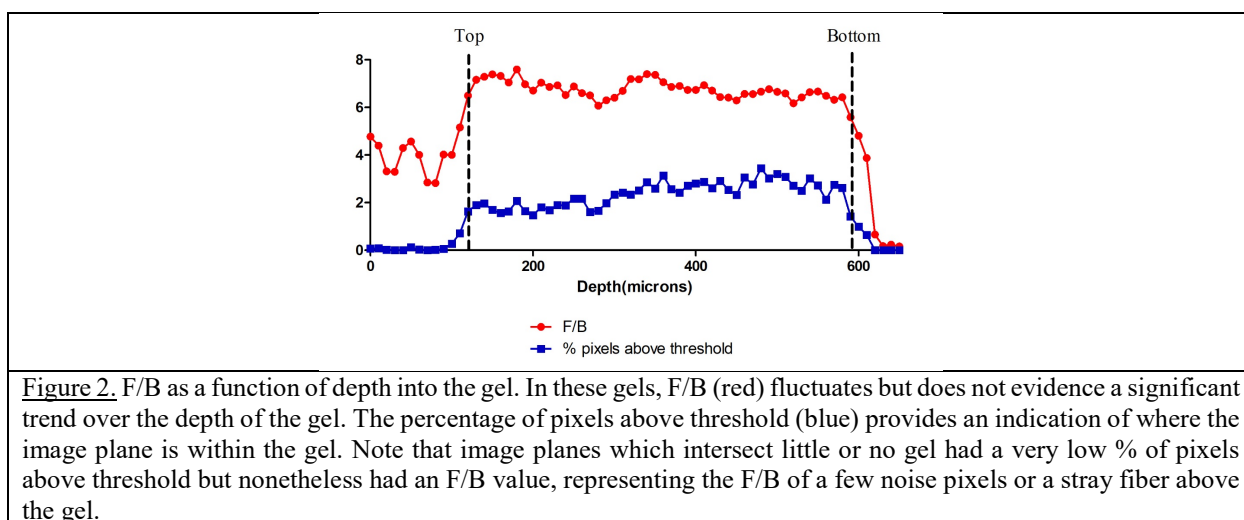
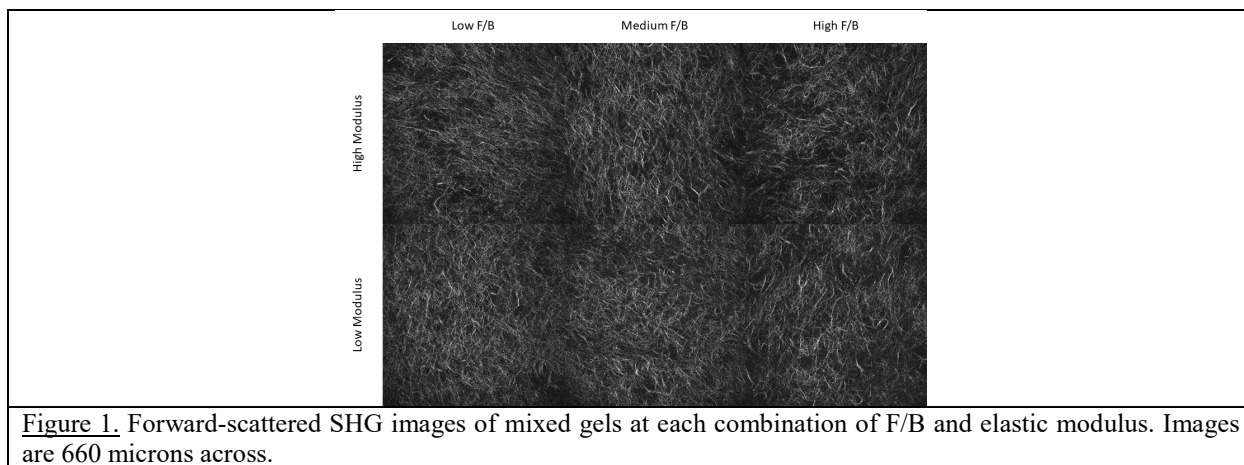
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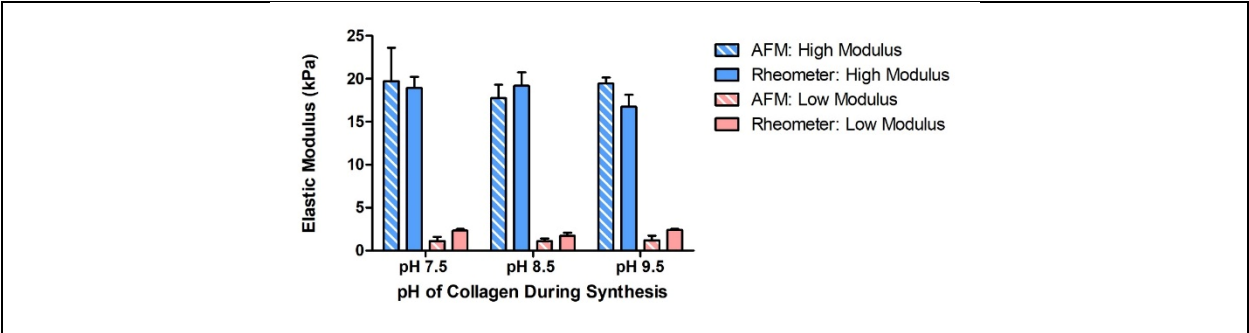


Figure 4. AFM (n=3) and rheometer (n=5) measurement of ‘high’ and ‘low’ modulus gels of various synthesis conditions. Data shown as averages $\pm$ SEM.

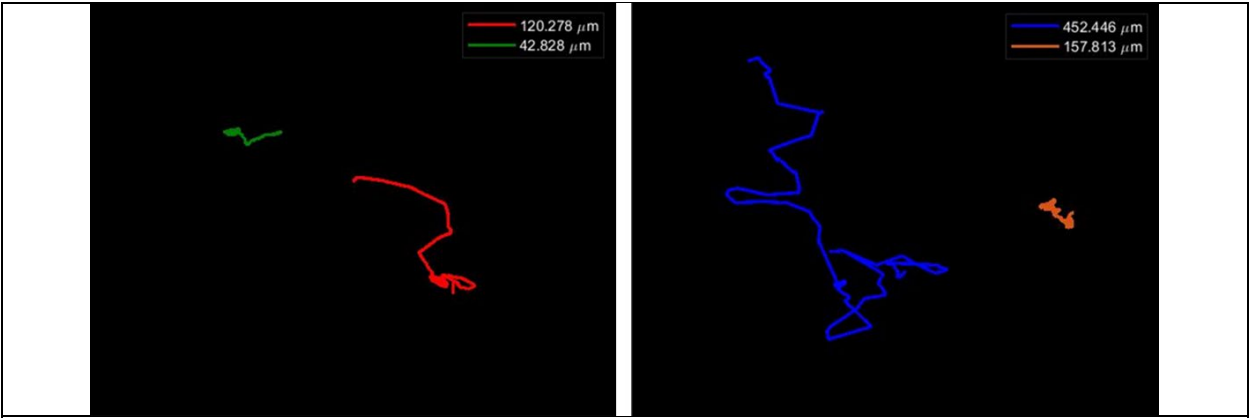


Figure 5. Sample traces of cell motility of two 67NR (left) and two 4T1 (right) cells on high elastic modulus, high F/B gels. For clarity, the two images are shown at different magnifications: the total distance traveled by each cell is recorded near each trace.

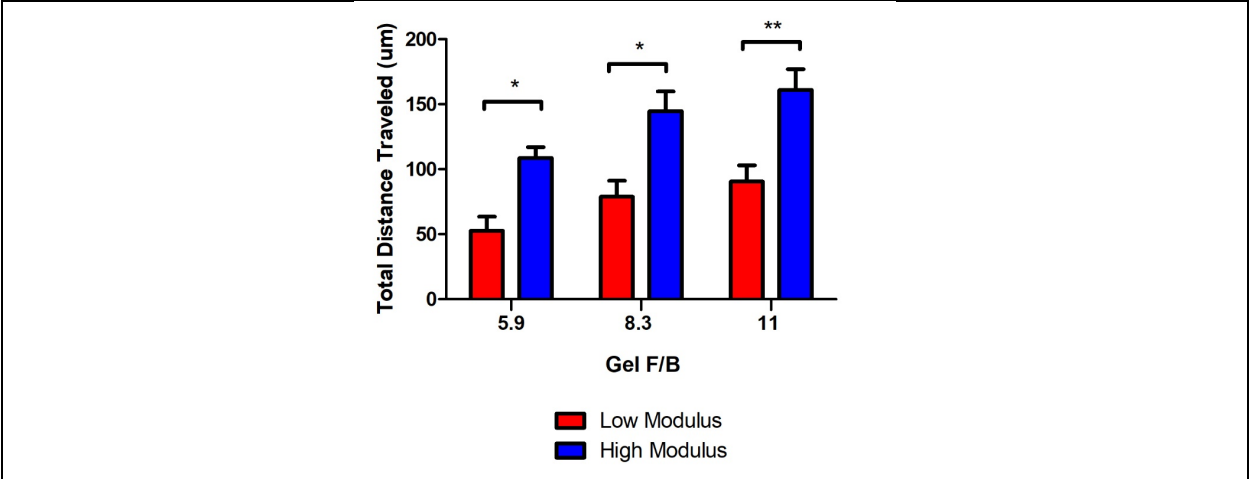


Figure 6. Effect of collagen fiber internal structure, as reported by F/B, and elastic modulus on tumor cell motility of 4T1 cells. Data shown as averages $\pm$ SEM (n = 3 gels). Red: Total distance traveled on high modulus gels. Blue: Total distance traveled on low modulus gels.

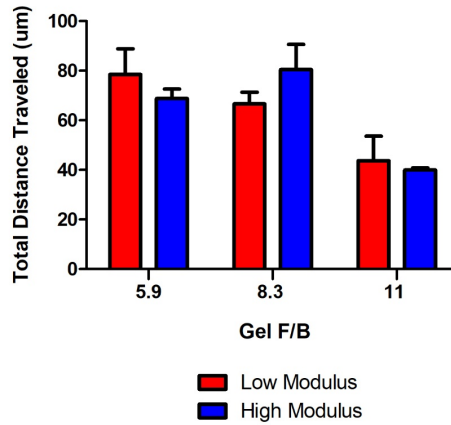
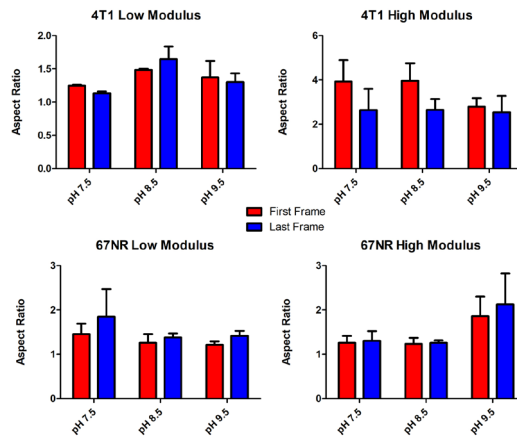


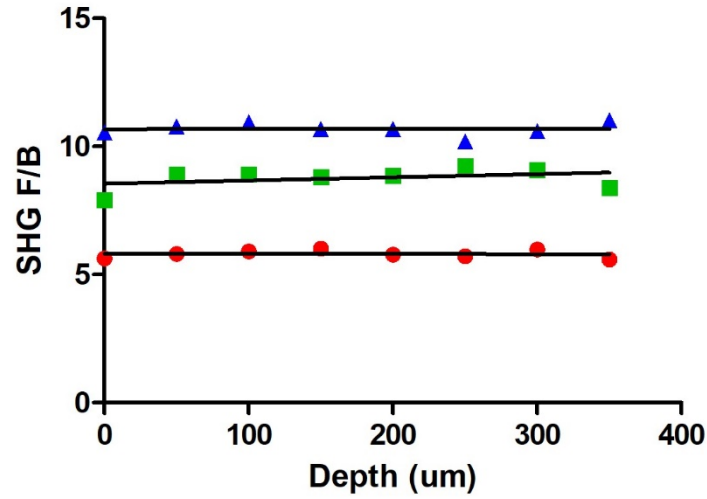
Figure 7. Effect of collagen fiber internal structure, as reported by F/B, and elastic modulus on tumor cell motility of 67NR cells. Data shown as averages $\pm$ SEM (n=3). Red: Total distance traveled on high modulus gels. Blue: Total distance traveled on low modulus gels.

Table 1. Number of each cell type analyzed in each of three experimental replicates for each F/B and elastic modulus condition.

