

Designing Ultrasound Image Analysis Techniques for DC101-treated Tumors in Mice

A Technical Report submitted to the Department of Biomedical Engineering

Presented to the Faculty of the School of Engineering and Applied Science
University of Virginia • Charlottesville, Virginia

In Partial Fulfillment of the Requirements for the Degree
Bachelor of Science, School of Engineering

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Spring, 2022

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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Abstract

Breast cancer is one of the most common cancers in women with more than 40,000 annual estimated deaths in the United States alone. Current treatment options such as surgery and radiation pose potentially extensive side effects. The use of focused ultrasound and lipid-shelled microbubbles in combination with anti-angiogenic drugs is a potential solution to difficult patient treatment processes. We developed a MATLAB program to analyze microbubble perfusion in ultrasound images of 4T1 breast cancer tumors in mice. To do this, tumor-bearing mice were anesthetized and held in an ultrasound imaging tank with the tumor submerged in the water next to an imaging transducer. A video was taken of the tumor on a singular plane to gather images over time. This program provides output for the fractional perfusion area. Additionally, we built a 3D modeling system that can produce three-dimensional images of tumors using cross-sectional ultrasound images of tumors. Using the same imaging setup as before, images were taken at separate planes 0.5mm apart. These image stacks were transformed into point clouds on ImageJ and finally converted to 3D models in ParaView. The two programs we developed can be used by researchers to gather more information about both microbubble perfusion and tumor vasculature normalization. Eventually, this will lead to more efficient chemotherapeutic drug delivery because less drug dosage will be needed. This means breast cancer treatment would be cheaper and reduce side effects and complications for patients.

Keywords: 4T1 breast cancer, DC101, anti-angiogenesis

Introduction

Breast cancer is the second leading cause of cancer-related deaths in women with over 43,000 estimated deaths for 2022, and is also the second most common cancer in women, making up 15% of all new cancer cases¹. Over the course of their lifetimes, approximately 12% of women in the United States will be diagnosed with breast cancer². Although innovative treatments and technologies have vastly improved survival rates throughout the years, these methods come with extensive side effects and complications.

Current treatments for breast cancer include chemotherapy, surgery, and hormonal therapy. Chemotherapy is a form of treatment that can be provided preceding tumor removal surgery as a method of reducing the size of the tumor by either killing cells that proliferate rapidly or preventing them from proliferating³. The chemotherapeutic drugs are ingested or injected and then travel through the blood and access the tumor through the tumor's vasculature, its system of blood vessels.

A method being developed to increase the effectiveness of chemotherapy is using an anti-angiogenic drug which will help normalize the irregularly formed blood vessels characteristic of tumors, which is caused by the unregulated blood vessel growth of cancer cells, specifically the vascular endothelial cells⁴. Unregulated vascular endothelial cell proliferation in tumor cells is in part associated with the high levels of vascular endothelial

growth factor (VEGF) signaling⁵. Activation of the VEGF signaling pathway promotes endothelial cell growth, increases vascular permeability, promotes degradation of vascular basement membranes resulting in increased cell migration, and inhibits signaling for cell apoptosis. DC101 is an antibody specific to VEGF-receptor-2 and serves as an anti-angiogenic drug that inhibits the VEGF signaling pathway⁶. Normalizing the tumor vasculature can make the transport of drugs through the blood more effective by increasing blood flow, limit the amount of off-site toxicity caused by leaky vessel structure, and decrease the chances for the cancer to metastasize due to cell migration⁴.

Imaging of tumors is also conducted through the use of lipid-based microbubbles which can noninvasively travel throughout the body. These microbubbles can be used as a contrast agent which allows imaging of a targeted tissue to be enhanced by changing the way that ultrasound waves pass through the body to a specific frequency which causes the agent to appear as a signal under the ultrasound⁷. Due to this characteristic of noninvasive travel throughout the body, lipid-based microbubbles have emerged as a possibility to be used for local chemotherapeutic drug delivery. Once microbubbles are injected with drugs and antibodies that allow them to target specific cells, they can be tracked with ultrasound as mentioned before. The frequency of the emitted ultrasound can be altered to burst the microbubbles and deliver its contents.

Current research on the effects of anti-angiogenic drugs such as DC101 on vascular normalization is mainly conducted through analyzing histological data which is primarily used to determine the size of endothelial cells as a measure of the effects of the anti-angiogenic treatment. However, they are unable to provide a direct view into the structure of the vasculature of the tumor in vivo, leaving room for further analysis using different methods.

