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TITLE: Understanding the Role of Matrix Microstructure in Metastasis

PRINCIPAL INVESTIGATOR: Edward Brown

RECIPIENT: University of Rochester
Rochester, NY 14642

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6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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U.S. Army Medical Research and Materiel Command
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13. SUPPLEMENTARY NOTES

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.

15. SUBJECT TERMS
Microscopy, metastasis

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Unclassified	Unclassified	Unclassified	Unclassified	17	**19b. TELEPHONE NUMBER** *(include area code)*

TABLE OF CONTENTS

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 first determine how that microstructure is defined by cells within the tumor, and the cues that influence those cells to do so. We hypothesize 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. 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.*

The Specific Aims of this three-year grant are as follows, and are unchanged from the original submission:

Aim 1: Determine how F/B is related to collagen properties in IDC ER+ LNN primary tumors.
- 1a. Evaluate F/B vs collagen I:III and I:V ratios in IDC tumor samples.
- 1b. Evaluate F/B vs collagen crosslink density in IDC tumor samples.

Aim 2: Determine what parameters influence F/B modification activity by the cells in a primary tumor.
- 2a. Alter collagen ratios (I;III and I:V) in collagen gels and quantify resultant F/B modification by tumor cells and by fibroblasts.

2b. Alter crosslink density in collagen gels and quantify resultant F/B modification by tumor cells and by fibroblasts.

2c. Test mechanisms by repeating 2a,b after inhibiting formation of new crosslinks, collagen synthesis, and MMP activity.

2d. Repeat 2a-c in decellularized tumor tissue.

Aim 3: Expand our ability to predict metastatic outcome in patient biopsy samples.

3a. Add quantification of collagen I:III and 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.

At the end of the first year of this three-year project we are well on track in pursuit of its goals. As this is a yearly report for a project with two Partnering PIs we will mark each accomplished task with the responsible PI, as required:

1. (Performed in Brown lab): Our first task was to apply for IRB approval for the various human sample work in the different Aims. We had never done this before therefore we chose to separate our IRB protocols, specifically first applying for the RSRB approval for the human samples work central to Aims 1 and 3, as it was based upon archival tissue and the process appeared less challenging. The human samples work in Aim 2 involved de-identified fresh tissue and we felt it would be more challenging for us to navigate the paperwork, so we did that second. We have filed for, and received, RSRB approval from both the University of Rochester and DoD IRBs for the work on archived human samples central to Aims 1 and 3:

7/11/2017: UR RSRB approval: RSRB00067440.

8/21/2017: Confirmation of Receipt of HRPO documents by DOD HRPO.

10/9/2017: UR RSRB approval of revisions in response to DOD review.

10/10/2017: Submission of DOD required revisions.

12/26/2017: DOD HRPO approval of Aims 1 & 3.

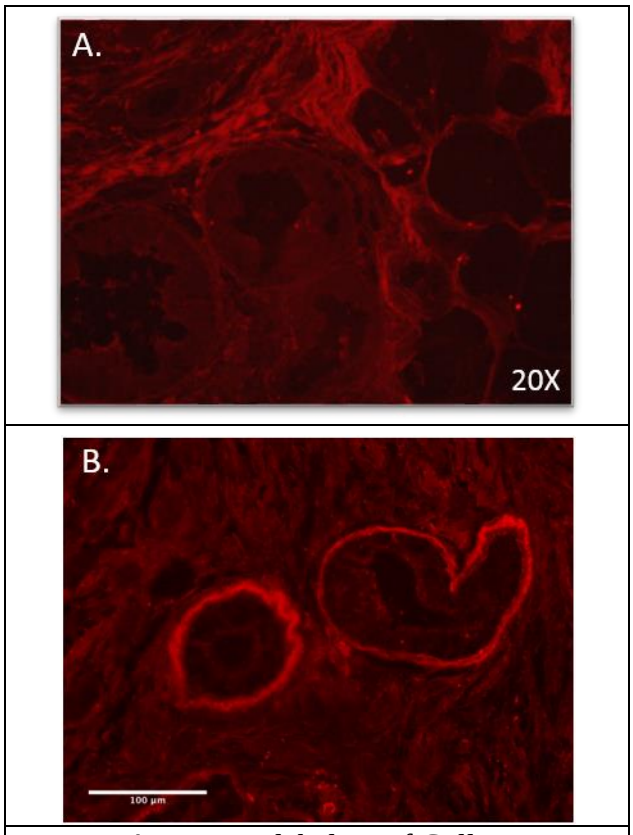

Figure 1A: Immunolabeling of Collagen I in archived FFPE human IDC samples. 1B: Immunolabeling of Collagen III in archived FFPE human IDC samples.

2. (Performed in Brown lab): We have made progress in Aim 1, specifically in immunofluorescence staining of collagen type I and III in archived formalin fixed paraffin embedded patient samples (FFPE). As shown in figure 1 after optimizing sample preparation conditions (especially deparaffinization and antigen retrieval) we have successfully imaged collagen I and III staining via fluorescence.