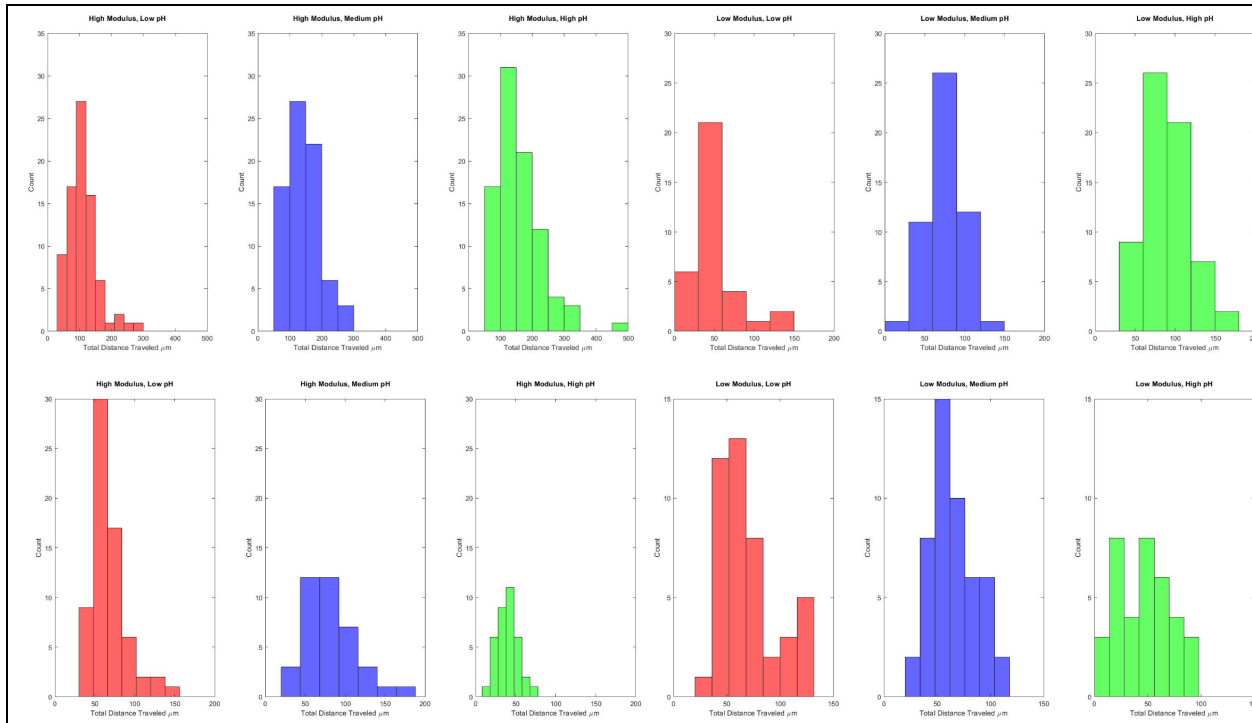
67 NR					
Low Modulus			High Modulus		
Low F/B	Medium F/B	High F/B	Low F/B	Medium F/B	High F/B
27	21	16	27	14	14
10	12	10	24	15	10
7	16	10	18	10	12
4T1					
Low Modulus			High Modulus		
Low F/B	Medium F/B	High F/B	Low F/B	Medium F/B	High F/B
10	16	22	25	21	27
17	15	22	23	27	32
13	21	21	32	27	30



Supplemental Figure 1. The average aspect ratio (longest dimension divided by largest width perpendicular to that dimension) of every cell analyzed. The average aspect ratio of cells on the first imaging time point (red) and last imaging time point (blue) was evaluated. A 2-way ANOVA with Bonferroni post-hoc test found no main effect of time in any of the four groups (4T1 Low and High modulus $p=0.95$ and 0.15 , respectively, and 67NR Low and High modulus $p=0.33$ and 0.71 , respectively). This suggests the cells had sufficient time to adhere before imaging started.



Supplemental Figure 2. SHG F/B plotted versus imaging depth for three gels with corresponding linear regression results. Each line represents a single region of a single gel. For the three lines shown: Red (pH 7.5) $p=0.91$, $R^2=2.2 \times 10^{-3}$, slope $=-5.6 \times 10^{-5}$. Green (pH 8.5) $p=0.38$, $R^2=0.13$, slope $=1.2 \times 10^{-3}$. Blue (pH 9.5) $p=0.97$, $R^2=2.6 \times 10^{-4}$, slope $=3.4 \times 10^{-5}$.



Supplemental Figure 3. Histograms of the total distance traveled for all cells analyzed (left: 4T1, right: 67NR) on each combination of gel parameters.

Second-harmonic generation directionality is associated with neoadjuvant chemotherapy response in breast cancer core needle biopsies

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Second-harmonic generation directionality is associated with neoadjuvant chemotherapy response in breast cancer core needle biopsies

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Abstract. Neoadjuvant chemotherapy (NACT) is routinely administered to subsets of breast cancer patients, including triple negative (TN) or human epidermal growth factor receptor 2-positive (HER2+) cancers. After NACT and subsequent surgical resection, 5% to 30% of patients have no residual invasive carcinoma, termed pathological complete response. Unfortunately, many patients experience little-to-no response after NACT and unnecessarily suffer its side effects. Methods are needed to predict an individual patient's response to NACT. Core needle biopsies, taken before NACT, consist of tumor cells and the surrounding extracellular matrix. We performed second-harmonic generation (SHG) imaging of fibrillar collagen in core needle biopsy sections as a possible predictor of response to NACT. The ratio of forward-to-backward scattering (F/B) SHG was assessed in the "tumor bulk" and "tumor-host interface" in HER2+ and TN core needle biopsy sections. Patient response was classified post-treatment using the Residual Cancer Burden (RCB) score. In HER2+ biopsies, RCB class was associated with F/B derived from the tumor-stromal interface, but not tumor bulk. F/B was not associated with RCB class in TN biopsies. These findings suggest that F/B from needle biopsy sections may be a useful predictor of which patients will respond favorably to NACT, with the potential to help reduce overtreatment. © The Authors. Published by SPIE under a Creative Commons Attribution 4.0 Unported License. Distribution or reproduction of this work in whole or in part requires full attribution of the original publication, including its DOI. [DOI: [10.1117/1.JBO.24.8.086503](https://doi.org/10.1117/1.JBO.24.8.086503)]

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1 Introduction

Neoadjuvant chemotherapy (NACT) is a presurgical option for select breast cancer (BC) patients, including many triple-negative breast cancers (TNBCs) or human epidermal growth factor receptor 2-positive (HER2+) patients. NACT is recommended based on pathological assessment of an initial core needle biopsy, which is routinely taken for diagnosis prior to any therapeutic or surgical intervention. Core needle biopsies utilize x-ray-guided stereotactic imaging, ultrasound guidance, or magnetic resonance imaging guidance to remove a sample of potentially diseased tissue that is then sectioned, stained, and used for diagnosis and determination of treatment options, including NACT. However, predicting who will and who will not benefit from NACT has proven to be challenging, exposing some patients to unnecessary toxicity and delaying administration of other life-saving treatments.¹ Methods to stratify patients based on expected response to NACT are greatly desired.

Core needle biopsies typically consist of tumor cells as well as the surrounding extracellular matrix (ECM). A major

component of the ECM is fibrillar collagen, which is capable of producing an intrinsic optical signal called second-harmonic generation (SHG). SHG is a nonlinear optical phenomenon that occurs when two identical photons scatter off a noncentrosymmetric material to produce a single photon of exactly twice the energy of the incoming photons and hence half the wavelength. The main source of SHG in solid tumors is fibrillar collagen, and the directionality of SHG emission is sensitive to its microscopic arrangement including fibril diameter, spacing, and packing disorder within collagen fibers, which we collectively call the "microstructure."²⁻⁷ The ratio of forward-emitted (where "forward" is the direction of the excitation laser) to backward-emitted SHG, or F/B ratio, is sensitive to the microstructure, which is inherent to individual collagen fibers and is distinct from the overall arrangement of ensembles of fibers.^{8,9}

SHG imaging has been used to study collagen remodeling in BC using a number of analysis methods.¹⁰⁻¹² Provenzano et al.¹³ identified "tumor-associated collagen signatures" (TACS) by assessing fiber density and alignment relative to the tumor boundary during early disease, *in-situ* breast carcinomas, and invasive BC. Using a manual approach, TACS-3 (fiber alignment orthogonal to tumor boundary) was shown to be prognostic of disease-free survival by Conklin et al.⁹ Polarization-based

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SHG reflects the pitch angle of α -helices within the collagen triple helix, which change with BC progression and has been applied to mouse models and human samples.^{2,14} Our group has previously shown that F/B measured from primary tumor samples is an independent prognostic indicator of the probability of remaining metastasis free for 10 years after tumor resection in a cohort of untreated estrogen receptor-positive (ER+), lymph node-negative (LNN) invasive ductal carcinoma (IDC) patients.¹⁵ Furthermore, we have shown that F/B from primary tumor samples is correlated with five-year survival in patients treated postoperatively with tamoxifen therapy.¹⁵

In this study, we assessed the relationship between Residual Cancer Burden (RCB) class, a measure of response to NACT, and collagen fiber orientation as well as microstructure (as reported by F/B) in different tumor regions of pre-NACT core needle biopsy sections from TNBC and HER2+ BC patients. We are interested in evaluating F/B as a useful prognostic indicator of response to NACT; therefore, we measured F/B in samples that are currently in the clinical workflow, specifically formalin-fixed, paraffin-embedded (FFPE) hematoxylin and eosin (H&E)-stained sections from core needle biopsies. Hence, the F/B we report here is not necessarily equal to F/B that would be measured in unprocessed fresh biopsy tissue, because various steps in processing and mounting may affect the F/B value.

1.1 Clinical Background

NACT is a well-established treatment option for the clinical management of patients with locally advanced BC (more than three positive lymph nodes or tumors >5 cm), patients who have medical contraindications to surgery at diagnosis but in whom surgery is anticipated at a later date (i.e., women diagnosed during late pregnancy), select cases of early stage BC patients in which breast-conserving surgery would be suboptimal, and select cases of TNBCs or HER2+ patients. NACT, which is administered preoperatively, is often used to downsize larger tumors, allowing breast-conserving surgery rather than mastectomy.¹ Types of NACT include combinations of anthracyclines (e.g., doxorubicin or epirubicin) and taxanes (e.g., docetaxel or paclitaxel), with other drugs such as fluorouracil and cyclophosphamide. In HER2+ patients, targeted therapies with drugs such as trastuzumab, pertuzumab, lapatinib, and/or bevacizumab are administered along with standard chemotherapy regimens, individualized to the patient. NACT has been shown to be as effective as adjuvant chemotherapy in terms of outcomes and is now considered as a possible standard option and a preferred approach for a select subset of BC patients.¹⁶ Multiple studies have shown that a patient's response to NACT is correlated with overall prognosis and disease-free survival.¹⁷ Specifically, patients who have a pathological complete response (pCR) after NACT exhibit better overall disease-free survival and better long-term outcome than those with residual disease (RD).^{1,18–22} In particular, pCR is most strongly correlated with better overall disease-free survival and better long-term outcome in TNBC and HER2+ BC patients.^{1,18,23,24} However, NACT has unpleasant and debilitating side effects, including, but not limited to, nausea, vomiting, fatigue, hair loss, infection, and mouth sores. Bone marrow reserves and renal function decrease with age, increasing the probability of myelosuppression, cardiodepression, peripheral neuropathy, and neurotoxicity.²⁵ Further, NACT has been shown to increase the density of tumor microenvironment of metastasis (TMEM) complexes in human breast tumors. TMEM complexes are thought

to be sites of tumor cell intravasation and their density is correlated with metastatic outcome in human mammary carcinomas.²⁶ For these reasons, there is an identified pressing need to administer NACT only to those who will benefit from it and not administer it to those who will not benefit.¹⁹

Though there are different definitions of pCR, many studies concede that true pCR includes a lack of residual invasive disease in both the breast and axillary lymph nodes.^{18,27–30} RCB, one method of quantifying RD in a resected specimen, is a score calculated based on the presence or absence of invasive tumor in the primary tumor bed, and presence or absence of tumor in the lymph nodes.^{20,21,31,32} Patients are sorted into one of four classes based on their RCB scores, where RCB-0 corresponds to pCR, RCB-I and RCB-II represent intermediate outcomes, and RCB-III indicates extensive RD.³¹ In numerous clinical trials and meta-analyses, RCB class has a significant positive correlation with overall survival and discriminates between patients with favorable and unfavorable prognoses.²¹ Specifically, RCB-0 and RCB-I have been shown to have the same positive five-year prognosis no matter the NACT, adjuvant chemotherapy, or pathological stage, while RCB-II and RCB-III have worse prognoses.³¹ To this end, an improvement in the ability to identify those BC patients who are most likely to have a class of RCB-0 or RCB-I, and therefore a better response to NACT, will assist decision-making regarding surgery, adjuvant chemotherapy, and follow-up care.¹⁹

2 Methods

2.1 Patient Samples

A total of 29 patients with HER2+ biopsy specimens and 27 patients with TNBC biopsy specimens, all with available HER2 and RCB results, were identified from the pathology files at the University of Rochester Medical Center (URMC) (2009 to 2019). Use of patient samples was approved by the Institutional Review Board at URMC. All TNBC patients received some type of NACT regimen which included standard combinations of a taxane, an anthracycline, cyclophosphamide, etoposide, 5-fluorouracil, methotrexate, and/or platinum therapy. All HER2+ patients received trastuzumab and/or pertuzumab with some type of NACT regimen which included standard combinations of a taxane, an anthracycline, cyclophosphamide, and/or platinum therapy. All biopsy samples collected prior to NACT administration were processed in the URMC pathology laboratory and mounted on slides as 5- μ m-thick FFPE sections, prior to routine H&E staining. After NACT administration and tumor resection, all patients received either partial or total mastectomy with appropriate surgical evaluation of lymph nodes. Postmastectomy tissues were processed in the URMC pathology laboratory as 5- μ m-thick FFPE sections and were H&E stained. All tumor H&E slides and immunohistochemistry stains were reviewed by at least two board-certified breast pathologists with manual interpretation of HER2 (rabbit antihuman HER2, Dako HercepTestTM). Fluorescence *in-situ* hybridization (FISH) was performed on all equivocal HER2 immunohistochemistry results (HER2 IQFISH pharmDx, FDA kit, Dako), and the FISH results were used in lieu of the immunohistochemistry for these cases.

The evaluation of the extent of RD following neoadjuvant therapy was performed on the post-treatment pathology material using the published method of Symmans et al.^{21,31,32} and the associated RCB online calculator. Briefly, the gross description

along with clinical imaging studies and specimen photographs were used to determine the two largest dimensions of the residual primary tumor bed in the resection specimen. Evaluation of microscopic sections was used to determine (1) the proportion of the primary tumor bed that contained metastatic carcinoma, (2) the number of axillary lymph nodes that contained metastatic carcinoma, and (3) the diameter of the largest metastatic deposit. This information was entered into the online calculator and the RCB score and RCB class were recorded.

2.2 Imaging

A Spectra Physics MaiTai Ti:sapphire laser (circularly polarized at the sample using a Berek Compensator, 100 fs pulses at 80 MHz, 810 nm, ~3 mW at the sample) was directed through an Olympus Fluoview FV300 scanner. The laser was focused through an Olympus UMPLFL20XW water-immersion lens (20 $\times$, 0.95 NA), which subsequently captured backward-propagating SHG signal. This SHG signal was separated from the excitation beam using a 670-nm dichroic mirror, filtered (HQ405/30 m-2P, Chroma, Rockingham, Vermont), and collected by a photomultiplier tube (Hamamatsu HC125-02). The forward-scattered SHG was collected through an Olympus 0.9 NA condenser, reflected by a 565-nm dichroic mirror (565 DCSX, Chroma, Rockingham, Vermont) to remove excitation light, filtered (HQ405/30 m-2P, Chroma, Rockingham, Vermont) and captured by an identical photomultiplier tube (Hamamatsu HC125-02). Quantification of “F” and “B” signals while scanning the excitation wavelength from 740 to 880 nm (i.e., just below to just above the emission filter bandpass) enabled us to estimate the extent of contamination of the SHG signal with two-photon-excited fluorescence, revealing that ~1% of the detected forward-scattered photons and ~10% of backward-scattered photons were from two-photon-excited fluorescence.

