Journal of Visualized Experiments

Imaging and Quantification of the Area of Fast Moving Microbubbles Using a High Speed Camera and Image Analysis

--Manuscript Draft--

Article Type:	Invited Methods Article - Author Produced Video
Manuscript Number:	JoVE61509R1
Full Title:	Imaging and Quantification of the Area of Fast Moving Microbubbles Using a High Speed Camera and Image Analysis
Section/Category:	JoVE Bioengineering
Keywords:	high speed imaging; cavitation; Dentistry; ultrasonic scaler; bubbles; microbubbles; imaging; high speed camera
Corresponding Author:	Nina Vyas, Ph.D University of Birmingham College of Medical and Dental Sciences Birmingham, West Midlands UNITED KINGDOM
Corresponding Author's Institution:	University of Birmingham College of Medical and Dental Sciences
Corresponding Author E-Mail:	N.Vyas@bham.ac.uk
Order of Authors:	Nina Vyas, Ph.D
	Mehdi Mahmud
	Qianxi Wang
	Damien Walmsley
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$3000)

Dear Sir/Madam,

Please find attached a revised manuscript entitled “Imaging and Quantification of the Area of Fast Moving Microbubbles Using a High Speed Camera and Image Analysis” for consideration by JoVE. We have made the requested changes to the manuscript and video.

In the paper, we demonstrate a method of creating an imaging setup using a high speed camera to image small objects, therefore it will be of interest to a broad base of the JoVE readership. We use image sequences and image analysis to visualise cavitation bubbles around a dental instrument. The paper should be of interest to readers in the areas of bubble imaging, ultrasonic cleaning and high speed imaging.

All authors have approved the manuscript and agree with submission to JoVE. The study was supported by a grant from the Engineering and Physical Sciences Research Council UK. The authors have no conflicts of interest to declare.

Thank you for your consideration of this manuscript.

Please address all correspondence to:

Professor Damien Walmsley

Telephone +44 (0) 121 466 5493 (Secretary); Fax +44 (0) 121 237 2825

Email a.d.walmsley@bham.ac.uk

The School of Dentistry, College of Medical and Dental Sciences

University of Birmingham

5 Mill Pool Way

Edgbaston, Birmingham

UK

TITLE:

Imaging and Quantification of the Area of Fast-Moving Microbubbles Using a High-Speed Camera and Image Analysis

AUTHORS AND AFFILIATIONS:

Nina Vyas[1], Mehdi Mahmud[2], Qianxi X Wang[2], A. Damien Walmsley[1]

[1]School of Dentistry, University of Birmingham, Birmingham, UK
[2]Department of Mathematics, University of Birmingham, Birmingham, UK

Email Address of Co-authors:
Nina Vyas (n.vyas@bham.ac.uk)
Mehdi Mahmud (mhm439@student.bham.ac.uk)
Qianxi Wang (q.x.wang@bham.ac.uk)

Corresponding Author:
A. Damien Walmsley
a.d.walmsley@bham.ac.uk

KEYWORDS:

Cavitation Bubbles, High-speed Imaging, Ultrasonic Scalers, Dental, Image Analysis

SUMMARY:

Cavitation microbubbles are imaged using a high-speed camera attached to a zoom lens. The experimental setup is explained, and image analysis is used to calculate the area of the cavitation. Image analysis is done using ImageJ.

ABSTRACT:

An experimental and image analysis technique is presented for imaging cavitation bubbles and calculating their area. The high-speed imaging experimental technique and image analysis protocol presented here can also be applied for imaging microscopic bubbles in other fields of research; therefore, it has a wide range of applications. We apply this to image cavitation around dental ultrasonic scalers. It is important to image cavitation to characterize it and to understand how it can be exploited for various applications. Cavitation occurring around dental ultrasonic scalers can be used as a novel method of dental plaque removal, which would be more effective and cause less damage than current periodontal therapy techniques. We present a method for imaging the cavitation bubble clouds occurring around dental ultrasonic scaler tips using a high-speed camera and a zoom lens. We also calculate the area of cavitation using machine learning image analysis. Open source software is used for image analysis. The image analysis presented is easy to replicate, does not require programming experience, and can be modified easily to suit the application of the user.

INTRODUCTION:

Imaging the motion of bubbles is important for various applications because it controls the

hydrodynamics of a system. There are many applications where this can be useful: in fluidized bed reactors[1,2], or for cleaning with cavitation bubbles[3,4]. The purpose of imaging bubbles is to understand more about the bubble dynamics or about the direction and motion of a cloud of bubbles. This can be done through observing structures imaged and also by using image analysis to obtain quantitative information, such as the size of the bubbles.

