

Detection and characterisation of three-dimensional specularly reflecting objects such as microbubbles through tissue using photometric stereo and other re-flectance microscopy techniques

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Scanning tunneling microscopy (STM) is the premier technique for atomic-scale imaging. Through preclinical and clinical studies, Acoustic Cluster Therapy (ACT) has demonstrated success in enhancing chemotherapy efficacy while reducing drug toxicity, compared to conventional passive transvascular drug delivery.[1][2][3] ACT consists of negatively charged microbubbles (Sonazoid™) and positively charged microdroplets, which form clusters via electrostatic interaction. These clusters, along with the administered drug, are activated by ultrasound to temporarily lodge in tumor capillaries, improving local drug delivery. However, the biomechanical mechanisms underlying ACT-induced enhancement of drug extravasation remain unexplored.

This study demonstrated a proof-of-concept imaging framework for detecting microbubbles and activated Acoustic Cluster Therapy (ACT) bubbles in tissue using reflectance microscopy. A customised imaging system enabled photometric stereo imaging. Optical clearing with 109.1 mM tartrazine (67 h, 8 rpm) improved light penetration and allows ability to observe the internal structure of the tissue and Sonazoid™ using confocal microscopy, while a 590 nm longpass filter provided subtle enhancement of bubble edge clarity. A multi-adaptive hough transform (mAHT) algorithm was developed for bubble detection. These approaches validated the combined use of photometric stereo, optical clearing, light filtering and algorithmic analysis. This integrated platform provides a foundation for future organ-on-a-chip studies aimed at understanding the biomechanical mechanisms of ACT-mediated drug delivery.

What was the aim of your project?

In this project, I aimed to carry out a proof-of-concept study to assess the potential of three methods (photometric stereo, optical clearing

with tartrazine and light filtering) and algorithms to improve ability to distinguish activated ACT microbubbles from surrounding tissue for eventual use in studying underlying biomechanical mechanisms of ACT.

How did you address the aim?

I tried three experimental methods along with imaging analysis to evaluate the potential of activated ACT bubbles detection through tissue.

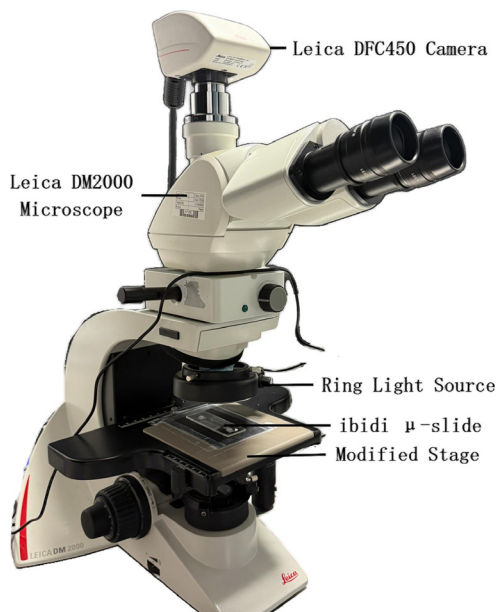


Figure 1. The basic setup of the microscope with main components labeled.

Instead of traditional transmission microscopy, reflectance microscopy was implemented with a modified imaging system (shown in Figure 1)

Experiments

Sonazoid™ was reconstituted with a microdroplet emulsion of perfluoromethylcyclopentane (PFMCP, F2 Chemicals Ltd., UK) to form PS101.

PS101 was then introduced into μ-Slide 1 Luer™ and activated with MI0.5 and 5MHz ultrasound energy.

Photometric Stereo was achieved by incorporating a ring light source, which through idealised ray diagram, shown as a reflected ring pattern from the specular bubbles.

Tartrazine of 109.1mM concentration was applied to vibratome cut chicken breast slices for optical

clearing. The tissue slices were soaked in the solution for 67h at room temperature on rocker at 8 rpm.

A longpass filter with cutoff wavelength at 590nm was selected considering its dimensions and availability. The analyser blanker of the microscope was modified by 3D printing a perfect-fit holder to accommodate the 25mm diameter unmounted 590nm longpass filter.

Algorithms

RGB images were converted to grayscale for automated channel extraction.

Intensity profiles along the y-axis were obtained for filtering effectiveness evaluation. mAHT featured with normal-guided voting was applied to identified circle centers and radii.

What did you find out?

Methods Validation

The combination of Photometric stereo and optical clearing results is promising with the ability to identify activated ACT bubbles within the ibidi μ-slide channel.

Bubbles at different depths of the channel were detected. The experiment was conducted with a 1000 μm thick tissue slice, which was compressed to 370 μm by the coverslip. However, the edges of the activated ACT bubbles were less well-defined compared with images obtained from thinner tissue slices.

Further combination of light filtering is discussed in the Algorithm section.