A microscopist who was blinded to the patient outcome (i.e., eventual RCB class) performed the SHG imaging and analysis. Three fields of view (FOVs) were taken in the “tumor bulk” (solid box, Fig. 1) of each 5- μ m-thick pretreatment core needle biopsy sample. The “tumor–host interface” was defined as the stroma immediately surrounding the tumor bulk. Three FOVs were also taken in the tumor–host interface, with the tumor bulk just out of the FOV (dashed box, Fig. 1). Forward- and backward-scattered SHG images were collected simultaneously. Examples of the H&E-stained FOV and corresponding SHG images are shown in Fig. 2. Images from these core needle biopsy sections revealed a random network of collagen fibers often encapsulating SHG-dark nodules containing highly cellular areas, consistent with our previous images of primary tumor sections.^{15,33} Interestingly, close inspection of forward (F) and backward (B) images often revealed the characteristic “segmented” appearance of fibrils in the B image relative to the F image (Fig. 2), as previously reported in other tissue types.^{4,8}

2.3 Image Analysis: Intensity Mask-Based F/B

A single value of F/B for a FOV was derived from the F and B images of that FOV using ImageJ^{34,35} as previously described.^{15,33} Briefly, an F image of forward-propagating SHG and a B image of backward-propagating SHG were collected in each FOV. All images were background-subtracted and a pair of masks was created to distinguish background pixels from collagen fibers. To produce the masks (one mask for the F image and one for the B image), a blinded user selected a threshold to best distinguish pixels within fibers from background pixels. The same two thresholds were used for all F and all B images in a single imaging session. All pixels above the lower threshold were set to 1 and those below the lower threshold to 0, producing binary F and B masks. These binary F and B masks were multiplied together to create a final mask of pixels within fibers. The background-subtracted F and B images were

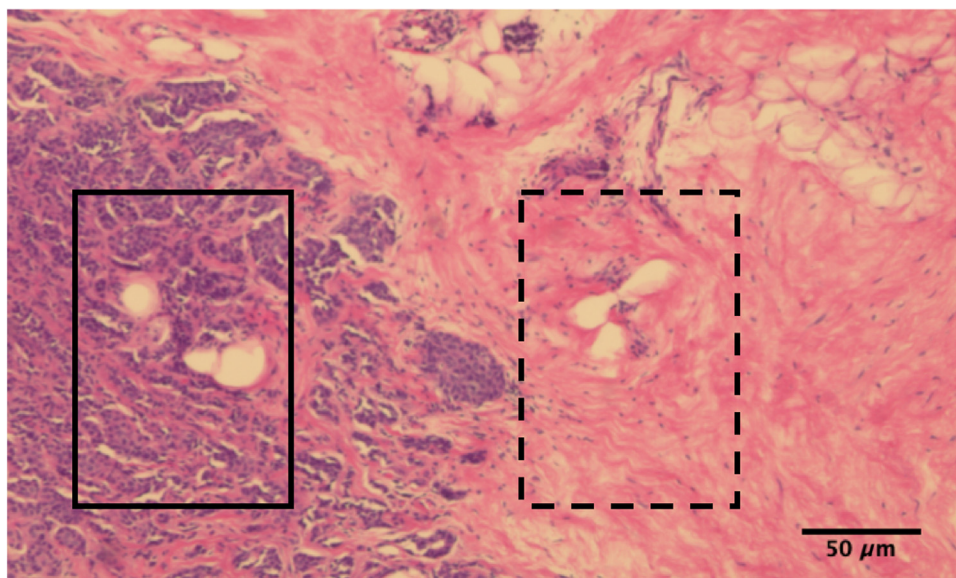


Fig. 1 Core needle biopsy sections contain tumor cells and surrounding stroma. Pretreatment core needle biopsy sections contain heterogeneous cellular and noncellular regions. We have defined both the tumor bulk (solid box), which consists primarily of tumor cell clusters, and the tumor–host interface (dashed box), the matrix that surrounds the cell clusters and consists mainly of collagen and structural proteins. Three images were taken in the tumor bulk and three in the tumor–host interface for each H&E-stained core needle biopsy.

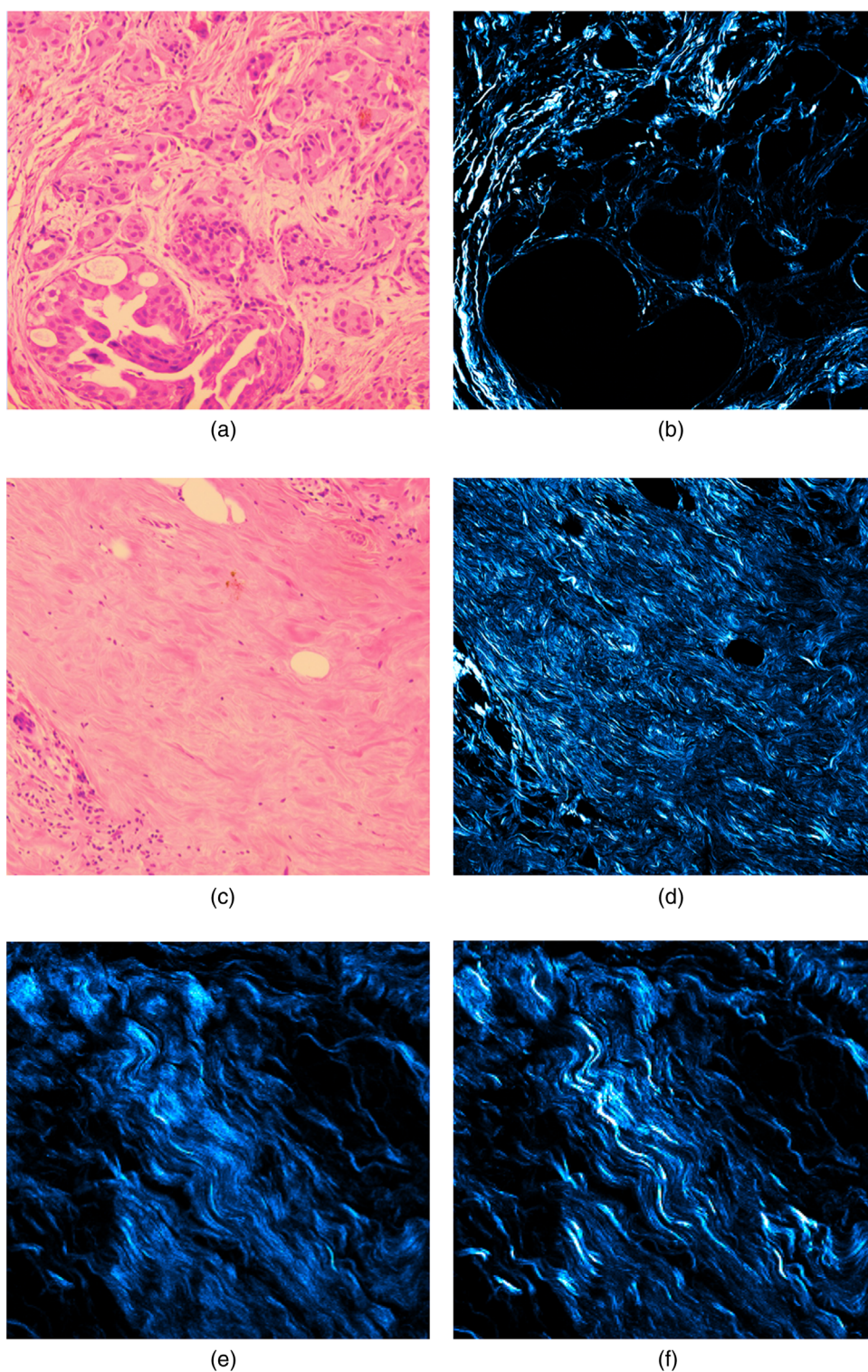


Fig. 2 Tumor bulk and tumor–host interface can be imaged with SHG microscopy. Core needle biopsy sections contain the bulk of the tumor and the tumor–host interface consisting mostly of collagen. Here, the same region of the tumor bulk is shown: (a) transmitted light microscopy (H&E stained) and (b) two-photon forward-scattered SHG image. In the same core, a FOV containing the tumor–host interface is shown: (c) transmitted light microscopy (H&E stained) and (d) two-photon forward-scattered SHG image of the same region. Note: (a)–(d) are $\sim 660\ \mu\text{m}$ across. (e) Backward-scattered and (f) forward-scattered SHG images reveal the fibrillar collagen network, often encapsulating SHG-dark cellular areas. Characteristic segmented fibrils appear in (e) the backward-scattered image, relative to (f) the corresponding forward-scattered image. Note: (e) and (f) are $\sim 420\ \mu\text{m}$ across.

divided to produce a single F/B image, which was multiplied by this final mask. The average value of the nonzero pixels from the resultant image yielded the average F/B of the entire FOV. The F/B values from the three FOVs in each location type were averaged to provide a single F/B value from the tumor bulk and one from the tumor–host interface for each patient.

2.4 Image Analysis: Coherency Mask-Based F/B

In addition to the selection of pixels based on an intensity threshold, we also investigated the selection of pixels based on their grouping into linear structures, as quantified by pixel “energy” and “coherency,” as previously described.³⁶ SHG images were background-subtracted and day-to-day variations in intensity were normalized, as described above. The open-source toolbox OrientationJ was used to select those pixels most relevant in this analysis. This toolbox enables the production of binary image masks where the pixels that are rejected or passed through the mask are selected based on their energy and/or coherency contribution. For this analysis, pixels passed by the mask were chosen to have a minimum of 2% of both normalized energy and coherency,³⁷ where the energy and coherency values are calculated from a 2×2 structure tensor that is determined for each pixel. The structure tensor is defined as

$$J = \begin{bmatrix} \langle f_x, f_x \rangle & \langle f_x, f_y \rangle \\ \langle f_y, f_x \rangle & \langle f_y, f_y \rangle \end{bmatrix},$$

where f_x and f_y are the partial derivatives of the intensity in the x and y directions, respectively.³⁷ This tensor is found for each pixel by using a cubic B-spline interpolation in both the x and y directions to find the continuous spatial derivative. Once the tensor values are known, the coherency is calculated based on the eigenvalues as

$$\text{coherency} = \frac{\lambda_{\max} - \lambda_{\min}}{\lambda_{\max} + \lambda_{\min}},$$

where $\lambda_{\max}$ is the largest eigenvalue and $\lambda_{\min}$ is the smallest. Then the energy of the tensor is found as the trace of the structure tensor or

$$\text{energy} = \langle f_x, f_x \rangle + \langle f_y, f_y \rangle.$$

Once the binary image masks were obtained for both the forward and backward images, they were multiplied together to produce a final mask. The background-subtracted forward and backward images were divided to produce a raw F/B image, which was multiplied by the final mask. All pixels of value zero were excluded, and the average of this image gave an average F/B for a particular FOV.

2.5 Image Analysis: Collagen Fiber Angle at Tumor Boundary

In addition to measuring F/B in each tumor region, we assessed the organization of collagen fibers at the tumor–host interface. Using the method of Provenzano et al.,¹³ the boundary between the tumor bulk and the interface was defined and then the angle of collagen fibers relative to the tangent of the boundary was measured every 30 μm . This analysis was performed in three imaged locations per biopsy section.

2.6 Calibration

For each imaging session, a background image of no sample and a reference F and B image of a dilute stock solution of isotropically emitting fluorescein isothiocyanate (FITC) were collected (to quantify day-to-day variations in sample alignment). Day-to-day variations in system alignment were normalized by dividing each F/B value by the F/B value measured for the FITC stock solution (expected F/B = 1) during that imaging session.

2.7 Statistical Analysis

Logistic regression was used to assess the association between F/B and binary response variable “RCB class >1.” (This is a variable that can be either 0 or 1. A value of “0” is assigned when RCB class is 0 or I, and a value of “1” if RCB class is II or III.) A likelihood ratio test was used to calculate the p -value for the test of whether the regression coefficient for F/B was zero. All graphs were generated using GraphPad Prism 8 and statistics were assessed using JMP Pro 14.

3 Results

We have previously shown that F/B from SHG images is prognostic of metastasis-free survival in a cohort of untreated ER+ LNN IDC patients. Further, a subset of these patients was administered postoperative (tamoxifen) therapy after they were diagnosed with distant recurrence and F/B of the primary tumor was correlated with five-year survival in these patients.¹⁵ As a result, we explored the relationship between F/B in core needle biopsy samples and a patient’s subsequent response to NACT. The goal of this study was to assess a potential relationship between F/B derived from different regions of pretreatment biopsy sections and NACT response. As RCB accounts for lymph node involvement as well as the primary tumor bed, it was used as an assessment of patient response to NACT. RCB-0 and RCB-I have been shown to have the same five-year prognosis no matter the neoadjuvant therapy, adjuvant therapy, or pathologic stage; therefore, RCB-0/I and II/III were grouped together for our comparison.³¹ We performed SHG imaging on pretreatment biopsy sections to see if we could distinguish patients who responded favorably to NACT from those with RD.

3.1 HER2+ Samples: Intensity Masks

When F/B is calculated from SHG images using masks that select collagen pixels and reject background based on pixel intensity, the average F/B from the tumor bulk was significantly different from that of the tumor–host interface [$F/B = 8.84 \pm 0.67$ (tumor bulk) or 15.35 ± 1.01 (tumor–host interface), $p < 0.0001$, data not shown]. Intensity mask-based F/B in each region type was subsequently stratified by the patient’s RCB class post-NACT after mastectomy. Samples were grouped as RCB-0/I or RCB-II/III in both the tumor bulk ($F/B = 8.87 \pm 0.88$ or 8.78 ± 1.05 , respectively) and the tumor–host interface ($F/B = 16.51 \pm 0.95$ or 12.32 ± 1.84 , respectively). When coded as a binary response (0/I or II/III), intensity mask-based F/B derived from the tumor–host interface was associated with RCB class ($p = 0.0246$; Fig. 3). However, intensity mask-based F/B was not associated with RCB class when derived from images of the tumor bulk ($p = 0.297$; Fig. 3).