Cavitation bubbles are gas or vapor entities that occur in a fluid when the pressure drops below the saturated pressure value[5]. They can occur when an acoustic field is applied to a fluid at ultrasonic frequencies. They repeatedly grow and collapse, and upon collapse can release energy in the form of high-speed micro-jets and shockwaves[6,7]. These can dislodge particles on a surface through shear forces and cause surface cleaning[8]. Cavitation bubbles are being investigated for surface cleaning in different industries, such as for semiconductors, food, and wound cleaning[9-12]. They could also be used to clean dental plaque from teeth and biomaterials such as dental implants[12,13]. Cavitation occurs around currently used dental instruments such as ultrasonic scalers and endodontic files and shows potential as an additional cleaning process with these instruments[14].

The oscillation of cavitation bubbles occurs over a few microseconds and therefore a high-speed camera is required to capture their motion by imaging at thousands of frames per second[8]. We demonstrate a method of imaging microbubble cavitation around dental ultrasonic scalers. The aim is to understand how cavitation varies around different ultrasonic scalers, so it can be optimised as a novel way to clean dental plaque.

Previous methods used to investigate the cavitation include sonochemiluminesence, which uses luminol to detect where cavitation has occurred[15,16]. However, this is an indirect technique and it is not able to visualize the cavitation bubbles in real time. Therefore, it is not able to accurately determine exactly where it happens on the instrument, and no information can be gained on the bubble dynamics, unless it is combined with other imaging techniques[17]. High-speed imaging can image not only the cavitation bubbles growing and collapsing but also the type of cavitation occurring: cavitation clouds, microstreamers and micro-jets[6,7,18]. These give more information about how the cavitation can clean surfaces.

We present a method of imaging cavitation microbubbles using a high-speed camera and calculating the mean area of cavitation occurring. This method is demonstrated using an example of cavitation occurring around different dental ultrasonic scaler tips, although the experimental and image analysis steps can be used for other applications, such as for imaging other macro and microbubbles.

PROTOCOL:

1. Instrument setup

1.1. Select the instrument or object to be imaged. In this experiment an ultrasonic scaler was imaged. Cavitation bubbles occur around the tips of ultrasonic scalers in water.

1.2. Select a micro positioning stage for the instrument to be imaged with XYZ translation and rotation. Place on a laboratory jack. Attach the instrument handle to the micro positioning stage

1.3. Select an optically transparent water container for imaging. The container used in these experiments was created with glass microscope slides.

1.4. Select an XY stage with a rotation platform. Place on a laboratory jack. Place the water container on the stage and fill with filtered water (reverse osmosis or distilled).

2. High-speed camera setup

2.1. Select a high-speed camera with the desired frame rate and resolution and a high intensity light source with a fibre light guide.

2.2. Attach a micropositioning sliding plate to the high-speed camera body and connect it to a tripod stand.

2.3. Select a lens with the desired resolution and focal length and attach this to the camera. For this experiment a zoom lens was used at a resolution of 8.4 µm/pixel.

2.4. Fill the imaging tank with water and position the tip of the instrument to be imaged in the water tank in the desired orientation.

2.5. After connecting the camera and loading the live view in the software, use low magnification to focus on the tip of the ultrasonic scaler, repositioning the light source if necessary. Position the instrument and the light source in front of the camera and focus. Adjust to the desired frame rate and brightness.

NOTE: A higher light intensity is required for imaging at high frames rates, short shutter speeds and/or high magnifications. Illumination can be provided in reflection mode or transmission mode. In this protocol the illumination is provided in transmission mode (bright field) using a high intensity cold illumination device.

2.6. Set an optimal frame rate and shutter speed for the high-speed camera. In this experiment the frame rate was 6400 fps with a shutter speed of 262 nanoseconds. A short shutter speed is required for fast moving bubbles such as cavitation bubbles to ensure that they are in focus.

2.7. Adjust the magnification of the zoom lens and the intensity of the light source so the background is white without being overexposed.

3. Calibration

3.1. Record the position of the tip (rotation in x-y stage, rotation angle of instrument for reproducibility).

3.2. To ensure the field of view is consistent for each repeat, choose a reference point and note down the coordinates. In this case the reference point was the tip of the ultrasonic scaler. It can then be repositioned in future experiments in the same place within the field of view.

3.3. If the pixel size is unknown, image a graticule with 10 µm markings at the set magnification and use image analysis software such as Fiji to calculate the resolution.

