Author's response to reviews

Title: Collagen reorganization at the tumor-stromal interface facilitates local invasion

Authors:

- Paolo P Provenzano (paolo.provenzano@gmail.com)
- Kevin W Eliceiri (eliceiri@wisc.edu)
- Jay M Campbell (jmcampbe@wisc.edu)
- David R Inman (drinman@wisc.edu)
- John G White (jwhite1@wisc.edu)
- Patricia J Keely (pkeely@wisc.edu)

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Author's response to reviews:

Dear Editor,

We are re-submitting our revised manuscript entitled "Collagen reorganization at the tumor-stromal interface facilitates local invasion" by Paolo P. Provenzano, Kevin W. Eliceiri, Jay M. Campbell, David R. Inman, John G. White, Patricia J. Keely for possible publication as an article in BMC Cancer. We have carefully addressed each of the reviewers' concerns and submit a point-by-point response to each of the reviewers' comments. Additionally, major changes to the text of the manuscript are highlighted with red font.

Overall, we believe that this manuscript is improved and contains novel biological information and utilizes innovative nonlinear imaging modalities that will be of great interest to researchers and clinicians in fields ranging from oncology and pathology to developmental and cancer cell biology to biophysics and engineering. Therefore, this manuscript is of broad interest to cancer researchers and as such will be valuable to the diverse readership of BMC Cancer.

I thank you for your time and look forward to hearing from you regarding our manuscript.

Best regards,

Paolo Provenzano and Patricia Keely

Collagen reorganization at the tumor-stromal interface facilitates local invasion
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Authors: Paolo P. Provenzano, Kevin W. Eliceiri, Jay M. Campbell, David R. Inman, John G. White, Patricia J. Keely

Response to reviewer #1: Dr. Guy Cox

We thank the reviewer for his suggestions and comment that "this is an excellent paper" that requires only minor revisions. We feel that our revised manuscript is significantly improved and addresses all of the reviewer's comments. A point by point response to the reviewer's comments is included below and changes to the text indicated with red font.

Minor Revisions:

1) "...The mathematical formulae and descriptions for MPE and SHG on page 5 seem to me to be superfluous...the only point the reader really needs to know...equivalent to confocal imaging in optical sectioning"

We agree that an in depth description of the theory behind MPE and SHG are not absolutely required for interpretation of the results and are in fact described in detail in the cited references. However, we have
found that although many biomedical scientists/readers do not fully understand the mathematical formulations, their presentation does help overcome the common misconception that SHG is a fluorescent event. By examining the formulas it is readily apparent that there is a fundamental differences between fluorescent events (such as MPE) and SHG that does not require a great deal of mathematical background to identify. As such we have included the formulas in the Methods section to help clarify the difference between these two phenomena.

Additionally, we have added the following text to page 3 of the manuscript for additional clarity: 
"...corresponding one-photon (half wavelength of 2PE) excitation [13, 18, 19]. In this 2PE process the fluorescence is dependent upon the square of the intensity (see Materials and Methods section), producing optical sectioning that makes it equivalent to confocal imaging in terms of restricting excitation to the plane of focus, but facilitates a much greater effective imaging depth and better cell viability ([16, 20]. SHG imaging, on the other hand...").

2) "On page 8 collagen structures shown in Figure 2...It would be helpful if these could be annotated on the micrographs"

We have added annotations and/or made changes to the figure text to further highlight key features on the micrographs. For instance we have altered the manuscript text and figure text for Figure 2 to indicate wavy vs. "straight" fibers and that radiating fibers are at the end of the duct (new text: ",...as well as radiating out from the duct (as can be seen predominantly at the top of the micrograph near the end of the duct")

3) "...more numerical data would be welcome..."

The analysis of collagen density has been expanded to include actual numerical results as well as additional information provided in the Methods section. The following text "...collagen was locally dense adjacent to (wrapped around) the epithelium as well as increased in the space extending from the duct resulting in a 2.53 +/- 0.24 and 2.79 +/- 0.34 (mean +/- SEM) fold increase in the area of collagen signal, as well as increased signal intensity around the duct, for heterozygous and homozygous col1a1tmJae mice relative to wild-type animals, respectively. Therefore, the col1a1 mouse model appears to be a promising candidate for studying the effects of increased collagen density..." has been added to page 9 of the Results section. Additionally the text "...Image analysis for combined 2PE-SHG was performed with ImageJ [29] and VisBio [30] software. Using ImageJ, differences in collagen density were quantified by measuring the area of collagen signal following density slicing from a constant threshold, and local and mean intensity were measured within these normalized areas. For TACS-1..." has been added to the Methods section for increased clarity.