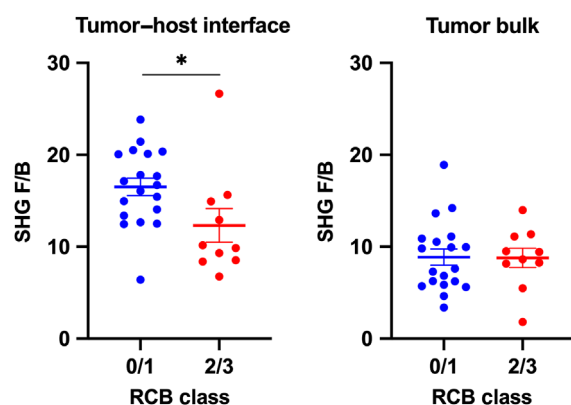


Fig. 3 Intensity threshold-based F/B is correlated with RCB class in HER2+ biopsy samples. Intensity threshold-based F/B was calculated from SHG images of the tumor bulk and tumor-host interface in each HER2+ core needle biopsy ($n = 29$). Each point is the average of three tumor bulk or tumor-host interface FOVs. Two categories of NACT response were determined using RCB class, grouped as RCB-0/I and RCB-II/III. F/B is correlated with RCB class in the tumor-host interface, but not in the bulk of the tumor. Error bars = standard error of the mean (SEM), $p = 0.0246$.

3.2 HER2+ Samples: Coherency Masks

The F/B was then derived using masks that select collagen pixels and reject background pixels based on their residence in linear structures (i.e., coherency). In each region type (tumor bulk and tumor-host interface), the F/B was coded as a binary response (0/I or II/III) post-NACT after mastectomy. Samples were again grouped as RCB-0/I or II/III in the tumor bulk ($F/B = 33.03 \pm 2.73$ or 35.12 ± 3.76 , respectively) and tumor-host interface ($F/B = 39.47 \pm 2.25$ or 35.66 ± 3.85 , respectively). Coherency mask-based F/B was not associated with RCB class when measured either in the tumor-host interface or tumor bulk ($p = 0.643$ and 0.346 , respectively; Fig. 4).

3.3 TNBC Samples: Intensity Masks

In TNBC needle biopsy samples, the average intensity mask-based F/B derived from the tumor bulk was significantly different from that of the tumor-host interface [$F/B = 7.48 \pm 0.55$

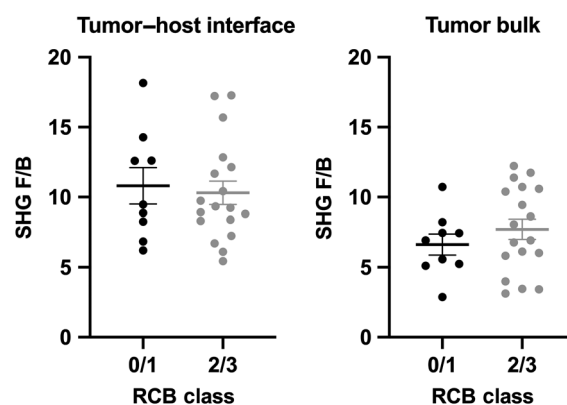


Fig. 5 Intensity threshold-based F/B is not correlated with RCB class in TNBC biopsy samples. Intensity threshold-based F/B was calculated from both tumor bulk and tumor-host interface in each TNBC core needle biopsy ($n = 27$). F/B is not correlated with RCB class in either region of TNBC biopsy samples. Error bars = SEM.

(tumor bulk) or 10.67 ± 0.69 (tumor-host interface), $p = 0.0008$, data not shown]. The F/B in each region was coded as a binary response according to a patient's RCB class post-NACT after mastectomy. Intensity mask-based F/B was not associated with RCB class 0/I or II/III in the tumor bulk (6.62 ± 0.75 or 7.72 ± 0.72 , respectively; $p = 0.329$; Fig. 5). Intensity mask-based F/B was also not associated with RCB class 0/I or II/III in the tumor-host interface (10.81 ± 3.89 or 10.31 ± 3.56 , respectively; $p = 0.73$; Fig. 5).

3.4 TNBC Samples: Coherency Masks

Coherency mask-based F/B in each region type was coded as a binary response by RCB class post-NACT after mastectomy. Samples were again grouped as RCB-0/I or II/III in the tumor bulk ($F/B = 29.98 \pm 4.20$ or 34.18 ± 2.98 , respectively) and tumor-host interface ($F/B = 35.99 \pm 3.81$ or 35.23 ± 2.57 , respectively). Coherency mask-based F/B was not associated with RCB class when derived from either the tumor-host interface or tumor bulk ($p = 0.400$ and 0.862 , respectively; Fig. 6).

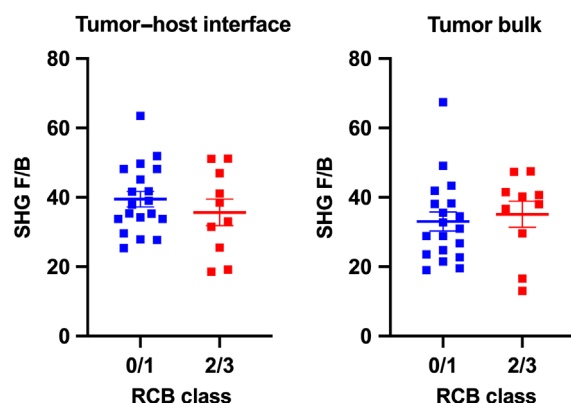


Fig. 4 Coherency-based F/B is not correlated with RCB class in HER2+ biopsy samples. Coherency-based F/B was calculated from tumor bulk and tumor-host interface in each HER2+ core needle biopsy ($n = 29$) using OrientationJ. Coherency-based F/B is not correlated with RCB class in either region of HER2+ biopsy samples. Error bars = SEM.

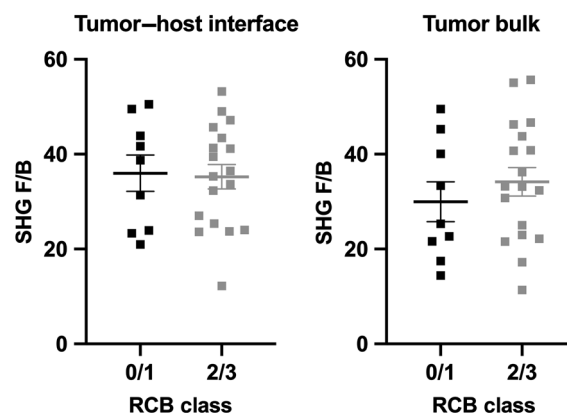


Fig. 6 Coherency-based F/B is not correlated with RCB class in TNBC biopsy samples. Coherency-based F/B was calculated from tumor bulk and tumor-host interface in each TNBC core needle biopsy ($n = 27$) using OrientationJ, as previously described. Coherency-based F/B is not correlated with RCB class in either region of TNBC biopsy samples. Error bars = SEM.

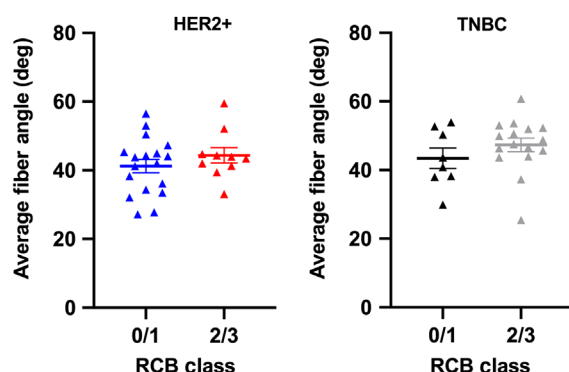


Fig. 7 Average fiber angle at the tumor boundary is not correlated with RCB class in HER2+ or TNBC biopsy samples. The collagen fiber angle relative to the tumor boundary was measured every $30\ \mu\text{m}$ in HER2+ ($n = 28$) and TNBC core needle biopsies ($n = 24$). The average fiber angle of each sample is not correlated with RCB class. Error bars = SEM.

3.5 HER2+ and TNBC Samples: Fiber Angle at Tumor Boundary

The average collagen fiber angle along the tumor boundary for each patient was coded as a binary response by RCB class post-NACT after mastectomy. Samples were again grouped as RCB-0/I or II/III in both HER2+ and TNBC patient groups. Average fiber angle was not associated with RCB class when measured in either HER2+ ($p = 0.298$) or TNBC samples ($p = 0.258$; Fig. 7).

4 Discussion

NACT has become a recommended treatment option for certain patients with locally advanced or inoperative BC. However, a significant number of patients do not respond to NACT and would instead benefit from immediate tumor resection and other therapies. The ability to predict patient response to NACT would greatly reduce overtreatment, mitigate side effects and cost, and optimize individual patient treatment regimens.

Solid tumors are heterogeneous, consisting of tumor cells as well as soluble growth factors, stromal cells, and the ECM.³⁸ The ECM is dynamic, responding to and influencing tumor cell behavior, and this relationship is integral to tumor chemoresistance and metastasis.^{38–42} Accordingly, we investigated the tumor ECM as a candidate for predicting NACT response in two subsets of BC patients that typically receive it—TNBC and HER2+ BC patients. We have previously shown that the F/B derived from SHG images is associated with metastasis-free survival in a subset of untreated IDC patients and progression-free survival in IDC patients treated with tamoxifen after a distant recurrence was diagnosed.¹⁵ Therefore, we used SHG imaging and F/B measurements to study the ECM and subsequent patient response to NACT.

We imaged core needle biopsy sections taken from IDC patients prior to NACT administration and assessed the relationship between SHG F/B and post-treatment RCB class. This technique is nondestructive, rapid, and fits into the typical pathology workflow, as imaging can be performed on the same sections used for diagnosis without additional staining or tissue processing. SHG imaging complements genetically based diagnostic tests by additionally assessing the ECM surrounding tumor cells. We first analyzed SHG F/B from pixels above a threshold brightness that served to distinguish pixels in collagen fibers

from background pixels. For comparison, we also assessed F/B using a method to identify pixels for analysis based on their grouping into linear arrangements. We found that intensity mask-based F/B was associated with NACT response in HER2+ BC patients, but not TNBC patients, while coherency mask-based F/B was not associated with NACT response in either patient group. Further, there was no association between NACT response and the average angle of collagen fibers relative to the tumor boundary. This suggests that the relationship between F/B and response to NACT may lie in the collagen microstructure (fibril properties) rather than the overall fiber alignment. It is worth noting that this statistically significant relationship was derived from images taken in the collagenous tumor–host interface rather than the tumor bulk (Fig. 3). This suggests the possibility that chemoresistant cells occupy the tumor–host interface rather than residing in the bulk of the tumor. Future improvements in the ability of F/B to predict RCB status might be found by more efficiently separating the small contaminating fluorescence signal from SHG (by using narrower bandpass filters, time-domain imaging, as well as exploring other excitation wavelengths), depositing tissue sections on antireflection-coated slides to more accurately measure the true F/B, or even assessing F/B from fresh tissue if that proves more predictive. However, any methodological modifications that would require alteration of the current clinical workflow should be carefully considered, as they may hinder adoption of any resultant predictive method.

Several possible mechanisms of chemoresistance may be responsible for the relationship between F/B and the outcomes we observed in the HER2+ population. Systemic drug delivery is controlled by vascular transport (flux via the vascular network), transvascular transport (flux through vessel walls and basement membranes), and interstitial transport (diffusion and convection through a tumor to target cells).⁴³ The ECM affects each of these transport mechanisms directly and/or indirectly and can be studied using multiphoton microscopy-based methods.^{43–47} Increased production of ECM components such as hyaluronan and collagen causes solid stress, constricting vasculature and normal cellular function. In addition, leaky and convoluted vessels combined with reduced interstitial pressure further hinder drug delivery via blood flow and diffusion.⁴⁸ Finally, collagen orientation and organization influence drug diffusion rates through the ECM by steric hindrance.^{49–51} In addition to affecting chemotherapy efficacy by influencing transport of drugs to target cells, collagen organization can influence the resistance of cells to that drug. This can occur via interactions between collagen and cancer cell receptors and activation of downstream signaling pathways.^{38,48}

5 Conclusions

NACT is often used to reduce tumors prior to operation in BC patients. The pCR to NACT is considered to be a positive prognostic indicator and is correlated with a good long-term outcome, particularly in HER2+ and TNBC patients. The RCB is a method of scoring a patient's response to NACT based on the amount of residual invasive carcinoma in the primary tumor bed and the presence or absence of invasive carcinoma in the lymph nodes. SHG imaging is a nonlinear optical method that utilizes intrinsic properties of fibrillar collagen in solid tumor ECM. Directional (F/B) scattering from fibrillar collagen is sensitive to microstructure properties, including fibril diameter, packing disorder, and spacing, and has been correlated with

metastasis-free survival response in a subset of IDC patients. SHG imaging was performed on two subsets of core needle biopsy sections taken from HER2+ and TNBC patients prior to NACT administration and microstructure analyzed two ways. Intensity mask-based F/B derived from the tumor–host interface, but not the tumor bulk, was associated with post-treatment RCB class in HER2+ biopsies. There was no association between coherency mask-based F/B and RCB class in HER2+ samples. In triple-negative biopsies, no association was found between F/B determined using intensity or coherency-based methods in either the tumor bulk or the tumor–host interface. The average fiber orientation relative to the tumor boundary was not correlated with RCB class in either HER2+ or TNBC patients. This pilot study suggests that intensity mask-based F/B may be a useful predictor of response to NACT in HER2+ patients and warrants further investigation. Development of a convenient F/B-based method to predict which patients will respond favorably to NACT will help to inform clinical decision-making for BC patients and their healthcare providers.

Disclosures

R.L.H., E.B.B., D.G.H., B.M.T., B.B., and D.E.D. are inventors on a provisional patent related to the methods used in the paper. All other authors declare that they have no competing interests.