4. High-speed video recording

4.1. Image the instrument without cavitation. This will be subtracted from the cavitation images in image analysis when calculating the area of the cavitation bubbles. Save the videos in a format such as TIFF so no image quality is lost.

4.2. Image the instrument operating with cavitation. Ensure there are sufficient frames for accurate analysis, for example 5 repeats with 500 frames each.

5. Image processing

5.1. Download Fiji[19] from the ImageJ website (https://imagej.net/Fiji). An ImageJ macro code has been provided which automatically does the image analysis steps described below and can also be changed to suit the application. The individual steps of the macro are described in steps 5.3-5.5.

5.2. Crop the image to remove any darker areas resulting from uneven illumination, if needed. Ensure that all images are cropped to the same size and at the identical point in the image.

5.3. Convert the images to binary by automatically thresholding using one of the auto thresholds. In this example the minimum auto threshold is used.

5.4. Run the fill holes command to remove any black pixels from inside the bubbles which were falsely segmented.

5.5. Calculate the histogram of the stack to show the number of pixels corresponding to the scaler and the cavitation in each frame.

5.6. In this case the pixels corresponding to the bubbles are white and have value 255. Save these measurements.

5.7. Repeat steps 5.3-5.6 for the video of the instrument operating without the bubbles.

5.8. Calculate the mean area of the ultrasonic scaler tip only from the results of the histogram.

5.9. Subtract the mean area of the instrument from each of the areas calculated from the videos of the bubbles around the scaler. The area of the bubbles is left to measure.

5.10. Visualize by subtracting the binary image of the scaler from the binary image of the scaler with bubbles using the image calculator in Fiji.

5.11. Calculate the mean and standard deviation of the area of the bubbles.

5.12. Convert the values from number of pixels to area (in this case μm^2) by multiplying by the pixel size squared. Calculate the size of each pixel by imaging a graticule with the high-speed camera at the same magnification as was used for imaging and use ImageJ to set the scale.

5.13. Plot the data. It is also possible to conduct statistical analysis to show any significant difference in the area of bubbles if comparing different conditions.

6. ImageJ macro

6.1. In the **ImageJ/Fiji** menu, go to **Plugins > New > Macro**. Ensure IJ1 Macro is checked under the language menu and copy and paste the following code. Click run to execute the macro (**Supplementary File**).

REPRESENTATIVE RESULTS:

The image analysis steps can be seen in **Figure 1** for one of the ultrasonic scaler tips tested. A FSI 1000 tip and a 10P tip were imaged inside a water tank with the cooling water turned off (Fig**ure 2**). Cavitation occurred near the bend of tip FSI 1000 at maximum power, and near the free end in tip 10P (**Figure 3** and **Figure 4**). The mean area of cavitation was 0.1 ± 0.07 mm^2 for the FSI 1000 tip and 0.50 ± 0.25 mm^2 for the 10P tip (**Figure 5**).

FIGURE AND TABLE LEGENDS:

Figure 1: High-speed imaging setup and image analysis steps (**a**) Schematic of the high-speed imaging setup used in the study. (**b**) Schematic of the image analysis steps used in the study, showing the raw images on the left of the scaler tip only and with cavitation, which were then binarized and subtracted from each other to calculate the area of the cavitation clouds.

Figure 2: Comparison between different tips High-speed image stills showing cavitation occurring around the two ultrasonic scaler tips tested (**a**) FSI 1000 (**b**) 10P.

Figure 3: Tip 10P high-speed images: High-speed image stills of tip 10P, from a video taken at 6400 frames per second. Cavitation can be seen around the free end of the tip.

Figure 4: Tip FSI1000 high-speed images: High-speed image stills of tip FSI 1000, from a video taken at 6400 frames per second. Cavitation can be seen around the middle of the tip.

Figure 5: Cavitation area image analysis results. The mean area of cavitation occurring around the FSI 1000 and 10P ultrasonic scaler tips calculated using the image analysis technique described. The error bars represent the standard deviation.

DISCUSSION:

The technique described in this paper enables imaging of fast-moving microbubbles with high spatial and temporal resolution. It can potentially benefit a wide range of scientific disciplines such as chemical engineering, dentistry and medicine. Engineering applications include imaging cavitation bubbles for cleaning surfaces, or for imaging bubbles in fluidized bed reactors. Biomedical applications include imaging cavitation around medical and dental instruments and imaging biofilm debridement from hard and soft tissue using cavitation bubbles. In this study we demonstrated the technique by imaging cavitation around two different dental ultrasonic scaler tips. The amount of cavitation varies between the two tips tested in this study, with more cavitation clouds observed around the free end of tip 10P. This has previously been linked to vibration amplitude[20]. The high-speed videos show that the FSI 1000 tip has less vibration, which is likely to be why there is less cavitation around this tip.