Confocal Microscopy

Confocal Images were obtained using Zeiss LSM980/ Airyscan 2 Confocal Microscope. As shown in Figure 3, tartrazine-treated tissue slices exhibited enhanced visualisation of internal structures and stronger reflective signals corresponding to Sonazoid™ microbubbles (bright and clustered red dots). These results indicate that optical clearing

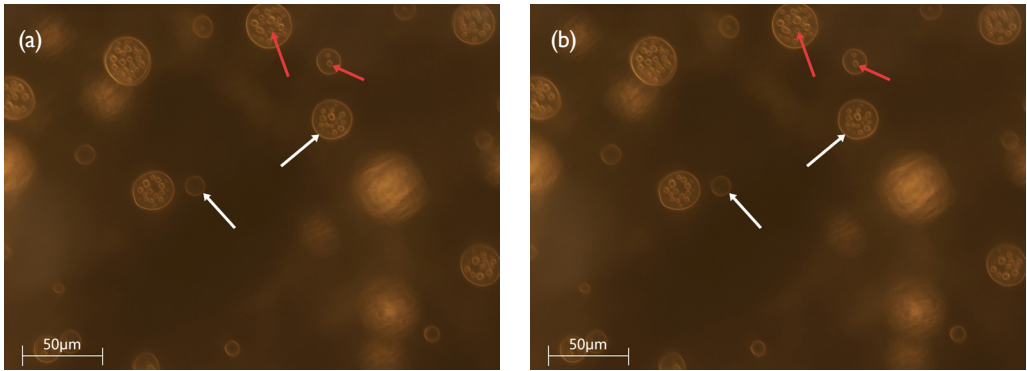


Figure 2. Images of identified activated ACT microbubbles in the tissue imaging setup of 1000 μm thick tissue slice using incoherent reflection imaging with the ring light source and optimal optical clearing without filtering. (a) top surface of ibidi μ -slide channel showing in-focus activated ACT bubbles (white arrows) and small bubbles (red arrows) (b) bottom surface of ibidi μ -slide channel showing activated ACT and smaller bubbles (white arrows).

with 109.1 mM tartrazine improves light penetration and contrast in thick tissue samples, supporting the subsequent experiment using reflected incoherent light microscopy.

Algorithm

Figure 4 showed the intensity profiling results for Figure 2b. As shown in Figure 4b, the ascending slope of a peak corresponded to one edge while the descending slope corresponded to the opposite edge and two closely adjacent peaks indicate a ring pattern detection. The filtered image profile (shown in Figure 4c) exhibited steeper gradients at the peak tips and

two closely adjacent peaks indicated the presence of a ring pattern. This finding further confirms the improvement in edge sharpness observed previously.

Bubbles located at the bottom of the channel showed bright and sharply defined ring patterns and were therefore considered more reliable subjects for automated detection compared with the less regular bubble patterns at the top. Application of the mAHT algorithm demonstrated robust automated detection performance across different tissue contexts. In the muscle fibre region (shown in Figure 5a), eight bubbles were identified with ring edges

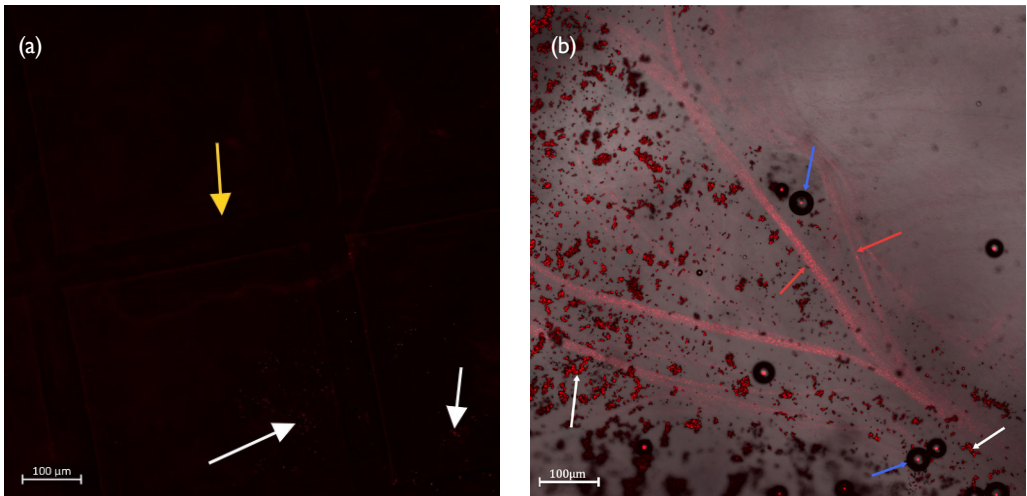


Figure 3. Comparison of images of control and tartrazine treated tissue slices captured by Zeiss LSM980/Airyscan 2 Confocal Microscope. Grid of well plate (yellow arrow), Sonazoid[™] (white arrows), large bubbles potentially air bubbles (blue arrows), and in-ternal tissue structure (red arrows). (a) 1000 μm thick chicken breast tissue without tartrazine treatment. (b) 1000 μm thick chicken breast tissue treated with Level A clear-ing experiment condition.