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RESEARCH ARTICLE

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Intratumoral heterogeneity of second-harmonic generation scattering from tumor collagen and its effects on metastatic risk prediction

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Abstract

Background: Metastases are the leading cause of breast cancer-related deaths. The tumor microenvironment impacts cancer progression and metastatic ability. Fibrillar collagen, a major extracellular matrix component, can be studied using the light scattering phenomenon known as second-harmonic generation (SHG). The ratio of forward- to backward-scattered SHG photons (F/B) is sensitive to collagen fiber internal structure and has been shown to be an independent prognostic indicator of metastasis-free survival time (MFS). Here we assess the effects of heterogeneity in the tumor matrix on the possible use of F/B as a prognostic tool.

Methods: SHG imaging was performed on sectioned primary tumor excisions from 95 untreated, estrogen receptor-positive, lymph node negative invasive ductal carcinoma patients. We identified two distinct regions whose collagen displayed different average F/B values, indicative of spatial heterogeneity: the cellular tumor bulk and surrounding tumor-stroma interface. To evaluate the impact of heterogeneity on F/B's prognostic ability, we performed SHG imaging in the tumor bulk and tumor-stroma interface, calculated a 21-gene recurrence score (surrogate for OncotypeDX®, or S-ODX) for each patient and evaluated their combined prognostic ability.

Results: We found that F/B measured in tumor-stroma interface, but not tumor bulk, is prognostic of MFS using three methods to select pixels for analysis: an intensity threshold selected by a blinded observer, a histogram-based thresholding method, and an adaptive thresholding method. Using both regression trees and Random Survival Forests for MFS outcome, we obtained data-driven prediction rules that show F/B from tumor-stroma interface, but not tumor bulk, and S-ODX both contribute to predicting MFS in this patient cohort. We also separated patients into low-intermediate (S-ODX < 26) and high risk (S-ODX ≥ 26) groups. In the low-intermediate risk group, comprised of patients not typically recommended for adjuvant chemotherapy, we find that F/B from the tumor-stroma interface is prognostic of MFS and can identify a patient cohort with poor outcomes.

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Conclusions: These data demonstrate that intratumoral heterogeneity in F/B values can play an important role in its possible use as a prognostic marker, and that F/B from tumor-stroma interface of primary tumor excisions may provide useful information to stratify patients by metastatic risk.

Keywords: Breast cancer, Metastasis, Second-harmonic generation, Multiphoton microscopy, Collagen, Tumor microenvironment, F/B, Prognosis

Background

Breast cancer is the most common invasive cancer in women, with the majority of deaths attributed to metastasis [1]. Breast tumors are typically classified using molecular and genetic markers as well as clinical staging systems. These biomarkers are analyzed to determine prognosis, predict response to therapies, and are also used as surrogates for outcome (i.e. measures of treatment effects that correlate with a clinical endpoint) in invasive breast cancers [2]. Such characterization may include expression levels of estrogen receptor (ER), progesterone receptor, and human epidermal growth factor receptor-2, and testing for other proliferation, invasion, and epithelial-mesenchymal transition markers. Tumors can exhibit heterogeneity, with different regions expressing different genetic aberrations [3–6] and differences in key biomarkers [7]. Heterogeneity in cell types is frequently seen in breast cancers and has been linked to poor patient prognosis independent of typical clinical variables including ER status, lymph node involvement, and tumor size [8]. Improving individualized treatments and overall patient survival therefore requires a better understanding of heterogeneity in the breast tumor microenvironment [9, 10]. Likewise, developing any new biomarker requires an understanding of its heterogeneity within tumors and the impact of that heterogeneity on its clinical utility.

Both cellular and noncellular components of the microenvironment facilitate primary tumor growth and metastasis [3, 11–13]. The ECM is an essential component of the solid tumor microenvironment, affecting cell biomechanics and signaling and therefore directly impacting metastatic potential. Microscopic ECM properties provide biophysical support and chemical cues necessary for normal cell function and are a result of constant matrix synthesis, modification, and degradation [14, 15]. As a major component of the ECM, collagen plays a critical role in cell migration and differentiation [14, 16]. Increased collagen deposition and crosslinking are associated with malignancy, and changes in collagen organization are thought to promote tumor cell invasion, possibly via increased protein deposition, increased tissue stiffness, and linearization of collagen fibers [17–24]. In more aggressive breast cancers, irregular tumor-stroma boundaries are seen with orthogonally-aligned fibers

facilitating cell invasion [18]. These altered collagen properties in turn affect biochemical signaling and tissue biomechanical properties, encouraging tumor cell proliferation, migration, and dysregulation of normal cellular activities [25].

Fibrillar collagen can produce an intrinsic optical signal called second-harmonic generation (SHG) [26]. SHG is a nonlinear optical phenomenon that occurs when two identical photons scatter off a noncentrosymmetric material, producing a single photon with exactly twice the energy of the initial photons [26]. In SHG images of excised primary breast tumor, the presence of collagen fibers oriented perpendicular to the tumor border has been shown to be prognostic of breast cancer progression and to enable tumor cell invasion [19, 27, 28], and in *in vivo* models of breast cancer, tumor cells can be observed locomoting along SHG+ collagen fibers [29]. Recent studies have demonstrated that SHG can be used, alone and in conjunction with two-photon excited fluorescence, to identify early stages of breast ductal carcinoma, collagen morphological changes during tumor progression, and to provide prognostic information on patient survival [30–32].

In addition to producing images whose properties provide insight into disease states, SHG polarization and scattering directionality can reveal important information about tissue. Polarization-resolved SHG has also been used to assess structural changes in ECM collagen during disease progression, including breast cancers [33–36]. This technique exploits the polarization of incident light to reveal information about collagen at the molecular level including helix pitch angles, fibril organization, and orientation. The directionality of SHG scattering from an individual fiber is sensitive to its internal structure, including fibril diameter, spacing, and disorder of fibril packing within the fiber [37–40]. We collectively call these three parameters the collagen fiber internal structure (FIS). One measure of SHG emission directionality is the ratio of forward-emitted to backward-emitted SHG (where “forward” is in the direction of the excitation laser), or the F/B ratio. F/B is sensitive to FIS, can be measured on a point-by-point basis or used to generate F/B images, and is distinct from the overall orientation of a fiber in an SHG image [19, 41]. SHG directionality imaging and analysis has been used

to distinguish healthy and diseased tissue in breast [42, 43], ovarian [44], lung [45], and basal cell cancers [46].

We have previously shown that the average F/B from SHG images of a cohort of untreated ER+, lymph node negative (LNN), invasive ductal carcinoma (IDC) samples is an independent prognostic indicator of metastasis-free survival time (MFS) [47]. The samples used in that study were tissue microarrays comprised of 1-mm tissue discs and as such, the F/B values prognostic of MFS in that study were taken from a small part of the excised primary tumor.

Recently, we performed SHG imaging on core needle biopsy sections taken from breast cancer patients prior to neoadjuvant chemotherapy (NACT) administration. As these tissue strips ($\sim 0.2 \times 1.5$ cm) span multiple regions of interest (ROIs), we selected two types of regions for study: the cellular tumor bulk and the surrounding tumor-stroma interface, consisting mainly of ECM proteins and stromal cells, each identified by a clinical pathologist. We found that F/B measured in the tumor-stroma interface, but not tumor bulk, was associated with Residual Cancer Burden class, one measure of NACT response [48]. These results from needle biopsy sections revealed that heterogeneity in collagen FIS affects the relationship between pre-treatment F/B and subsequent NACT response.

Prompted by that discovery, in this study we investigated how heterogeneity in primary tumor excisions affects the ability of F/B to predict MFS in untreated IDC patients. We evaluated the association between collagen FIS (as reported by F/B) and MFS in two regions associated with tumor tissue in primary tumor excisions. We then used both regression trees [49] and Random Survival Forests (RSF) [50] to further explore this association and the combined prognostic ability of F/B and a widely used 21-gene recurrence score. Our results reveal that heterogeneity in SHG F/B measurements within breast tumor samples is significant and must be considered when evaluating this method as a possible predictor of metastasis and further suggest that F/B measured in appropriate tumor regions may add prognostic information to currently used genomic methods.

Methods

Patient samples

Slides prepared from post-biopsy primary breast cancer surgical excisions from 95 different patients were used from a collection at the Erasmus Medical Center (Rotterdam, Netherlands). The studies on secondary use of archived tissues was approved in writing by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, Netherlands (MEC 02.953) and was performed in accordance with the Code of Conduct (The Code for Proper Secondary Use of Human Tissue) of the Federation of Medical Scientific Societies in The Netherlands. Primary

tumor excisions were formalin-fixed and paraffin-embedded, (FFPE) mounted on slides as 5 μ m sections and stained with hematoxylin and eosin (H&E). Multiple regions were imaged within a single 5 μ m section from each patient in this study. Patients were tested for ER status using immunohistochemistry, where the cutoff for receptor positivity was 10% positive tumor cells. All patients were ER+ and LNN and had not been treated with NACT or adjuvant hormonal nor chemotherapy. Other primary tumor characteristics are summarized in Supplementary Table 1. Some patients received radiation therapy, which has been shown not to affect distant metastases [51]. No other treatment was received before nor after excision of the primary tumor. We note that these are historical samples and clinical practice in the Netherlands at the time was more focused on monitoring patients. Thus, these cases are uniquely suitable for a purely prognostic study.

Follow-up data on patient outcomes were recorded every 3 months for 2 years, every 6 months for years 3–5, and every 12 months afterwards. Gene expression data for these patients are archived in the GEO repository (ncbi.nlm.nih.gov/geo/) as part of databases GSM2034 and GSM5327. In order to study F/B in the context of the 21-gene OncotypeDX® score, we used a surrogate 21-gene score (S-ODX) calculated from these gene expression data using the publicly available Recurrence Online tool (www.recurrenceonline.com), an online analysis tool to determine breast cancer recurrence scores and hormone receptor status using microarray data [52].

In this study, we wanted to evaluate the effect of heterogeneity on F/B's prognostic ability in a clinical setting, with the hope of producing a clinically relevant technique for predicting metastasis. Therefore, we performed SHG imaging and F/B analysis using samples that were already within the clinical workflow, specifically the typical FFPE H&E sections generated from primary tumor excisions, as opposed to fresh, unprocessed tumor tissue. Using these FFPE H&E sections also allows us to easily identify clinically relevant regions within the tumor that inform pathologists' diagnoses and recommendations. Consequently, we note that the F/B value we report here is not necessarily equal to F/B that would be measured in unprocessed fresh tissue because various steps in processing and mounting may affect the F/B value.

Imaging

A Spectra Physics MaiTai Ti:Sapphire laser (circularly polarized at the sample using a Berek Compensator, 100 fs pulses at 80 MHz, 810 nm, ~ 4 mW at the sample) was directed through an Olympus Fluoview FV300 scanner. The laser was focused through an Olympus UPLFL20XW water-immersion lens (20x, 0.95 NA), which subsequently

captured backward-propagating SHG signal (i.e. the B image). This backward-propagating SHG signal was separated from the excitation beam using a 670 nm dichroic mirror, filtered (HQ405/30 m-2P, Chroma), and collected by a photomultiplier tube (Hamamatsu H10492–003). The forward-scattered SHG (i.e. the F image) was collected through an Olympus 0.9 NA condenser, reflected by a 565 nm dichroic mirror (565 DCSX, Chroma) to remove excitation light, and captured using an identical filter (HQ405/30 m-2P, Chroma) and identical photomultiplier tube (Hamamatsu H10492–003) with minimal autofluorescence captured, as previously described [48].

Image analysis

User-defined thresholds

Image pairs were analyzed using Fiji, as we have previously described [47, 48, 53]: in summary, to produce a forward-to-backward scattering ratio (F/B) for a given ROI, two masks (one for the F image and one for the B image) were created by a blinded observer selecting a threshold for each F and each B image that best distinguished pixels within fibers from background pixels. Pixels above the threshold were set to 1 and those below to 0, producing binary F and B masks. The binary masks were multiplied together to create a final mask of pixels within collagen fibers. In this final mask, pixels are assigned a value of 1 only if the value of that pixel is 1 in both the F and B masks (i.e. located within a collagen fiber), and 0 if otherwise. The background-subtracted F and B images were divided to produce a single F/B image, which was multiplied by this final mask (the product of the binary F and B masks). The average value of the nonzero pixels from the resultant image yielded the average F/B of the entire ROI.

Histogram-based thresholding

To reduce possible user bias in image analysis, histogram-based automatic image thresholding (“Otsu’s method”) was also performed [54]. This method separates pixels into two classes: foreground pixels above a selected threshold (i.e. collagen fiber) and background pixels. The algorithm steps through all possible thresholds, calculates the variance of each pixel class and selects the threshold that minimizes the sum of foreground and background variances. A threshold was selected for each image using Otsu’s method with a scaling factor of 0.6 implemented in MATLAB (Mathworks, Inc.). Foreground pixels were converted to 1 and background to 0 to produce binary F and B masks, which were multiplied by the background-subtracted F/B image to produce a single F/B value as described above.

Adaptive thresholding

While histogram-based segmentation succeeds in calculating thresholds with minimal user input (reducing possible

bias), this method does not consider heterogeneity (i.e. spatial variation in intensity) within the images. A real-time adaptive thresholding method was used to account for these variations as follows [55]. Our goal continues to be to generate a mask whose pixel values are 1 if they are in collagen fibers and 0 if they are background pixels. In adaptive thresholding, we do this by considering each pixel in the image and assigning that pixel a value of 1 or 0, depending on its value relative to the average intensity of the pixels in a window centered on the pixel in question. A small window size is desirable as it will be more responsive to variations in image intensity, allowing somewhat dim collagen pixels in regions whose collagen is overall very dark to be assigned a value of 1. However, if the window is too small, it could be contained within regions that are entirely collagen-free, thus assigning background pixels a value of 1. Hence our first task is to identify the smallest window size for each image that will *not* fit entirely into collagen-free regions. For this initial task we first binarize the entire image based upon whether each pixel is greater than or less than an arbitrary value (0.6x the average pixel intensity of the entire image). Then, a series of progressively smaller windows are scanned across the binarized image and the percentage of nonzero pixels calculated for every possible position of the window in the image. The first window size that, when applied to any location in the image, has < 5% nonzero pixels is selected as the smallest allowed window size for that image. This window size is next applied to the original image and a mask is generated whereby each pixel in the image is assigned a value of 1 or 0 depending upon whether it is less than or greater than 0.6x the average pixel value in the window surrounding it. This algorithm was implemented in MATLAB (Mathworks, Inc.) and a binary mask produced for each image. The resultant F and B masks were then multiplied by the background-subtracted F/B image to produce a single F/B value as described above.