One limitation of the image analysis method is that the image subtraction technique to remove the area of the scaler is not completely accurate because the scaler is oscillating and therefore the subtraction may leave some areas of the scaler falsely segmented as bubbles. However, this has been accounted for by averaging the area from a large number of frames (n=2000). This would not be a problem for applications where the object to be subtracted is stationary. For studies where the moving object to be subtracted has a much higher variance, we recommend synchronizing the movements in both videos before subtracting for accurate results. In the current study, we did not synchronize the oscillations but since the vibration was low, we can assume that the oscillations correspond well to each other in these two measurements.

The image thresholding is accurate because the brightfield illumination provides a uniform background with good contrast. It is critical to ensure that the background is uniform and does not contain any other objects which could be falsely segmented. The thresholding method can be modified by using other automatic thresholds to suit the application. Manual thresholding, where the user sets the threshold value, is also possible but is not recommended as it reduces the reproducibility of the results, since different users will select different threshold values.

Image analysis has been used for many other bubble imaging studies. These also use a similar method of backlighting to get optimum contrast between the bubbles and the background, and thresholding to segment the bubbles[21-24]. The method shown in the current study can also be generalized to use for many different bubble imaging applications, which are not limited to only high-speed imaging. High-speed imaging has been used for cavitation bubbles generated in water and also around instruments such as endodontic files and ultrasonic scalers[12,25-28]. For example Rivas et al. and Macedo et al. used a high-speed camera attached to a microscope, with

illumination provided by a cold light source to image cleaning with cavitation, and to image cavitation around an endodontic file[17,29]. Bright field illumination provides more contrast between the background and the bubbles, making it possible to use simple segmentation techniques such as thresholding, as demonstrated by Rivas et al. for imaging and quantifying cavitation erosion and cleaning over time[29]. Dark field illumination makes thresholding more difficult due to the higher variation in grey scales[4,30]. Image analysis has been used in other studies to gather more information about bubbles[1,2]. Vyas et al. used a machine learning approach to segment cavitation bubbles around an ultrasonic scaler[20]. The method described in the current paper is quicker because it uses simple thresholding so it is less computationally intensive, and bubbles occurring above and below the scaler can be analysed. However, the thresholding method used in the current paper is only accurate if the background is uniform. If it is not possible to obtain a uniform background during imaging, other image processing techniques can be used such as the use of background subtraction using a rolling ball radius to correct for uneven illumination, filtering using median or Gaussian filters to remove noise, or also using machine learning based techniques[20,31].

In conclusion, we present a high-speed imaging and analysis protocol to image and calculate the area of a microscopic moving object. We have demonstrated this method by imaging cavitation bubbles around an ultrasonic scaler. It can be used for imaging cavitation around other dental instruments such as endodontic files and it can be easily adapted for other non-dental bubble imaging applications.

ACKNOWLEDGMENTS:
The authors are grateful for funding from the Engineering and Physical Sciences Research Council EP/P015743/1.