The objective of this research project is to develop alternative methods to breast cancer research. This will be done by developing an image analysis software in MATLAB that will collect data from ultrasound images and output the fractional area of a tumor that is accessible to perfusion to help measure the effects of anti-angiogenic drugs on vascular normalization. Furthermore, a 3D modeling procedure will be developed whereby cross-sectional images of the tumor's vasculature can be taken and modeled to provide a clearer physical view of the tumor vasculature in a holistic fashion.

Materials and Methods

Inoculation and Imaging

25 BALB/c mice were used to collect data that could be run through the MATLAB program, as well as to generate 3D models of the breast cancer tumors. All 25 mice were used for the MATLAB program, with 6 of these also being used to acquire 3D model data. Within the two categories, the mice were also evenly split into groups of those treated with DC101 and those treated with the control IgG.

The mice were all injected with 4 hundred thousand 4T1 breast cancer cells which were allowed to grow over a period of 2 weeks. During the 3 weeks, the mice were given 10 mg of either DC101 or IgG per kg of mouse mass every 3 days starting at 7 days after inoculation with imaging being done on the 14th day.

After the growth period, the mice were imaged using an ultrasound system made by the Price Lab that can hold the subject submerged in a water tank next to a transducer, allowing for stable imaging of the mice. Separate imaging techniques were used depending on whether the images were going to be used for the 3D model or the MATLAB program. For both techniques, imaging would begin shortly after the microbubbles were injected into the tail vein of the mouse. For the procedure for imaging for the 3D modeling, cross-sectional images were taken beginning at one end of the tumor and then subsequent images would be taken moving through the tumor at 0.5 mm intervals until the

other end of the tumor was reached. For the MATLAB program imaging, images of the tumor were taken at 15 Hz beginning after a short high-frequency ultrasound pulse that would burst all the bubbles inside the tumor. This allowed the images to track the progression of microbubbles as they reperused through the tumor vasculature. Images would be taken over the course of 1 minute, resulting in approximately 2500 images in total.

Image Analysis Software

The MATLAB code must be directed to the folder storing the series of images to be analyzed. Once the directory is found, the program will prompt the user to manually highlight the region of the image that represents the tumor vasculature. The code will then run on the designated region and determine the baseline signal intensity from the ultrasound images that is to be considered significant enough to count as microbubble signaling when calculating the fractional perfusion area of the tumor. After determining the signal intensity for the images, the program will show a graph of the video intensity over time and ask the user to determine in which frame microbubbles began to reperuse through the tumor after the bursting pulse was used. The program will then prompt the user to select either an exponential or linear fit for the fractional perfusion plot based on the shape of the curve which must be approximated by the user. This information will be used to output the fractional perfusion area versus the image number. The graph will also contain lines in red representing the average fractional perfusion area with a solid line, standard deviations with dashed lines, and the start frame for reperfusion in a vertical dashed line. The minimum signal strength considered significant based on the video intensity is represented as a solid black line, with horizontal dashed lines providing the standard deviations and the vertical dashed line representing the start frame. Using this graph, data can be collected on each tumor's relative perfusion area.

3D Modeling System

For the 3D modeling system, cross-sectional ultrasound image stacks were first imported into ImageJ and converted to grayscale. The images were then thresholded to clearly distinguish between microbubble signaling and surrounding tissue. A plugin called *Export Point Cloud* was then used to convert the thresholded images into a point cloud. Point clouds are a collection of individual points plotted in 3D space. The settings were adjusted so the output file was in a format that could be acceptable by the open-source 3D modeling program called ParaView. This program accepted the point cloud files and used the data to generate a 3D

model of the tumor vasculature. For our visualization purposes, we used the Point Gaussian representation with enlarged spheres representing each pixel of the thresholded images. The entire z-axis was scaled to approximately 5.5 mm to approximate the average size of the tumors before rendering out the model for viewing.

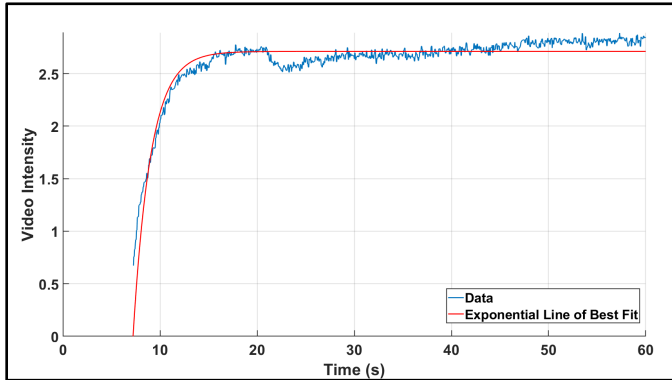


Fig. 1. Video intensity serves as a baseline for the signal intensity that is considered significant in calculating the fractional perfusion area.