3. (Performed in Brown lab): We have also made progress in Aim 3, and thus far have successfully accrued 64 IDC breast cancer cases with associated 10 year recurrence data and clinical data from patient records, all curated by our collaborating pathologist Dr. Bradley Turner. This was done by developing an IRB-approved workflow for acquiring blocks using approved personnel, passing them and associated pathology records to our collaborating pathologist, as well as query of electronic records by approved/trained personnel for outcome data. Preparation of slides from archived blocks and verification of pathology, clinical, and outcome data produces a single curated and de-identified "case" for subsequent optical imaging and statistical analysis. We must now continue using this workflow to acquire the additional 171 cases to reach our target n=235.

4. (Performed in Brown lab): Based upon lessons learned during our first RSRB experience we have filed for RSRB approval from the U. of Rochester for the de-identified fresh tissue work of Aim 2 (2/20/2018) and are awaiting their response. This application requests discarded tissue from IDC ER+ patients from our collaborator Dr. Rachel Farkas' surgical practice and requires coordination with the Rochester Cancer Library, an initiative at the University of Rochester to collect discarded tumor tissue. For this approval, a letter of memorandum was written detailing our coordination with Rochester Cancer Library (RCL). For our study when discarded tissue < 2 cm, all tissue is ours, while when discarded tissue > 2 cm it will be shared with the RCL.

5. (Performed in Kuo lab): We have also made progress in other parts of Aim 2. Aims 2a, 2b, and 2c require the synthesis of mixed collagen/polyacrylamide gels enabling gel elastic modulus to be controlled independently of collagen microstructure. In figure 2 we see the result of development work, demonstrating that we are able to make mixed collagen/PA gels with different elastic modulii. Note that the atomic force microscope-based modulus measurement is pivotal to much of Aim 2.

6. (Performed in Kuo lab): Aim 2c, 2d, 1b, and 3a will require the imaging of crosslink density (hydroxylysyl pyridinoline (HP); lysyl pyridonoline (LP)) using 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 3 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.

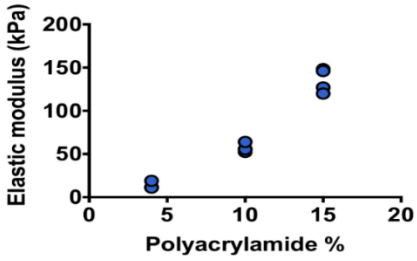

Figure 2. Polyacrylamide (PA) gels functionalized with collagen type I were made with different formulations and tested with atomic force microscopy (AFM) to obtain elastic modulus. A 5 μm radius spherical tip probe was used with an indentation rate of 6 μm/s. Results for n=3 show each formulation consistently yields PA gels with specific mechanical properties.

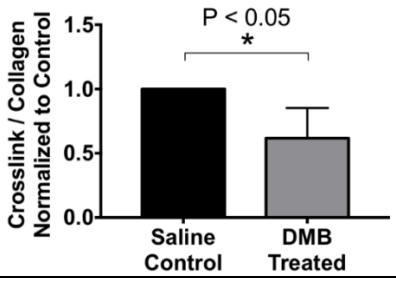

Figure 3. To optimize imaging methods to evaluate lysyl oxidase (LOX)-mediated collagen crosslinks of tissues, we imaged embryonic chick tendons using methods we previously reported with the same tissues (Marturano et al., 2014 Acta Biomaterialia). Based on our preliminary experiments in an un-related project, decamethonium treatment (DMB) of the tendons reduces LOX-mediated crosslinking of tissues. Here, we show that the fluorescently detected crosslinks in tendon were indeed reduced after DMB treatment as compared to vehicle control.

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.

We expect to finish Aim 1 and determine how F/B is related to collagen properties in IDC tumors. We expect to finish part of Aims 2a-c and gain insight into tumor cell modulation of microstructure in mixed collagen/PA gels. We plan to perform this work simultaneously with Aim 2d (answering these questions in decellularized tissue) since availability of fresh tissue will be sporadic and *in vitro* work will progress when fresh tissue is unavailable. We also expect to finish sample acquisition for Aim 3.

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).

Nothing to Report

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.*

Nothing to Report.

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
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report.

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.

Nothing to Report.

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).*

  Nothing to Report.

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.*

Nothing to Report.

- **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.

Nothing to Report.

- **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 has 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 has provided general oversight in all aspects of data generation and analysis with particular focus on mixed gel generation/analysis and crosslink density analysis.

Continued on Next Page:

Name:	Danielle Desa
Project Role:	Graduate Student
eraCommons	N/A
Nearest person month worked:	12
Contribution to project:	Ms. Desa has assisted with IRB protocol generation as well as immunolabeling and imaging, and creation of a curated human samples dataset.

Name:	Sabrina Pan
Project Role:	Graduate Student
eraCommons	N/A
Nearest person month worked	9
Contribution to project:	Ms. Pan has been developing and optimizing 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 been collaborating 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 and creation of a curated human samples dataset.

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.

Dr. Ping Tang, who was originally listed as a collaborator on the grant at 5% effort, left our institution. In consultation with Amanda Carrera USAMRAA Grants Management Specialist amanda.c.carrera.civ@mail.mil I replaced her with Dr. Bradley Turner. Both are practicing breast cancer pathologists, as required for 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.*

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

n/a

9. APPENDICES: n/a