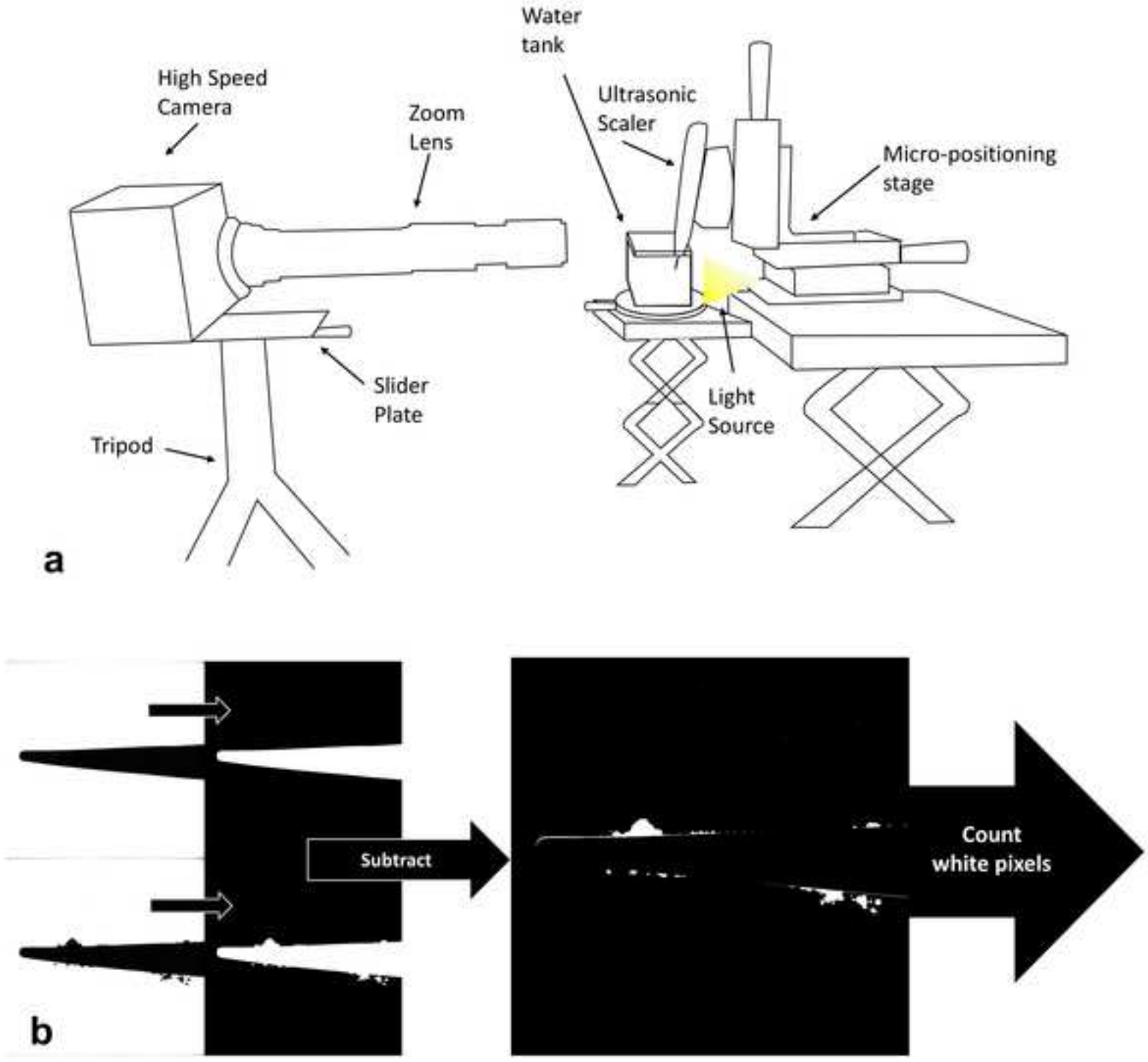
DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1 Asegehegn, T. W., Schreiber, M., Krautz, H. J. Investigation of bubble behavior in fluidized beds with and without immersed horizontal tubes using a digital image analysis technique. *Journal of Power Technologies.* **210** (3), 248-260 (2011).
2 Busciglio, A., Vella, G., Micale, G., Rizzuti, L. Analysis of the bubbling behaviour of 2D gas solid fluidized beds: Part I. Digital image analysis technique. *Chemical Engineering Journal.* **140** (1), 398-413 (2008).
3 Versluis, M. High-speed imaging in fluids. *Experiments in Fluids.* **54** (2), 1-35 (2013).
4 Matsumoto, H., Yoshimine, Y., Akamine, A. Visualization of irrigant flow and cavitation induced by Er: YAG laser within a root canal model. *Journal of Endodontics.* **37** (6), 839-843 (2011).
5 Young, F. R. *Cavitation* (World Scientific, 1999).
6 Brennen, C. E. *Cavitation and Bubble Dynamics*. (Cambridge University Press, 2013).
7 Leighton, T. *The acoustic bubble*. (Academic Press, 2012).
8 Verhaagen, B., Rivas, D. F. Measuring cavitation and its cleaning effect. *Ultrasonics*