All graphs were generated using GraphPad Prism 5 and statistical analysis performed using Prism 5 or R.

Calibration

For each imaging session, an F and B image of no sample (for background) and a reference F and B image of a dilute stock solution of fluorescein isothiocyanate (FITC) were collected (to quantify day-to-day variations in laser alignment, detector alignment, etc.). Variations were normalized by dividing each patient sample’s F/B value by F/B of the measured FITC value for that imaging session.

Results

To illustrate the heterogeneity in collagen FIS, we sequentially acquired H&E and SHG images from one sample by holding the *y* position of the ROI constant

and moving across the tissue section in x . The resultant series of images (typically $660 \times 660 \mu\text{m}$ ROIs for SHG) were stitched together to form image strips (Fig. 1 and Supplementary Fig. 1) [56]. In the H&E image, our collaborating breast pathologist marked different regions including the tumor bulk and surrounding tumor-stroma interface (solid and dashed boxes, respectively) as well as uninvolved tissue (circle). To produce the accompanying F/B image we calculated F/B in the SHG strip using user-defined thresholds to set background pixels to zero as described above. Variations in collagen FIS, as reported by F/B, are clearly visible in both the tumor bulk (solid box) and tumor-stroma interface (dashed box).

Collagen fiber internal structure differs between tumor regions

We next investigated whether the observed variations in F/B can be found systemically between biologically relevant, identifiable regions in the tumor. In collaboration with breast pathologists, we previously identified two region types in breast cancer core needle biopsies: the highly cellular tumor bulk and the tumor-stroma interface directly adjacent (i.e. within $\sim 660 \mu\text{m}$, one microscope field of view), which is comprised mainly of ECM proteins. In that study of core needle biopsies, we observed that F/B from the tumor-stroma interface, but not the tumor bulk, of core needle biopsy sections is associated with NACT response in certain breast cancer patients [48]. As a result, in this study we chose to evaluate F/B in the same two types of regions (tumor bulk and tumor-stroma interface) in sections from primary tumor excisions. Examples of these regions are shown in both H&E and SHG in Fig. 2.

An observer blinded to patient outcome was trained by a pathologist to recognize these regions in primary excisions and acquired 3 images in the tumor bulk and 3 in the tumor-stroma interface of each slide. The F/B values for each of the 3 ROIs within each region type were averaged to produce a single F/B for tumor bulk

and one for tumor-stroma interface in each patient. In most of the excisions ($n = 92$ of 95), a clear tumor-stroma interface was available for imaging. In agreement with our previous results for core needle biopsy samples imaged prior to NACT [48], we found a significant difference between measured bulk and interface F/B values, indicating heterogeneity in collagen FIS between these two regions of the tumor ($p < 0.0001$, Fig. 3). Additionally, to put these values in context we imaged further away from tumor bulk (at least one full field of view, $\sim 660 \mu\text{m}$). This was possible in 60 of the 95 slides, and an example region is circled in Fig. 1. The F/B values from these “far” regions (17.7 ± 6.60) were significantly greater than the tumor bulk (t-test, $p < 0.0001$, data not shown) and tended to be slightly greater than the tumor-stroma interface (t-test, $p = 0.07$, data not shown).

F/B generated from user-defined thresholds and its relation to metastasis-free survival

Our previous discovery that F/B is an independent prognostic indicator of metastasis-free survival time was generated using a tissue microarray in which 1-mm diameter discs were available for each patient and minimal information on intratumoral heterogeneity was obtainable [47]. Armed with larger tissue sections and the realization that F/B significantly varies between different tumor regions, one naturally then asks if assessing F/B in certain regions can improve its prognostic ability. Based on the observed significant FIS differences between the tumor bulk and tumor-stroma interface, we assessed the prognostic ability of F/B measured in each of those tumor regions.

Patients were first listed in order from lowest to highest based on $\ln$ F/B (natural log of F/B) measured in tumor bulk and then divided into four equal groups based on this ordering. The first group (Quartile 1, or Q1) contained patients with the lowest F/B (corresponding to values < 2.11 , $n = 24$). Q2 consisted of patients with $\ln$ F/B from 2.11–2.35 ($n = 24$), Q3 with $\ln$ F/B from 2.35–2.56 ($n = 24$) and Q4 with the highest $\ln$ F/B

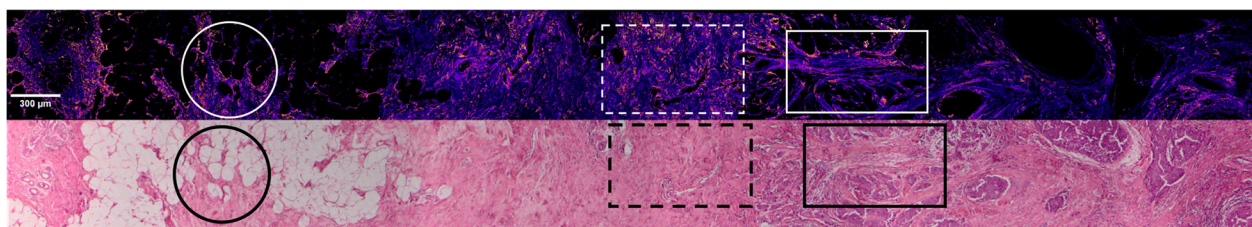


Fig. 1 Collagen F/B is heterogeneous within primary breast tumor tissue. SHG F/B images (a series of adjacent ROIs extending along the x -axis) and matching H&E images were stitched end-to-end to form a composite ROI for a representative sample. Examples of tumor bulk (solid box), tumor-stroma interface (dashed box), and uninvolved tissue (circle) are shown. The F/B values were plotted in a false color “heatmap” (low F/B values = dark blue, high F/B values = yellow) to illustrate differences in F/B within the tumor region including tumor bulk and tumor-stroma interface, indicating intratumoral heterogeneity in F/B

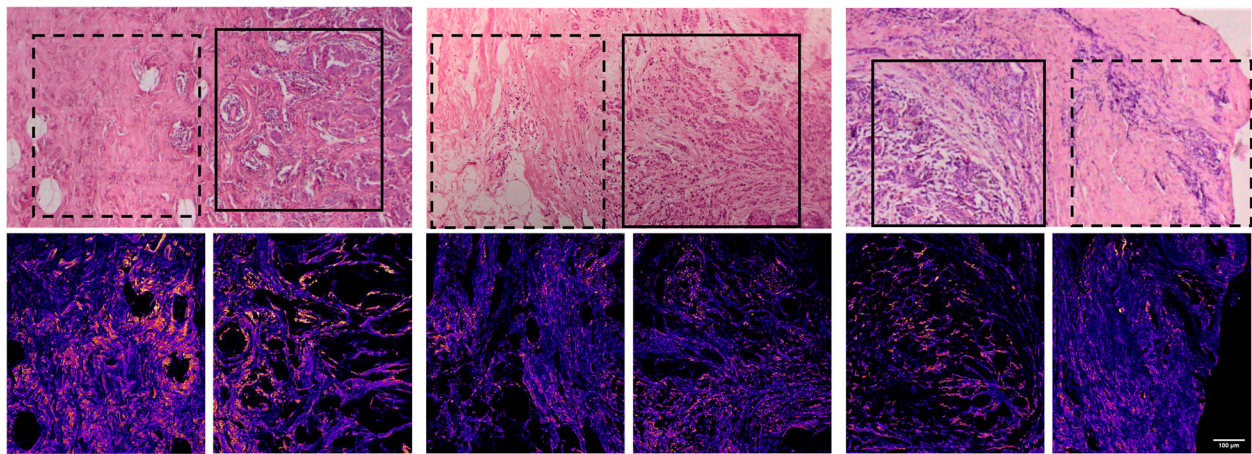


Fig. 2 Collagen features vary between the tumor bulk and the tumor-stroma interface of primary tumor excisions. Primary tumor excisions contain both tumor bulk (solid boxes) and tumor-stroma interface (dashed boxes). Tumor bulk consists of tumor cell clusters surrounded by individual SHG-producing fiber bundles. The tumor-stroma interface is comprised mainly of closely packed collagen fibers and individual stromal cells adjacent to the tumor bulk. Representative SHG F/B and matching H&E images from 3 individual patients are shown. The scale bar applies to all images in this figure

values, > 2.56 ($n = 23$). A Kaplan-Meier plot was then generated for the tumor bulk (Fig. 4). A second plot (Fig. 4) was generated for $\ln$ F/B derived from the tumor-stroma interface in a similar manner (Q1 $\ln$ F/B: < 2.43 , Q2 $\ln$ F/B: $2.43-2.69$, Q3 $\ln$ F/B: $2.69-2.97$, Q4 $\ln$ F/B: > 2.97 , $n = 23$ in each quartile). Tests for a linear association between $\ln$ F/B and the log-relative risk of MFS were first carried out by fitting separate Cox regression models relating MFS to each of these two measures, with corresponding partial likelihood ratio test p -values of 0.05 (tumor bulk) and 0.0008 (tumor-stroma interface). A Cox regression model including both measures was additionally fit to these same data and demonstrated no effect of $\ln$ F/B for tumor bulk ($p = 0.63$) when simultaneously accounting for $\ln$ F/B from the tumor-stroma interface ($p = 0.0046$). In all cases, the

regression models demonstrate an empirical trend of decreasing relative risk with increasing F/B, whether taken from tumor bulk or tumor host-interface. Taken in combination, these results suggest that F/B from the tumor-stroma interface has a stronger explanatory effect for MFS than does F/B from the tumor bulk.

F/B generated using histogram-based thresholding and its relation to metastasis-free survival

One possible reason for the different prognostic ability of F/B from tumor-stroma interface versus tumor bulk is that the two regions appear differently to the outcome-blinded observer who must choose intensity thresholds to select bright pixels within collagen fibers and reject dark pixels in background regions. For example, regions in the tumor bulk typically contain well-defined individual fiber bundles (solid boxes, Fig. 2) while regions in the tumor-stroma interface typically contain closely packed collagen fibers (dashed boxes, Fig. 2). The observer's selection of thresholds may be influenced by these differing image features, possibly affecting the resultant F/B and hence its prognostic ability.

To reduce this possible user bias in calculating F/B, we next generated masks for distinguishing collagen pixels from background pixels using two less subjective techniques. First, a histogram-based technique (Otsu's method) was used to separate pixels into foreground (collagen fibers) and background and generate a resulting binary mask. These masks were then applied to the background-subtracted F and B images and average F/B values were calculated as described above. We again found a significant difference between F/B calculated from tumor bulk versus tumor-stroma interface, indicating

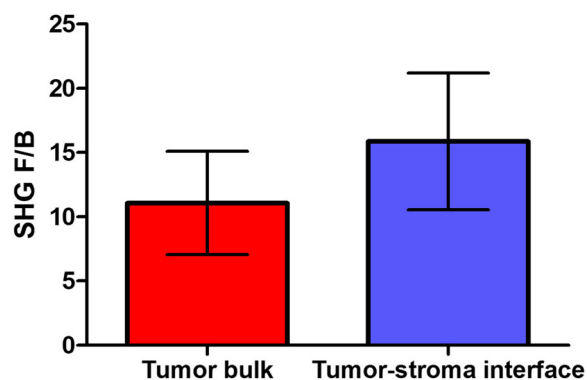


Fig. 3 F/B in tumor-stroma interface versus tumor bulk. Collagen fiber internal structure, as represented by F/B, is significantly different between tumor bulk and the tumor-stroma interface in IDC ER+ LNN excised primary tumors. Error bars = SD, t -test, $p < 0.0001$, $n = 92$

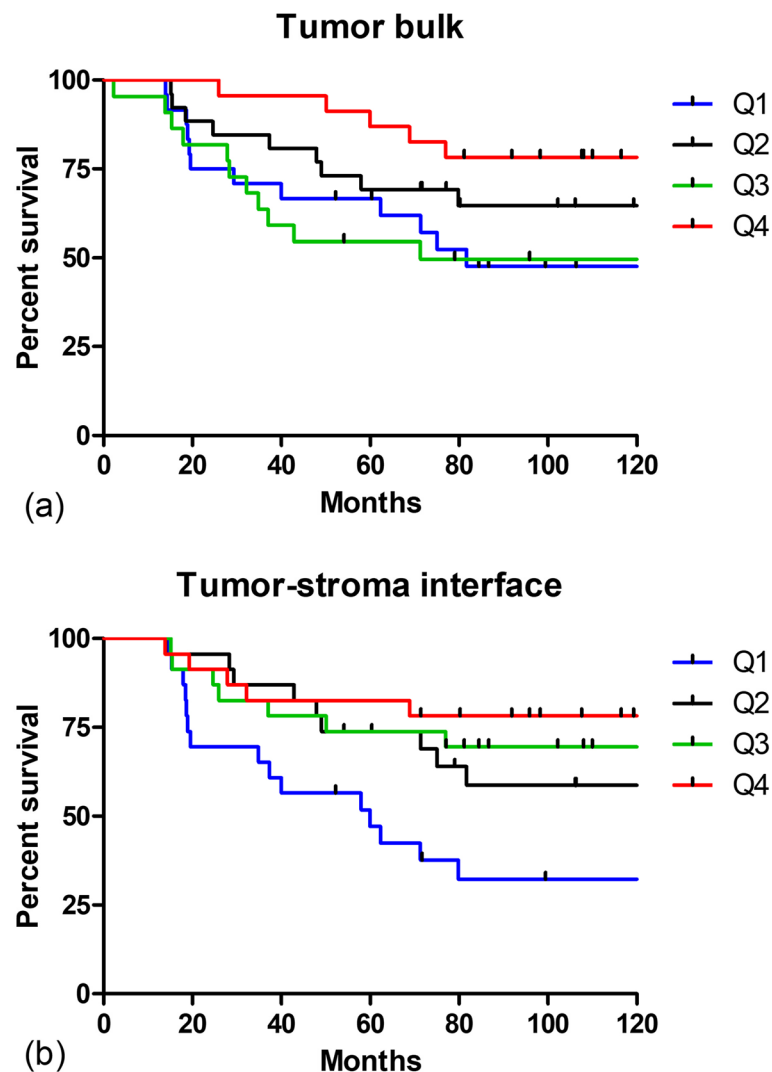


Fig. 4 F/B measured in tumor-stroma interface and bulk of primary tumor sections and relation to MFS. SHG F/B values were produced by a user-defined threshold for each individual image from the **a**, tumor bulk and **b**, tumor-stroma interface. Patients were split into four equal quartiles (Q1 = lowest F/B) based on F/B, and the percentage of each quartile surviving without metastasis then plotted versus time. Tick marks represent censoring events caused when a patient dies of a cause other than cancer or is lost to follow-up. Partial likelihood ratio tests for ln F/B: $p = 0.05$ (tumor bulk, $n = 95$) and $p = 0.00008$ (tumor-stroma interface, $n = 92$)