4) "The last sentence of the Results section..."

This sentence has been edited to read: "Hence, our results suggest that in order to facilitate local invasion, cells at the tumor boundary contract and align collagen fibers, perhaps with the assistance of proteolytic cleavage to facilitate matrix reorganization, and then invade along aligned collagen structure to expand the tumor and later metastasize" to be more clear.

Response to reviewer #2: Thomas Dittmar

We thank the reviewer for his suggestions and comments. We feel that our revised manuscript is significantly improved and addresses all of the reviewer's comments. A point by point response to the reviewer's comments is included below and changes to the text indicated with red font.

3) Are the data sound and well controlled?

"Figures 1-3": Bars should appear in black"

We have changed the color of the scale bar.

"Bar is missing in Fig. 1A, 2Ba..."

We have added scale bars to these figures as is relevant.

"Can the authors explain why some data are presented as grayscale images whereas other data are shown
in color?"

The authors chose to present the images in the mode that allows the easiest visual interpretation of each piece of data.

"The major point of criticism is that due to SHG imaging only reflection images (as described by Hegerfeldt et al....are shown, thus making it extremely difficult to detect tumor tissue and ever single tumor cells...the tumor cells must be visualized...or by detecting NADP(H)"

Second harmonic generation (SHG) is a polarization phenomena occurring after multiphoton excitation that depends on the structure of the material and is not equivalent to the confocal reflectance microscopy technique utilized by Hegerfeldt. Although some similar information regarding collagen structure can be obtained with both techniques, they are fundamentally different. In the case herein the SHG signal arising from collagen is maximal with two-photon excitation at 890-900 nm and the "emission" wavelength is exactly half of the excitation wavelength (i.e. energy conserved). Fortunately, this is the excitation maximum for FAD allowing endogenous imaging of cellular autofluorescence from the cytoplasm (dark round structures are the nucleus surrounded by a "ring" of FAD signal from the cytoplasm). Additionally since the SHG signal is conserved (445-450 nm in this case) while the FAD emission follows fundamental decay behavior with higher wavelength (lower energy) emission (peak at 530 nm), the emission signals can be differentiated after simultaneous excitation. Although the reviewer is concerned that imaging of collagen structure by SHG does not allow simultaneous imaging of tumor cells in proximity, in fact the authors are able to image tumor cells when combined MPE/SHG is utilized. This approach is analogous to the reviewer's suggestion to image NADH in the cells to produce and endogenous signal from live cells. Using FAD emission to detect cells, tumor cells that have been identified that have invaded into the stroma and examples of this are annotated on Figures 5 and 6. In particular, Figure 5F is a rather clear example of an individual tumor cell attached to a collagen fiber.

Included in this comment, the reviewer suggests that we perform immunofluorescent staining to image tumor cells, but to do so would negate the power of this approach, as part of the appeal is the fact that we are imaging fresh, unfixed tissue using endogenous signals.

"Figure 4: The tumor-stromal boundary is missing in Fig. 4Bb and Fig 4Cb, Cc"

The reviewer is correct that the signal from SHG is stronger than that from endogenous cellular autofluorescence (see previous comment), however the tumor cells can be visualized in these figures. The authors encourage the reviewer to magnify the digital figures and possibly alter the brightness and contrast of their system (which will be an available option due to the online nature of open access) and examine the boundary at this magnification and brightness. The boundary is present with cells intermingling with the stromal boundary. We chose not to draw the tumor-stromal boundary in all of the panels, as we felt the line obscures some of the image.

"Figure 5: The authors should use a different color to visualize..."

The authors agree that highlighting boundaries and features with yellow works very well in Figure 4. However when the authors attempted to utilize yellow in Figure 5 it did not show well. This is because Figure 5 is much brighter than Figure 4. As such, using yellow in Figure 5 does not show up well due to the essentially white background. Hence, the authors tried a number of colors and found that red shows the best for this figure.