Sonochemistry. **29**, 619-628 (2016).
9 Oulahal-Lagsir, N., Martial-Gros, A., Boistier, E., Blum, L., Bonneau, M. The development of an ultrasonic apparatus for the non-invasive and repeatable removal of fouling in food processing equipment. *Letters in Applied Microbiology.* **30** (1), 47-52 (2000).
10 Gale, G. W., Busnaina, A. A. Roles of cavitation and acoustic streaming in megasonic cleaning. *Particulate Science and Technology.* **17** (3), 229-238 (1999).
11 Erriu, M. et al. Microbial biofilm modulation by ultrasound: Current concepts and controversies. *Ultrasonics Sonochemistry.* **21** 15-22 (2014).
12 Van der Sluis, L., Versluis, M., Wu, M., Wesselink, P. Passive ultrasonic irrigation of the root canal: a review of the literature. *International Endodontic Journal.* **40** (6), 415-426 (2007).
13 Vyas, N., Sammons, R. L., Addison, O., Dehghani, H., Walmsley, A. D. A quantitative method to measure biofilm removal efficiency from complex biomaterial surfaces using SEM and image analysis. *Scientific Reports.* **6**, 32694 (2016).
14 Walmsley, A. D., Lea, S. C., Felver, B., King, D. C., Price, G. J. Mapping cavitation activity around dental ultrasonic tips. *Clinical Oral Investigations.* **17** (4), 1227-1234 (2013).
15 Price, G. J., Tiong, T. J., King, D. C. Sonochemical characterisation of ultrasonic dental descalers. *Ultrasonics Sonochemistry.* **21**, 2052-2060 (2014).
16 Felver, B., King, D. C., Lea, S. C., Price, G. J., Damien Walmsley, A. Cavitation occurrence around ultrasonic dental scalers. *Ultrasonics Sonochemistry.* **16**, 692-697 (2009).
17 Macedo, R. G. et al. Sonochemical and high-speed optical characterization of cavitation generated by an ultrasonically oscillating dental file in root canal models. *Ultrasonics Sonochemistry.* **21**, 324-335 (2014).
18 Reuter, F., Lauterborn, S., Mettin, R., Lauterborn, W. Membrane cleaning with ultrasonically driven bubbles. *Ultrasonics Sonochemistry.* **37**, 542-560 (2017).
19 Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. *Nature Methods.* **9** (7), 676-682 (2012).
20 Vyas, N. et al. High-speed Imaging of Cavitation around Dental Ultrasonic Scaler Tips. *PLoS One.* **11** (3), e0149804 (2016).
21 Ahmed, F. S., Sensenich, B. A., Gheni, S. A., Znerdstrovic, D., Al Dahhan, M. H. Bubble dynamics in 2D bubble column: comparison between high-speed camera imaging analysis and 4-point optical probe. *Chemical Engineering Communications.* **202** (1), 85-95 (2015).
22 Honkanen, M. Reconstruction of three-dimensional bubble surface from high-speed orthogonal imaging of dilute bubbly flow. *Proceedings of Computational Methods in Multiphase Flow V, New Forest, UK.* 469-480 (2009).
23 do Amaral, C. E. et al. Image processing techniques for high-speed videometry in horizontal two-phase slug flows. *Flow Measurement and Instrumentation.* **33**, 257-264 (2013).
24 Lau, Y., Deen, N., Kuipers, J. Development of an image measurement technique for size distribution in dense bubbly flows. *Chemical Engineering Science.* **94**, 20-29 (2013).
25 Matsumoto, Y., Yoshizawa, S. Behaviour of a bubble cluster in an ultrasound field. *International Journal for Numerical Methods in Fluids.* **47** (6-7), 591-601 (2005).
26 Peeters, H. H., Iskandar, B., Suardita, K., Suharto, D. Visualization of removal of trapped air from the apical region of the straight root canal models generating 2-phase intermittent counter flow during ultrasonically activated irrigation. *Journal of Endodontics.* **40** (6), 857-861 (2014).