FIS heterogeneity between two regions of the tumor (t -test, $p < 0.0001$, $n = 92$). Patients were then divided into four quartiles based on ln F/B from each region (tumor bulk: Q1 ln F/B: < 2.73 , $n = 24$; Q2 ln F/B: $2.73-3.40$, $n = 24$; Q3 ln F/B: $3.40-3.75$, $n = 24$; Q4 ln F/B: > 3.75 , $n = 23$; and tumor-stroma interface: Q1 ln F/B: < 3.37 , Q2 ln F/B: $3.37-3.64$, Q3 ln F/B: $3.64-3.97$, Q4 ln F/B: > 3.97 , $n = 23$ in each quartile) and Kaplan-Meier plots were generated (Fig. 5). As before, tests for a linear association between ln F/B and the log-relative risk of MFS were carried out by fitting separate Cox regression models respectively relating MFS to F/B from tumor bulk ($p = 0.01$) and tumor-stroma interface ($p = 0.0009$). The Cox regression model including both measures demonstrated no effect of ln F/B for tumor bulk

($p = 0.50$) when simultaneously accounting for ln F/B from the tumor-stroma interface ($p = 0.0058$). In all cases, the regression models again demonstrate an empirical trend of decreasing relative risk with increasing F/B, whether taken from tumor bulk or host-interface. When taken in combination, these results continue to suggest that F/B from the tumor-stroma interface has a stronger explanatory effect.

F/B generated using adaptive thresholding and its relation to metastasis-free survival

While Otsu's method succeeds in reducing the influence that an observer has on the process of selecting pixels for analysis, it does not account for heterogeneity in intensity within images, which may be critical when

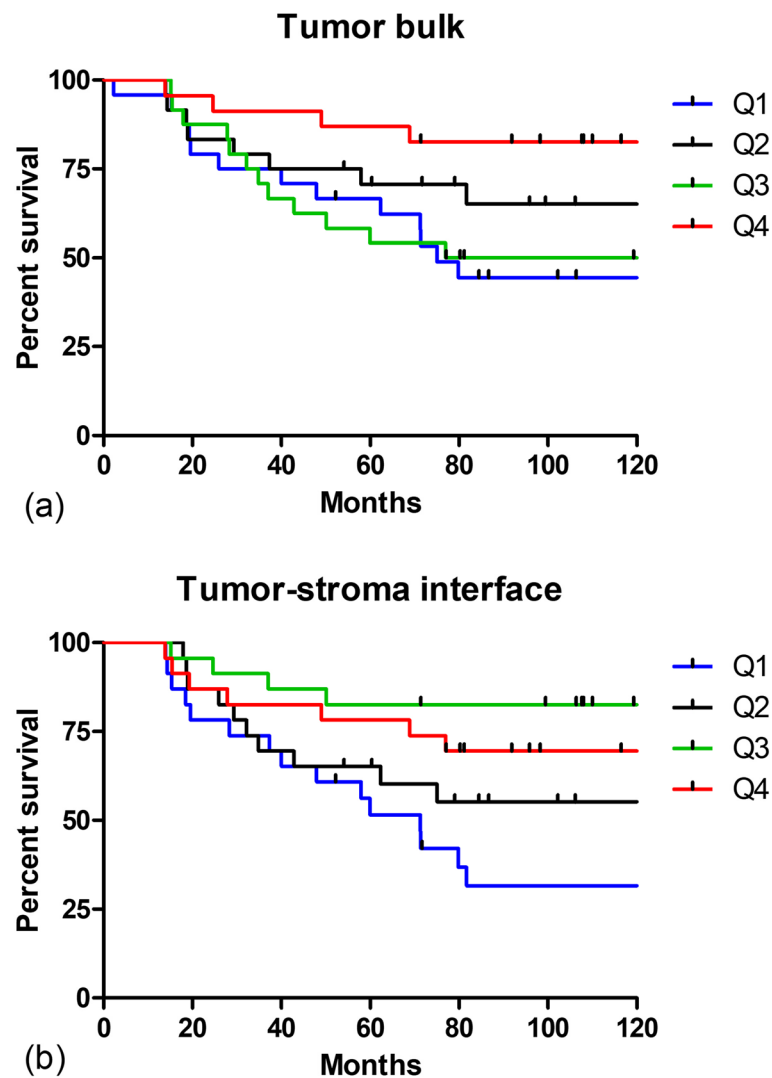


Fig. 5 F/B generated using histogram-based thresholding and its relation to MFS. SHG F/B values were produced using binary masks generated by a histogram-based thresholding method for each individual image taken in the **a**, tumor bulk and **b**, tumor-stroma interface. Patients were split into four equal quartiles (Q1 = lowest F/B) based on F/B, and the percentage of each quartile surviving without metastasis then plotted versus time. Tick marks represent censoring events caused when a patient dies of a cause other than cancer or is lost to follow-up. Partial likelihood ratio tests for $\ln$ F/B: $p = 0.01$ (tumor bulk, $n = 95$) and $p = 0.0009$ (tumor-stroma interface, $n = 92$)

assessing heterogeneity in tumor ECM. Therefore, we next used an adaptive thresholding method that compares each pixel to the average of a surrounding window to determine its contribution to the binary mask. We calculated F/B using these masks and again found a significant difference between average F/B in tumor bulk and tumor-stroma interface (t -test, $p < 0.0001$, $n = 92$). Patients were divided into four quartiles based on $\ln$ F/B from each region (tumor bulk: Q1 $\ln$ F/B: < 2.01 , $n = 24$; Q2 $\ln$ F/B: 2.01 – 2.39 , $n = 24$; Q3 $\ln$ F/B: 2.39 – 2.75 , $n = 24$; Q4 $\ln$ F/B: > 2.75 , $n = 23$; and tumor-stroma interface: Q1 $\ln$ F/B: < 2.68 , Q2 $\ln$ F/B: 2.68 – 2.93 , Q3 $\ln$ F/B: 2.93 – 3.17 , Q4 $\ln$ F/B: > 3.17 , $n = 23$ in each quartile) and

Kaplan-Meier plots were generated (Fig. 6). Tests for a linear association between $\ln$ F/B and the log-relative risk of MFS were again carried out by fitting separate Cox regression models respectively relating MFS to F/B from tumor bulk ($p = 0.4$) and tumor-stroma interface ($p = 0.002$). The Cox regression model including both measures demonstrated no effect of $\ln$ F/B for tumor bulk ($p = 0.9$) when simultaneously accounting for $\ln$ F/B from the tumor-stroma interface ($p = 0.0028$). The regression models demonstrate an empirical trend of decreasing relative risk with increasing F/B from the tumor host-interface. The combined results once again suggest that F/B from the tumor-stroma interface has a stronger explanatory effect.

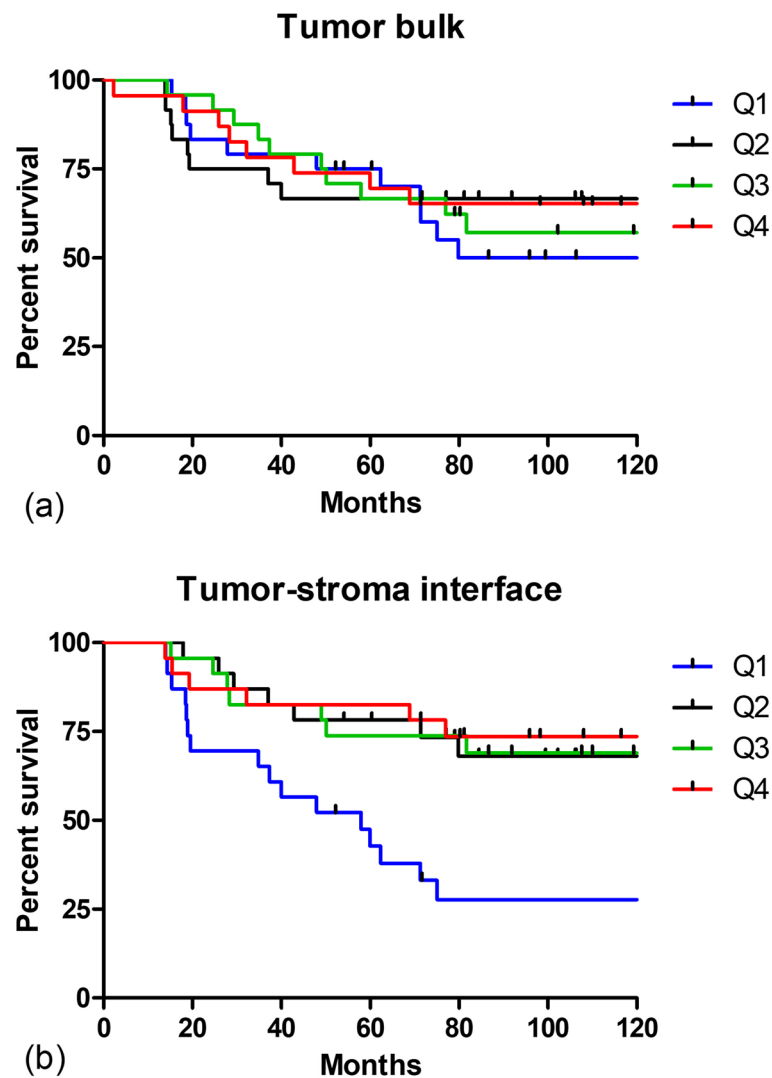


Fig. 6 F/B generated using adaptive thresholding and its relation to MFS. An adaptive thresholding method was used to create binary masks used to calculate SHG F/B in the **a**, tumor bulk and **b**, tumor-stroma interface. Patients were split into four equal quartiles (Q1 = lowest F/B) based on F/B, and the percentage of each quartile surviving without metastasis then plotted versus time. Tick marks represent censoring events caused when a patient dies of a cause other than cancer or is lost to follow-up. Partial likelihood ratio test $p = 0.4$ (tumor bulk, $n = 95$) and $p = 0.002$ (tumor-stroma interface, $n = 92$)

F/B and 21-gene recurrence scores

One possible clinical use of F/B measurements would be to identify people who are at risk for experiencing future metastases and are therefore candidates for adjuvant therapy, should be considered for clinical trials, etc. The standard of care for the IDC ER+ LNN patient cohort at our institution (University of Rochester Medical Center) uses the 21-gene recurrence score assay OncotypeDX®. Using the latest post-Trial Assigning Individualized Options for Treatment (TAILORx) criterion, patients with a recurrence score of 26 and above are recommended for adjuvant chemotherapy while patients with a score of below 26 are not [57]. In order to study F/B in the context of OncotypeDX®, we used the Recurrence Online tool that uses gene expression data

archived online in the GEO repository to calculate a surrogate 21-gene score (S-ODX) [52]. To evaluate the effect of heterogeneity on the ability of F/B to predict metastasis in conjunction with the S-ODX score, we utilized two related approaches to analyze these data: regression trees, and a Random Survival Forest (RSF) algorithm, which both derive a data-driven predictive regression model [50, 58–60]. In this case, both algorithms constructed a predictive model for MFS considering as input parameters the S-ODX score, F/B from tumor bulk calculated with the three image analysis methods, and F/B from tumor-stroma interface calculated with the three image analysis methods. The regression tree approach selected two of the input parameters (S-ODX and tumor-stroma interface F/B as calculated

using adaptive thresholding) and divided patients into 3 risk categories based upon those two inputs. Importantly, this algorithm does not use prespecified cut-points to derive risk groups; rather, the data are used to determine these groupings using an appropriate search algorithm and the results (Fig. 7) show, similar to earlier results (Figs. 4, 5 and 6), that the lowest 25% of the tumor-stroma interface F/B values identify a subgroup having the worst MFS experience. Furthermore, in the group having higher tumor-stroma interface F/B values (i.e., $\ln F/B \geq 2.675$) the S-ODX score subdivides this group into moderate (S-ODX ≥ 25.5) and low risk groups with respect to MFS. This regression tree approach does not deem tumor-stroma interface F/B calculated using the other two image analysis methods, nor any of the tumor bulk F/B measurements, as adding additional useful information in determining patient risk. The RSF algorithm does not produce a single tree; however, it produces measures of variable importance, and consistent with the regression tree approach, identifies F/B from the tumor-stroma interface calculated using adaptive thresholding and S-ODX as the two most influential predictors.

To determine if F/B can further stratify patient groups once their genomic score has been calculated, we next separated our patient cohort into two groups based upon their S-ODX score relative to the TAILORx cutoff (0–25

for low-intermediate and ≥ 26 for high-risk patients) [57]. We then generated Kaplan-Meier plots of $\ln F/B$ from tumor-stroma interface (calculated using adaptive thresholding) as described above (Fig. 8). Due to the low number of patients in each of the resulting graphs, we plotted the Q1 cohort versus the combined Q2-Q4 cohort to view the trends. F/B from tumor-stroma interface demonstrates prognostic ability in the S-ODX < 26 cohort, but not in the S-ODX ≥ 26 cohort (partial likelihood ratio test, $p = 0.008$ and $p = 0.4$, respectively).