Results

MATLAB Imaging

Ultrasound images of the inoculated mice were first examined using the MATLAB imaging analysis software. Data could not be properly collected for 2 of the mice and, as such, only 23 were used for this analysis. Examples of the fractional perfusion graph output are shown in Figure 2 and Figure 3. As was expected, the DC101-treated mouse showed a relatively higher fractional perfusion area compared to the IgG-treated mouse as is shown in Figure 4. This is consistent with our expectations as the anti-angiogenic drug DC101 would have normalized the tumor vasculature and allowed for more microbubble perfusion when compared to the IgG control.

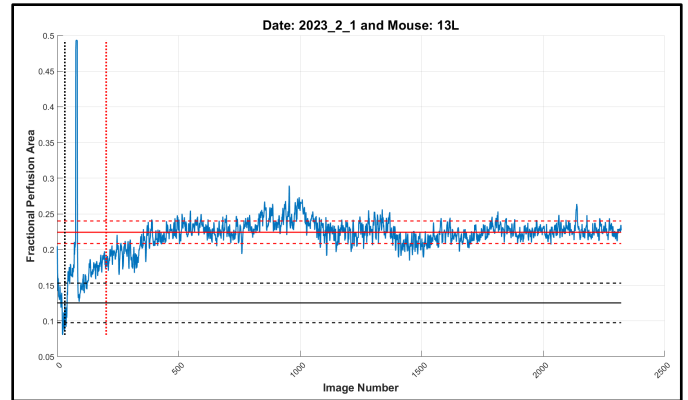


Fig. 2. Mouse 13L, which was treated with DC101, showed a relatively higher fractional perfusion area of 0.227.

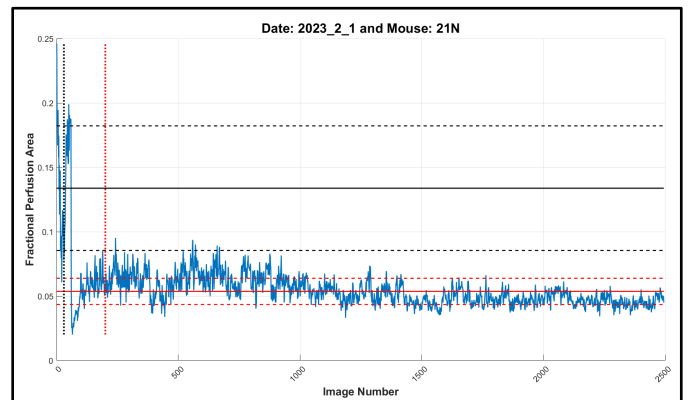


Fig. 3. Mouse 21N, which was treated with IgG, showed a relatively lower fractional perfusion area of around .052.

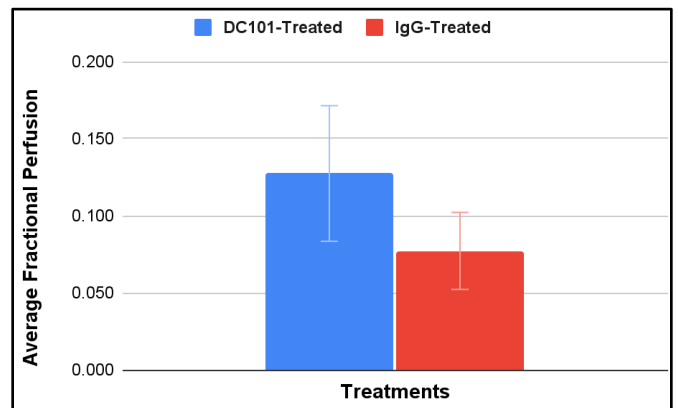


Fig 4. Bar Graph representing the average fractional

perfusion areas for the two conditions with corresponding error bars.

Furthermore, these findings are reinforced when looking at the compiled data from all 23 inoculated mice as seen in Table 1 and Table 2, which shows an average higher fractional perfusion area for mice treated with DC101.

Mice	Fractional Perfusion
1N	0.166
2R	0.122
8L	0.109
10RR	0.089
11N	0.158
12R	0.104
13L	0.227
17R	0.102
20RR	0.171
22R	0.081
24B	0.115
25RR	0.086
AVG	0.127

Table 1. Fractional perfusion area for DC101 treated mice along with average.

Mice	Fractional Perfusion
3L	0.124
5RR	0.106
7R	0.051
9B	0.069
14B	0.105
15RR	0.058
16N	0.065
18L	0.066
19B	0.061
21N	0.052
23L	0.092
AVG	0.077

Table 2. Fractional perfusion area for IgG treated mice along with average.