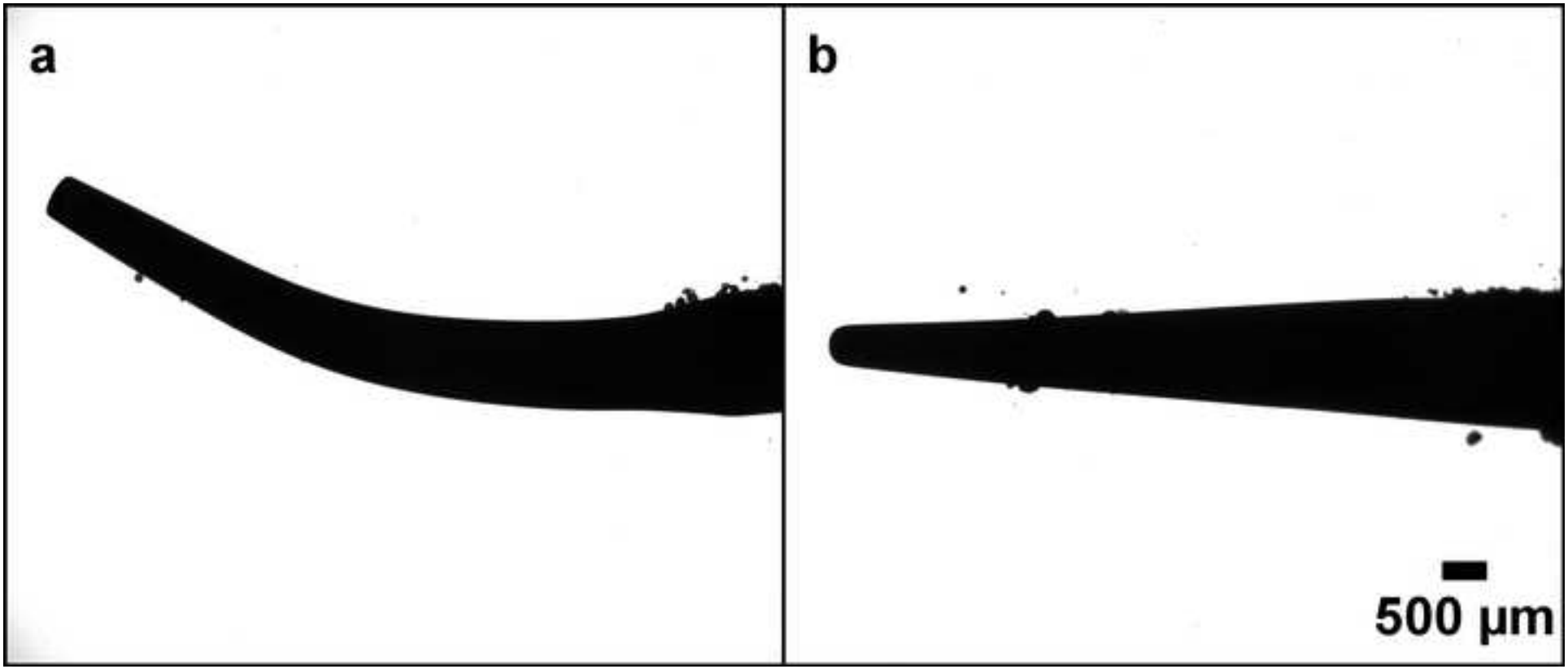
27 Halford, A. et al. Synergistic effect of microbubble emulsion and sonic or ultrasonic agitation on endodontic biofilm in vitro. *Journal of Endodontics.* **38** (11), 1530-1534 (2012).
28 Kauer, M., Belova-Magri, V., Cairós, C., Linka, G., Mettin, R. High-speed imaging of ultrasound driven cavitation bubbles in blind and through holes. *Ultrasonics Sonochemistry.* **48**, 39-50 (2018).
29 Rivas, D. F. et al. Localized removal of layers of metal, polymer, or biomaterial by ultrasound cavitation bubbles. *Biomicrofluidics.* **6** (3), 034114 (2012).
30 Pishchalnikov, Y. A. et al. Cavitation Bubble Cluster Activity in the Breakage of Kidney Stones by Lithotripter Shockwaves. *Journal of Endourology.* **17** (7), 435-446 (2003).
31 Sternberg, S. R. Biomedical image processing. *Computer.* (1), 22-34 (1983).