"Especially higher magnification images (Fig. 5E, 5F) should be confirmed by appropriate in-vitro data...Thus visualization of tumor cells is necessary

As we interpret the reviewer's comment, he is concerned that it is difficult to see if the cells are truly in contact with the collagen fibers (i.e., bound to the fibers). However, only biochemical data would demonstrate a receptor/ligand sort of interaction. There is a wealth of literature demonstrating that indeed such interaction occurs between specific integrin and proteoglycan receptors on mammary tumor cells and collagen (for example: Keely et al, Journal of Cell Science, 1995). Moreover, functional data confirms that mammary tumor cells migrate directly along collagen fibers (Wang et al, Cancer Research 2002). Consistent with these published findings, the elongated, adherent phenotype of the cell along the collagen fiber is strong indication that the cells are interacting with the fibers. In this case, immunostaining would do no better to demonstrate such an interaction. As discussed above the FAD signal arises in the cytoplasm resulting in dark spots (nuclei) and bright spots (signals from the cytoplasm) with some cells brighter than others. Hence visualization of tumor cells is possible and presented for live tissue.
To further address the functional interaction between tumor cells and the collagen fibers, we had already included in vitro data, as presented in Fig. 7, in which tumor explants are cultured in a randomly organized collagen matrix. These tumor cells migrate out from the explant along collagen fibers. Furthermore, in this tumor explant system the matrix has been reorganized by the tumor cells directly indicating the expected physical connection between the cells and stroma is present.

"All scale bars are missing in Fig. 5"

Scale bars have been added to each panel of the image.

"Figure 6: same criticism as for Fig. 5...tumor cells (visualization) is pretty difficult. Tumor cells must be visualized. The tumor stromal boundary should be marked...higher magnification...marked by a rectangle...scale bars are missing"

As discussed above, we are examining endogenous fluorescence to view tumor cells and their stromal interaction in their native environment. Although SHG produces a very strong signal, tumor cells are visible. The tumor stromal boundary was not marked nor rectangles drawn since in this case such markings mask key features in the images. Instead, these key features, such as the state of the tumor-stromal boundary, are more thoroughly annotated and discussed in the figure text. In regards to rectangles to identify regions of magnification, our trial to include such rectangles rendered panel 5A a confusing mess, so we did not make this change. However, since the structures are rather apparent, identifying the magnified region in the original image is not difficult. The addition of scale bars to each panel should assist the reader in interpreting the scale.

"Figure 7: In Fig. 7C and 7D the interactions between tumor cells and collagen fibers should be analyzed by staining the beta-1 integrin expression of the tumor cells."

This is not a trivial experiment and is in fact quite difficult in this system. For instance, staining in 3D is not straightforward, no antibody against beta-1 integrin exists that stains mouse tissue well, and fixation interferes with the collagen SHG signal. Moreover, staining for beta-1 does not provide a significant addition to the major points of manuscript and is therefore outside the scope of this manuscript. In the case of what we are demonstrating with respect to matrix reorganization, staining beta-1 integrin is not required to indicate a cell-matrix interaction is present. The in vitro data in Figure 7 clearly demonstrates that the cells themselves reorganize a random matrix, indicating that a cell-matrix interaction exists.

Minor Concern "Page 8...Fig. 4...must be...Fig. 2..."

Yes, thank you for identifying this error. The error has been corrected.

Minor Concern "Page 11...Which group of cells do the authors mean..."

This refers to regions were TACS-3 is noted as indicted by the text "This invasive tumor morphology was seen in regions of tumors where collagen fibers are primarily aligned in the direction of cell invasion (see histogram in 4C, in which the angle of the collagen fibers relative to the tumor boundary distributes around 90degrees). Furthermore, at regions where TACS-3 is noted...".

Minor Concern "Page 12...invading cells are difficult to see...authors indicate some matrix disorganization was present but did not show appropriate result."