Running a one-tailed two-sample T-test on this data, checking for the DC101 data set average being significantly greater than the IgG dataset average, returned a p-value of 0.0016. This demonstrates a statistically significant difference between the two averages, at a confidence level of $\alpha = 0.05$.

3D Modeling

Subsequently, cross-sectional ultrasound image stacks of 6 mice were used to develop 3D models of the tumor vasculature. An isometric view of one such model is shown in Figure 5. A clear distinction was seen in the perfusion volume between DC101 and IgG-treated mice, though it may be difficult to clearly see this in the isometric view. Unfortunately, a more quantitative analysis of the two

conditions was unable to be performed at this time and further methods will need to be developed in order to do so.



Fig. 5. 3D model of tumor vasculature for Mouse 3L, which was treated with IgG.

Discussion

Conclusions

The MATLAB imaging analysis software was able to effectively take a range of ultrasound images over time to provide valuable information about both video intensity and fractional perfusion area. Video intensity is important because it provides a baseline for where the signal is considered significant enough to calculate fractional perfusion area. The fractional perfusion area displays the amount of tumor that is accessible to microbubbles, which represents where unobstructed blood flow can occur.

Given the potential uses of microbubbles and anti-angiogenic drugs in the treatment of breast cancer tumors, this image analysis technique will allow researchers to understand how microbubble perfusion will occur in tumors. Eventually, this knowledge can be used to optimize how drugs are delivered. If healthcare providers can more accurately deliver chemotherapeutic drugs to tumors, they would need less dosage overall. This would make chemotherapy cheaper since they would purchase less drugs. Additionally, this would be able to reduce the side effects and complications that are affiliated with extended cancer treatments.

The 3D modeling system used cross-sectional ultrasound imaging to create 3D models of tumor vasculature. This information provides researchers with a more visual description of the structure of a breast cancer tumor. When used with the MATLAB imaging analysis software, this offers a more comprehensive understanding of how the tumor can be potentially treated.

Limitations

Throughout the imaging process, there were issues that may have limited the validity of the gathered data. Overall, the quality of the ultrasound images were poor compared to previous batches of images taken by the Price Lab. The poor quality made it more difficult for the MATLAB imaging analysis software to reliably determine where there was microbubble signaling in images and where there wasn't. This means the DC101 treated samples produced somewhat lower fractional perfusion areas than expected. Tied to this, an error occurred in the output of the graphs whenever the average fractional perfusion area was too low, resulting in the displayed minimum signal intensity to appear above the average fractional perfusion area. This does not appear to affect the actual calculation of the average perfusion area, but only appears when printing the plot, requiring adjustments to how the data is being displayed to correct this issue.

Next, some images were unintentionally taken at the wrong plane that the tumor was not at. This led to inconsistent spikes in the signaling, which translated to skewed data in some groups. This issue was somewhat mediated by manually removing images that were taken on the wrong plane, but this solution is not ideal as it also results in the loss of small amounts of data in the process.

Furthermore, in some of the ultrasound images, there were also artifacts that may have been produced by either technical imaging errors or natural interactions between ultrasound and the mice. These may have produced images that are inaccurate and falsely represent the tumors, and some of the artifacts were likely interpreted by the program as microbubble signaling which would also serve as a source of error.

For the 3D modeling system, the z-axis was scaled to be about 5.5 mm in accordance with the method that was used to gather the images. However, this value is still only an approximation. Although the 3D tumor models are accurate in representing the tumors' vasculatures, they are not fully to scale.

Future Steps

Moving forward, the MATLAB imaging analysis software could incorporate other parameters that provide additional information about microbubble perfusion. One of these potential parameters is microbubble velocity. Gathering quantitative information about how rapidly microbubbles can travel to and throughout a tumor would indicate the rates at which blood is flowing in different tumors. Depending on the type of tumor and drug, this could be optimized to make treatments much more effective.

There are also changes to explore in our 3D modeling system that could create more detailed and accurate 3D models. When using ParaView to generate 3D models from point clouds, there are a variety of complex options and variables that can be utilized in the software. While we were able to produce accurate 3D models, it is very likely that if we were able to optimize the variables we could have produced more accurate models. Lastly, it would be valuable to determine the fractional perfusion area in the 3D modeling system as well. This could be used to verify the fractional perfusion area values determined from the MATLAB imaging analysis system.

End Matter

Author Contributions and Notes

The initial MATLAB imaging analysis software was developed by Andrew Thim at the Price Lab which was then built upon in this research project.

The authors declare no conflict of interest.

Acknowledgments

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