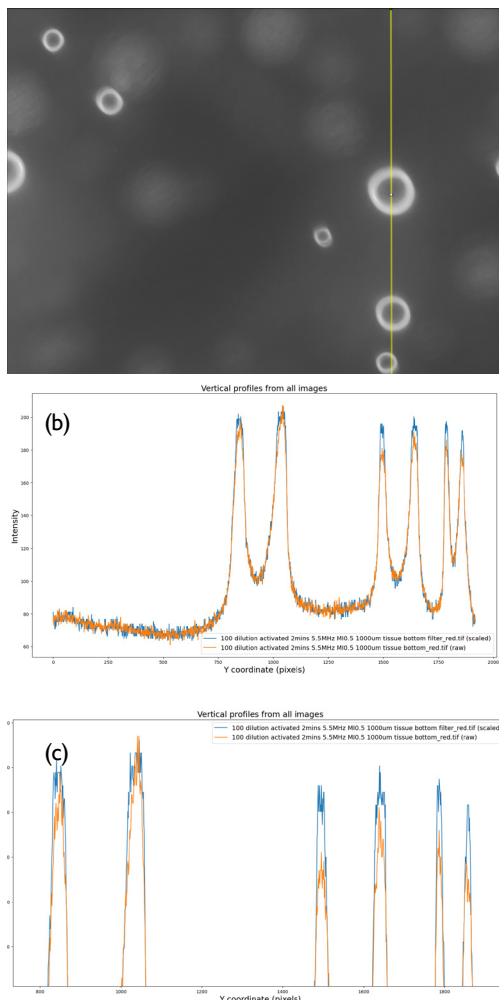


Figure 4. Intensity profiling of Figure 2b (a) column of interest for intensity profiling (b) Algorithm extracted and rescaled intensity profile along the column (c) zoom-in plot of the peaks for comparison.

clearly distinguishable from background texture of the fibres. In the tendon-rich region (shown in Figure 5b), three bubbles were detected. Although the background exhibited a foggy appearance due to strong scattering from tendon structures, the algorithm was still able to identify the bubbles at the edge of the area.

What did you learn from participating in this project?

Through this project, I gained a deeper understanding of the mechanisms of optical imaging and the practical setup of a microscope. I learned how individual components work together to

generate high quality images for analysing purpose. These hands-on experiences not only strengthened my technical ability to operate and troubleshoot a customised microscope system but also enhanced my appreciation of the principles underlying reflectance and confocal imaging. I also acquired a wide range of research skills through experiments, refined my ability to communicate scientific ideas effectively during weekly group meetings, daily discussions and formal presentations and even learned knowledge beyond the scope of my project through on-site lectures and visiting of laboratories. Furthermore, through this work, I learned to consider each challenge as an opportunity for research in depth and also developed a mindset

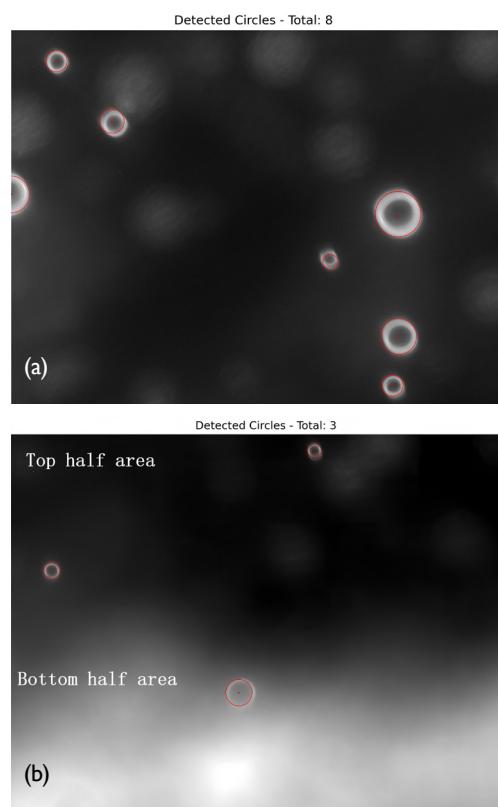


Figure 5. Bubble detection with Multi-AHT algorithm using images extracted of the bottom 100x dilution with Milli-Q water of activated ACT bubbles illuminated with ring light source through optically cleared 1000 μm thick chicken breast tissue at different areas of the tissue. (a) Detected bubbles at bottom of the channel through 1000 μm thick chicken fibre area. (b) Detected bubbles at bottom of the channel through 1000 μm thick chicken breast with heterogeneous structure ('white foggy' area in the bottom half of the image was of unknown origin but associated with structure that, unlike the top half, remained uncleared).

of being critical and thorough while avoiding unnecessary complexity when resolving issues.

How has this project affected your long-term goals?

This project has strongly enhanced my determination of pursuing a PhD through a well-coordinated and friendly working atmosphere. This project has shown me how powerful imaging technologies can be when combined with innovative biological models. I am inspired by the potential of integrating advanced microscopic imaging and image analysis with microfluidic devices to study dynamic biological processes in real time. Such approaches could enable more precise modelling of physiological environments and provide deeper insight into mechanisms underlying disease progression or therapeutic response. Therefore, I aim to contribute to the advancement of microfluidics devices combined with state-of-the-art microscopy to improve both fundamental research and clinical applications.

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