The ability to see invading cells is discussed above, and is demonstrated in Figures 5, 6, and 7. In regards to matrix disorganization, it is clear from examining images such as 5E that the collagenous stroma is not well organized and grouped in well arranged collagen fibers associated with normal conditions. We have added the following text to the Results section "In certain cases, some matrix disorganization was also observable (see Fig. 4Cc and 5E) possibly indicating proteolytic cleavage of collagen [40, 43]".

Discussion: The authors thank the reviewer for his statement that the Discussion in well balanced and well written.

In regards to using TACS-1 as a standard hallmark for locating small tumors, and the reviewer comments that it is like looking for a needle in haystack. We were able to identify small tumors this way, however we have removed the word "diagnostic" from the statement "Importantly, the presence of increased was diagnostic...". In regards to the needle in haystack analogy, the reviewer is correct, it is like a needle in haystack, which is exactly why hallmarks such as TACS-1 that produce a very strong signal could provide
so much utility in locating abnormal cellular structures and as such may provide a useful tool, along with the other TACS, for clinical pathology someday. Moreover, these techniques may prove to be feasible in humans. Multiphoton fiber optic probes have already been reported and research in this area is rapidly growing.

"Conclusions should be revised. The authors should not go into detail, which mechanism might be responsible for matrix organization. Several pathway are known to induce and maintain cell migration...additionally, these factors...induce...MMPs, which are crucial for reorganization of the extracellular matrix."

The authors agree with the reviewer that a number of mechanisms are at play in tumor cell invasion and migration. The authors' statements regarding GTPase contributions are not meant to rule out these other mechanisms. In fact, quite the opposite is true. Our contention is that GTPases play a role amongst the other mechanisms such as growth factors, integrin signaling, MMP secretion and activity. The concluding statements have been edited and now read "matrix reorganization (possibly assisted by proteolytic cleavage [40, 43] to release collagen fibers) to help facilitate local invasion. This matrix reorganization would require enhanced contractility and motility of the tumor cells, which may explain the increased presence of Rho and ROCK, in invasive cancers (see [53] and references therein). Although a number of mechanisms, such as growth factor and integrin signaling and protease secretion and activity, are associated with invasion and metastasis it seems likely that GTPase regulated motility events are also involved these processes. Hence, the mechanisms behind local invasion may include matrix reorganization through GTPase mediated tumor cell contractility (authors unpublished observations), leading to an aligned matrix that facilitates local invasion." to better convey the authors' interpretations of the data and literature and how GTPases may play a role along with the more well studied mechanisms such as EGF signaling.

Response to reviewer #3: Claudia Binder

We thank the reviewer for recognizing the difficulty in studying the tumor-stromal interaction both in vivo and in vitro and the promise that in vivo imaging may provide. We feel that our revised manuscript is significantly improved and addresses all of the reviewer's comments. A point by point response to the reviewer's comments is included below and changes to the text indicated with red font.

Major Compulsory Revisions

1) "The experimental conditions have to clarified. The authors make a strong point that the investigations have been performed in live tissue. So supposedly, the experiments have been done with live animals...".

Thank you for identifying a key point that requires more clarity in the manuscript. The tumors are in fact live tissues but imaging is not being performed intravitally. The tumors are freshly harvested live tissues that are maintained during imaging of fresh, unfixed, non-sectioned tissues. We have made multiple changes to the text to clarify this point. Additionally, the Abstract now reads "Imaging of the tumor-stromal interface in live tumor tissue ex vivo..." and we have included a section to the Methods section that reads "For live tissue imaging with multiphoton microscopy, mammary tissues and tumors were harvested and live tissue maintained in buffered media at 37degreesC. All tissues were imaged immediately following tissue harvest. Following live cell imaging, all tissues and tumors were fixed in formalin and tumors confirmed by histology".

2) "...the authors come to rather far-reaching conclusions regarding the diagnostic utility of these methods (e.g. Abstract), it would be necessary to present the respective evidence in the form of a larger series of carcinomas investigated in comparison with benign glands in all states of development...Thus the investigation numbers should be clearly indicated in "Methods" and conclusions restricted to the proven findings."