Discussion

Here we assessed the heterogeneity in F/B within IDC ER+ LNN patients, and the impact of that heterogeneity on possible use of F/B as a prognostic marker. We found that the heterogeneity in F/B within an individual tumor is not entirely random, as F/B measured in the cellular tumor bulk was statistically significantly different from F/B measured in the collagenous tumor-stroma interface. This agrees with our previously published study of core needle biopsy sections taken from IDC patients before NACT administration and subsequent tumor excision [48]. This suggests that the biological relevance of the collagen in the two tumor regions may be different, that the FIS of one region may have a different impact

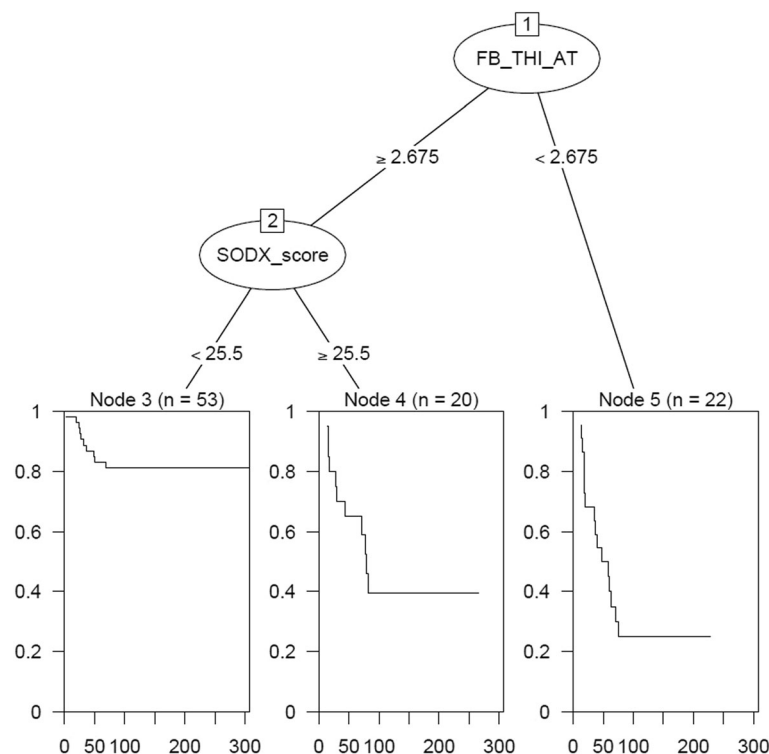


Fig. 7 Regression tree derived using the method of Leblanc & Crowley. When given the SODX score and all six methods of generating F/B as inputs, this algorithm selects F/B from the tumor-stroma interface, calculated using the adaptive thresholding method ("FB_THI_AT"), and SODX score ("SODX_score"), as predictors of MFS. The RSF method (results not shown) identifies the same two predictors as having the highest variable importance in predicting MFS

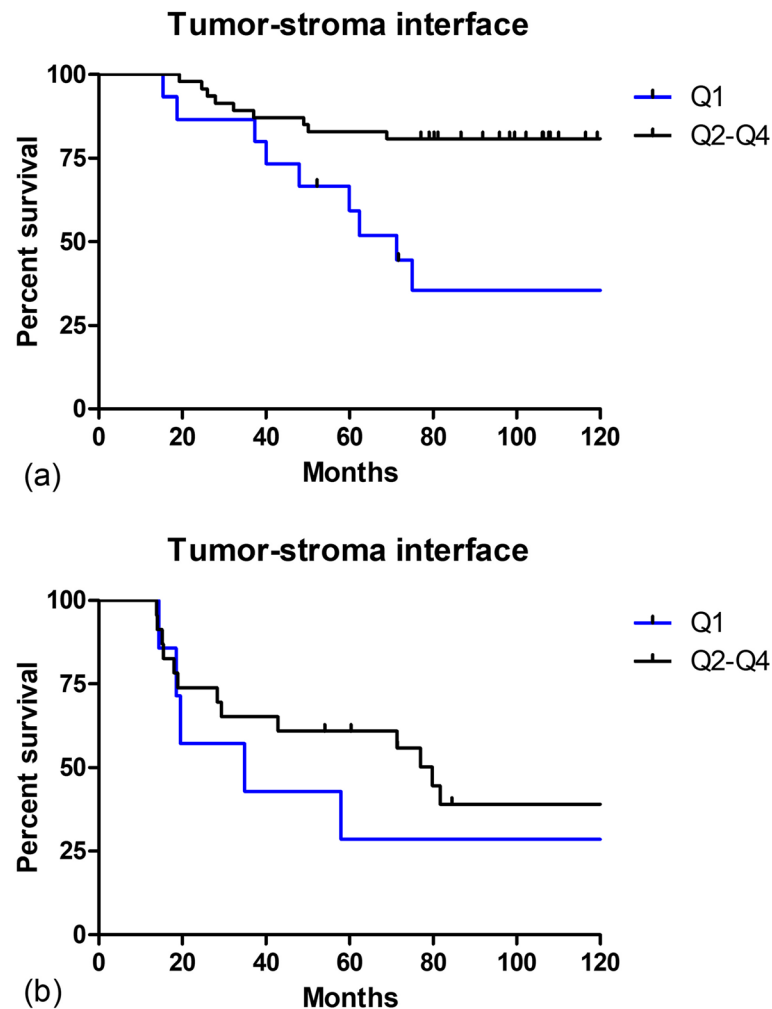


Fig. 8 F/B is prognostic of MFS in the tumor-stroma interface of primary excisions with S-ODX < 26. Patients were divided by S-ODX score and then each group was split into four equal quartiles (Q1 = lowest F/B) based on F/B. The three higher quartiles (Q2-Q4) were pooled, and the percentage of each quartile surviving without metastasis then plotted versus time for these two groups (Q1 and Q2-Q4). **a**, S-ODX < 26 and **b**, S-ODX ≥ 26. Tick marks represent censoring events caused when a patient dies of a cause other than cancer or is lost to follow-up. Partial likelihood ratio test **a** $p = 0.008$, $n = 62$, **b** $p = 0.4$, $n = 30$

on metastasis than the other, and that the prognostic ability of F/B may vary between the two regions. Therefore, we assessed the relationship between SHG F/B and MFS in both these regions and, using user-defined thresholds to select collagen pixels for analysis, observed that F/B derived from the tumor-stroma interface has a stronger explanatory effect than does F/B from the tumor bulk.

Numerous automated image analysis and deep learning techniques have been developed to study breast cancer progression and improve diagnoses [61–63]. We implemented two image processing techniques with the goal of reducing user involvement in generating collagen fiber masks. Histogram-based thresholding (Otsu's method) places pixels into foreground or background categories and finds the threshold between the groups that minimizes the

sum of their variances. This reduces user input but does not account for heterogeneity in intensity within individual images. Adaptive thresholding compares each pixel's value to the surrounding pixel average in a defined window. Because the window size is generally smaller than the image and is selected in an automated fashion for each individual image, this technique better preserves spatial variation and distinct contrasting features [55]. These methods also found the same relationships between F/B and metastatic outcome as F/B determined from a blinded user-defined threshold: for both methods, the combination of graphical analyses and Cox regression modeling found that F/B measured in the tumor-stroma interface had a stronger explanatory effect than F/B measured in the tumor bulk (Figs. 5, 6). This suggests that the difference in prognostic ability between the two regions is not due to the outcome-

blinded observer setting thresholds in a different manner when faced with the two differently appearing types of regions, and confirms the impact of heterogeneity in the prognostic ability of F/B. Interestingly, in all image analysis methods F/B from the tumor-stroma interface appears to be identifying a cohort of patients (Quartile 1) with poor outcomes relative to the rest of the studied population.

We are interested in evaluating the possible use of F/B as a predictor of post-excision metastatic outcome, where it may inform the clinical decision-making process as to the nature of post-excision therapy. To further probe the effects of heterogeneity on the use of F/B for metastasis prediction, we evaluated the contribution of F/B from tumor-stroma interface and from tumor bulk on a survival prediction model. We developed models using both the Leblanc-Crowley regression tree and Random Survival Forest algorithms. These methods consider all input predictors and attempt to find the best prediction model using (possibly only some of) these inputs. One product currently used to assist in these treatment decisions is OncotypeDX®, a 21-gene score that is part of the current standard of care at our institution when planning post-excision treatment and is a known predictor of MFS [64]. Therefore, as inputs we included F/B from the tumor-stroma interface (3 image analysis methods), F/B from the tumor bulk (3 image analysis methods), as well as a 21-gene score inferred from Affymetrix data that is an economical surrogate for OncotypeDX® score [52], or 7 total inputs for each patient. The resultant regression tree (Fig. 7) finds that none of the three values of F/B from tumor bulk contributes to classifying patients based on risk. However, F/B from tumor-stroma interface (derived using adaptive thresholding) is first selected to split patients into groups based on recurrence risk, followed by S-ODX. The result is three risk groups: 1) patients with $\ln F/B < 2.65$ (highest risk), 2) patients with $\ln F/B \geq 2.675$ and $S-ODX \geq 25.5$, and 3) patients with $\ln F/B \geq 2.675$ and $S-ODX < 25.5$. This reveals that F/B from the tumor-stroma interface and S-ODX contribute to classifying patients' metastatic risk, but that F/B from the tumor bulk does not, again demonstrating the effect of intratumoral heterogeneity on the possible use of F/B as a predictor of metastasis. Note that the selection of tumor-stroma interface F/B calculated using adaptive thresholding, and rejection of tumor-stroma interface F/B calculated using the other two methods, should not be interpreted as a strong endorsement of one image analysis method over the other, as the three are highly correlated (range 0.83–0.9) and hence are providing similar information. Interestingly, the regression tree split group 2 and 3 based upon an S-ODX value of 25.5, which is close to the TAILORx cutoff of 26.

Any tool to help predict metastasis and assist with treatment decisions is likely to be applied in combination

with the now well-established genomic scores. To further understand how F/B can support genomic methods for guiding treatment decisions we divided our patient samples into two cohorts based upon the value of their S-ODX score relative to the TAILORx cutoff of 26 (separating low-intermediate and high-risk groups). Because our regression tree determined that F/B from tumor-stroma interface, but not bulk, is necessary to classify patients based on metastatic risk, we generated Kaplan-Meier plots of F/B from tumor-stroma interface (calculated using adaptive thresholding as it was selected by the RSF algorithm) (Fig. 8). F/B from tumor-stroma interface demonstrates prognostic ability in the $S-ODX < 26$ cohort, but not in the $S-ODX \geq 26$ cohort (partial likelihood ratio test $p = 0.008$ and 0.4 , respectively). The $S-ODX < 26$ cohort represents patients in the genomic low- and intermediate-risk groups, patients who will likely not be recommended for adjuvant chemotherapy. The F/B value from tumor-stroma interface appears to identify a subgroup of these patients with poor clinical outcome (Fig. 8). This suggests that after assessment of patients with a 21 gene risk score, F/B from tumor-stroma interface may be useful in further stratifying patients with low or intermediate recurrence scores, therefore providing a tool to better identify patients in need of adjuvant treatment, enrollment in clinical trials, or more intensive monitoring.

Conclusions

Most breast cancer-related deaths are due to metastases. A tumor's metastatic ability is affected by the microenvironment, including the extracellular matrix. The ratio of forward-to-backward-scattered (F/B) second-harmonic generation (SHG) photons can be used to study collagen fiber internal structure and has been shown to be an independent prognostic indicator of metastasis-free survival in invasive ductal carcinoma patients. These results demonstrate that, within a cohort of 95 untreated ER+ LNN IDC patients, intratumor heterogeneity has a significant impact on the possible use of F/B as a tool to predict metastatic outcome. They also suggest that F/B specifically from the tumor-stroma interface of primary tumor excisions may provide information, independent of cell-based morphology or genomic methods, to further stratify patients by metastatic risk and identify those in need of post-operative treatment. This assessment can be performed on the FFPE H&E slides already within the clinical workflow but, naturally, this implies that the slides used to determine F/B therefore contain tumor-stroma interface. Due to the low number of patient samples ($n = 95$), these results should be considered exploratory and they provide the impetus for additional research to confirm/replicate these findings.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12885-020-07713-4>.

Additional file 1: Table S1. Primary tumor characteristics and associations with F/B. Primary tumor characteristics were measured and recorded after tumor resection, including progesterone (PgR) and human epidermal growth factor-2 (HER2) receptor expression, tumor stage, and tumor size in mm. Also shown is the association between these clinical variables and F/B from the tumor bulk and tumor-stroma interface produced using three analysis methods (individual thresholds, histogram-based thresholds, and adaptive thresholds) was assessed using Mann-Whitney tests (*p*-values listed).

Additional file 2: Figure S1. Full F/B heatmap and matching H&E for a representative primary tumor excision section. a) SHG F/B images (a series of adjacent ROIs extending along the x-axis) and b) matching H&E images were stitched end-to-end to form a composite ROI. These files are high definition versions of Fig. 1.

Abbreviations

B: Backward-propagating SHG; ECM: Extracellular matrix; ER: Estrogen receptor; F: Forward-propagating SHG; FFPE: Formalin-fixed paraffin-embedded; FIS: Fiber internal structure; FITC: Fluorescein isothiocyanate; F/B: Forward-to-backward scattering ratio; H&E: Hematoxylin & eosin; IDC: Invasive ductal carcinoma; LNN: Lymph node negative; MFS: Metastasis-free survival time; RSF: Random Survival Forest; NACT: Neoadjuvant chemotherapy; ROI: Region of interest; SHG: Second-harmonic generation; S-ODX: Surrogate OncotypeDX® score; TAILORx: Trial Assigning Individualized Options for Treatment

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Not Applicable.

Authors' contributions

Conceived and designed experiments: EBB, RLH, BMT. Performed experiments: DED. Analyzed data: DED, RLS, WW. Provided samples and patient data: MS, JWMM. Wrote manuscript: DED, EBB, RLS. All authors have read and approved the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request. Genomic data for the patients used here are available from the GEO repository (in the GSE2034 and GSE5327 datasets) while the resultant S-ODX score is available from http://www.recurrenceonline.com/?q=GSE_download.

Ethics approval and consent to participate

The studies on secondary use of archived tissues was approved in writing by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, Netherlands (MEC 02.953) and was performed in accordance to the Code of Conduct (The Code for Proper Secondary Use of Human Tissue) of the Federation of Medical Scientific Societies in The Netherlands.

Consent for publication

Not applicable.

Competing interests

RLH and EBB are inventors on patent #US10,765,376 related to the methods in this paper. All other authors declare they have no competing interests.

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