Reviewer's report

Title: Comparison of mammary gland imaging techniques and applications: reflectance confocal microscopy, GFP Imaging, and ultrasound

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Reviewer: James C Lacefield

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General

This manuscript qualitatively compares three imaging technologies with respect to their capabilities for depicting the morphology of mammary glands, lymph nodes, and tumors in mouse models of breast cancer. A whole organ culture experiment demonstrates that reflectance confocal microscopy, including treatment with a 1% acetic acid solution for contrast enhancement, does not affect the viability of the mammary gland. Whole organ culture, transplantation, and surgical recovery experiments demonstrate that expression of green fluorescent protein and surgical exposure for in-vivo fluorescence microscopy does not alter mammary gland development. High-frequency ultrasound is shown to be effective for non-invasive detection of small preneoplastic lesions.

The manuscript provides a good visual presentation of potential applications of the three imaging techniques in mouse cancer models. The observations about the features that were, and were not, well depicted by each technique are consistent with similar studies showing other applications of the methods. The demonstration that each technique is non-destructive and so can readily be incorporated into longitudinal studies is the most significant contribution of the manuscript.

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Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

[Page 8]: Explain why the mouse used for the metastasis assay was not sacrificed until one month after ultrasound imaging. This approach seems to reduce the confirmatory value of the necropsy, since the tumor burden was likely to have been much different when the ultrasound data were acquired.

[Figure 7(g)]: One would expect to be able to identify necrotic regions in a tumor using ultrasound as the tissue liquefies into a homogeneous mass, but this example is unconvincing. The depth of field of the 55-MHz VisualSonic transducer extends only about 0.7 mm above and below the 4.5-mm focal depth, so the top half of that tumor is out of focus, which probably explains a great deal of the apparent variation in image brightness. Unless the necrotic region can actually be outlined in the ultrasound image, Figs. 7(g)-(h) should be omitted
from the paper.

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Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

[Page 3, 2nd paragraph]: A word, perhaps "In", is missing from the beginning of the first sentence.

[Page 4, 2nd paragraph]: Strictly speaking, confocal microscopy should not be termed "non-invasive" since the limited penetration depth (a few hundred microns) prevents imaging through the skin. Preferable terms are "minimally invasive", if imaging of biopsy specimens is contemplated, or "non-destructive" in the context of in-vivo imaging with surgical exposure.

[Page 6, 2nd paragraph]: In the last sentence, clarify that a greater acoustic impedance mismatch yields greater reflected pressure magnitude or intensity. The current wording of the sentence can be misinterpreted as implying a greater impedance mismatch produces an increased number of reflected waves.

[Page 6, 3rd paragraph]: "Doppler" is a proper noun.

[Page 12, 1st paragraph]: Specify that sizes, or cross-sectional areas, of the lesions were measured. Were images specifically acquired in the plane showing the largest cross-sectional area of each lesion?

[Page 12, 2nd paragraph]: The objective of the transplantation experiment should be stated, since its purpose is not clear in the current presentation until the reader reaches the Results section.

[Figure 6(c) legend]: The attribution of the hypoechoic appearance of the HANs to "denser tissue" requires clarification. This statement is only accurate if it is interpreted as referring to cell number density, as opposed to mass density, such that those tissue regions are homogeneous on the scale of the ultrasound wavelength (e.g., a low mass density, homogeneous inclusion would also be hypoechoic). Confusion of some readers is especially likely since the relationship between mass density and acoustic impedance was mentioned on p. 6. The wording used in the legend of Fig. 7(l) is preferable.

[Figure 7(a)-(c)]: Outline the lesions using a dashed rather than a solid line. Precisely identifying the tumour boundary is the most challenging aspect of measuring tumor growth, and the solid lines prevent the reader from assessing how clearly the lesion margins are visualized in ultrasound images.

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Discretionary Revisions (which the author can choose to ignore)

[Title]: Consider specifying that the manuscript addresses preclinical or murine mammary gland imaging. The in-vivo microscopy techniques requiring surgical
exposures are unlikely to be used routinely in humans, and many of the observations about ultrasonography are dependent upon the high resolution provided by the small-animal scanner.

[Figure 6(d)]: Fitting a straight line to a comparison of area data to volume data is an unwieldy way to present the lesion size results. Estimating lesion volumes from the 2-D ultrasound images, either by measuring major and minor axes diameters and assuming the lesion is ellipsoidal or by numerically integrating the volume enclosed when a 2-D lesion boundary is rotated about an axis, would enable an "apples-to-apples" comparison. Also, logarithmic axes would allow the data from the small lesions to be shown more clearly, and it is worth mentioning (perhaps in the Conclusions) that 3-D ultrasound imaging can be performed if direct measurements of lesion volume are desired.

**What next?:** Unable to decide on acceptance or rejection until the authors have responded to the major compulsory revisions

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

I acknowledge in-kind research support from VisualSonics Inc., the manufacturer of the ultrasound imaging system used in the studies reported in this manuscript.