Although we respectfully disagree with the reviewer regarding the diagnostic utility of MPE and SHG, as well as other nonlinear optical imaging approaches, for clinical pathology of live tissue biopsies as well as the great potential associated with MPE, SHG, and FLIM through fiber optic probes, the authors' have removed the word "diagnostic" from the abstract. Additionally, although the Polyoma and Wnt models do not initially induce tumorigenesis in the exact same manner as most human breast cancers, their well-characterized phenotypes are very transferable to human cancer. As such they provide an excellent model to understand tumor growth, invasion, and metastasis.
Furthermore, we have added specific details to the Methods section regarding the number of animals used in the study. The following text has been added:

"For MPE and SHG imaging of live unfixed, intact (not sectioned), non-stained glands, and tumor explants within collagen gels, as well as hematoxylin and eosin stained histology slides we used an optical workstation [27] that was constructed around a Nikon Eclipse TE300. For live tissue imaging, twenty mammary tissues including nine from the Col1a1tmJae strain (3 wt, 3 heterozygous, 3 homozygous), and tumors from Wnt-1 (n = 10, plus wild-type controls) and PyVT (n = 20) mice were harvested and live tissue maintained in buffered media at 37 degrees C. All tissues were imaged immediately following tissue harvest and a Ti:sapphire laser (Spectra-Physics-Millennium/Tsunami) excitation source producing around 100fs pulse widths and tuned to 890-900nm was utilized to generate both multiphoton..."

"For histology, formalin-fixed paraffin-embedded samples from eight B6129SF2/J mice and eight Col1a1tmJae mice were sectioned and stained for hematoxylin and eosin, trichrome, and picrosirius red using standard techniques. Additionally, all tissues imaged with MPLSM were subsequently fixed and processed for histology to confirm the presence of tumors and characterize the tumor morphology. Sample preparation for scanning and transmission electron microscopy (SEM and TEM, respectively) was performed by fixing whole mammary glands in 2.5% formaldehyde/2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for 1hr at room temperature (RT), after which sample were placed in fresh fixative overnight at 4 degrees C. Samples were then washed in cacodylate buffer and postfixed in 1.5% osmium tetroxide at RT for 1.5hrs. Samples (SEM n = 8 glands and TEM n = 6 glands) were again washed..."

Minor Revisions

1) "From most of the figure legends it not clear which kind of mouse had been investigated..."

We have added details to the figure text and/or Methods to specify which mouse strain was investigated in each figure.

2) "Spelling errors"

These have corrected

3) "PyVT should be PyMT"

We are utilizing the abbreviation PyVT to conform to the Jackson Laboratory abbreviation since the animals were originally obtained from that source. As such, the PyVT abbreviation allows the reader to locate the strain and its relevant information via the original distributor. Furthermore, this abbreviation is not uncommon although both PyMT and PyV-MT are also commonly used. Therefore, we have added the following text "...and MMTV-polyoma middle-T (abbreviated PyVT following the Jackson Laboratory title but is also commonly abbreviated as PyMT or PyV-MT; colony founder mice originally obtained from Jackson Laboratory were provided..." has been added to the Methods section for increased clarity.

4) "The histological findings should be specified according to the usual classification of breast malignancies..."

We have added the following text "Analysis of tumor bearing Wnt-1 mice, which progress through hyperplasia, mammary adenocarcinoma, and invasive (metastatic) ductal carcinoma, revealed multiple epithelial clusters..." and "...we utilized the well established and characterized PyVT mouse model. This model bears resemblance to many aspects of human cancer, is reliably invasive and metastatic following defined, progressive, and reliable histological grades from hyperplasia to adenoma, and then to early and late carcinoma, and therefore provides a good model for studying human disease" to help put the data in context.

5) "high localization probably meaning high density?"

Yes, thank you. We have corrected with error.

6) "Results contain a lot of interpretations..."unpublished observations" should be omitted if these are not own observations..."

We have edited the manuscript to eliminate any non-essential discussion. Additionally the "unpublished observations" are the authors'. We have changed the text to read "authors' unpublished observations" and
the suggested reference from the Journal of Clinical Investigation is cited and discussed in the manuscript reporting density-related tumor data in detail.

7) "In the conclusion, the statements on tumor cell contractility.....are speculative and not underlined by the presented experiments."

We have added the note "authors' unpublished observations" associated with a portion of this conclusion as we have data sets in hand that are continuing to grow but are beyond the scope of the current manuscript and therefore are not included herein.