Figure



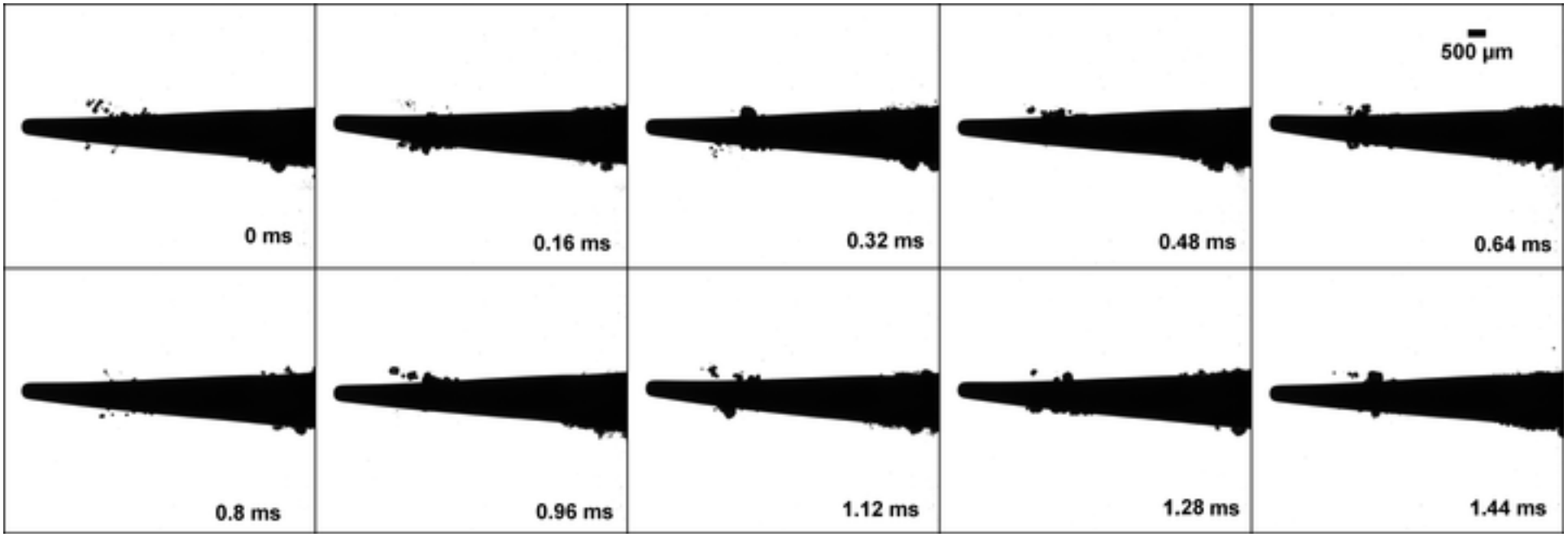
Figure

Click here to access/download;Figure;Figure 2 results.tif



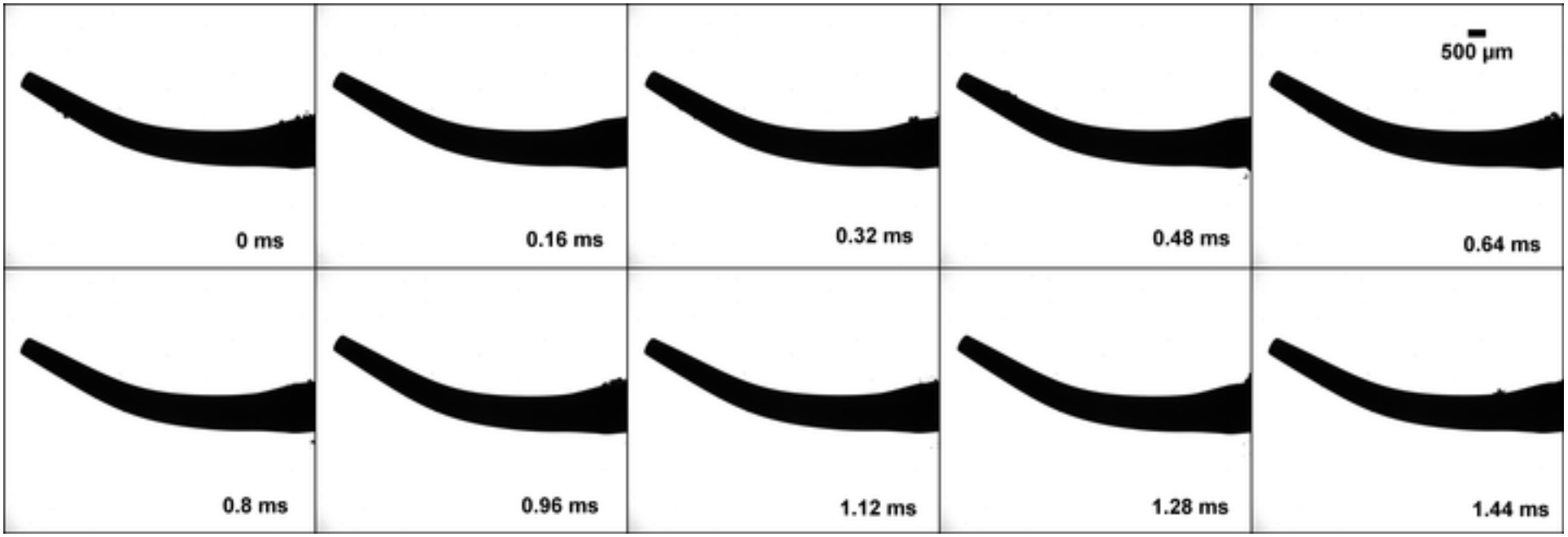
Figure

Click here to access/download;Figure;figure 3 Montage 10P.tif



Figure

Click here to access/download;Figure;figure 4 Montage FSI 1000.tif



Figure

Click here to access/download;Figure;figure 5 mean cavitation area updated high dpi.TIF

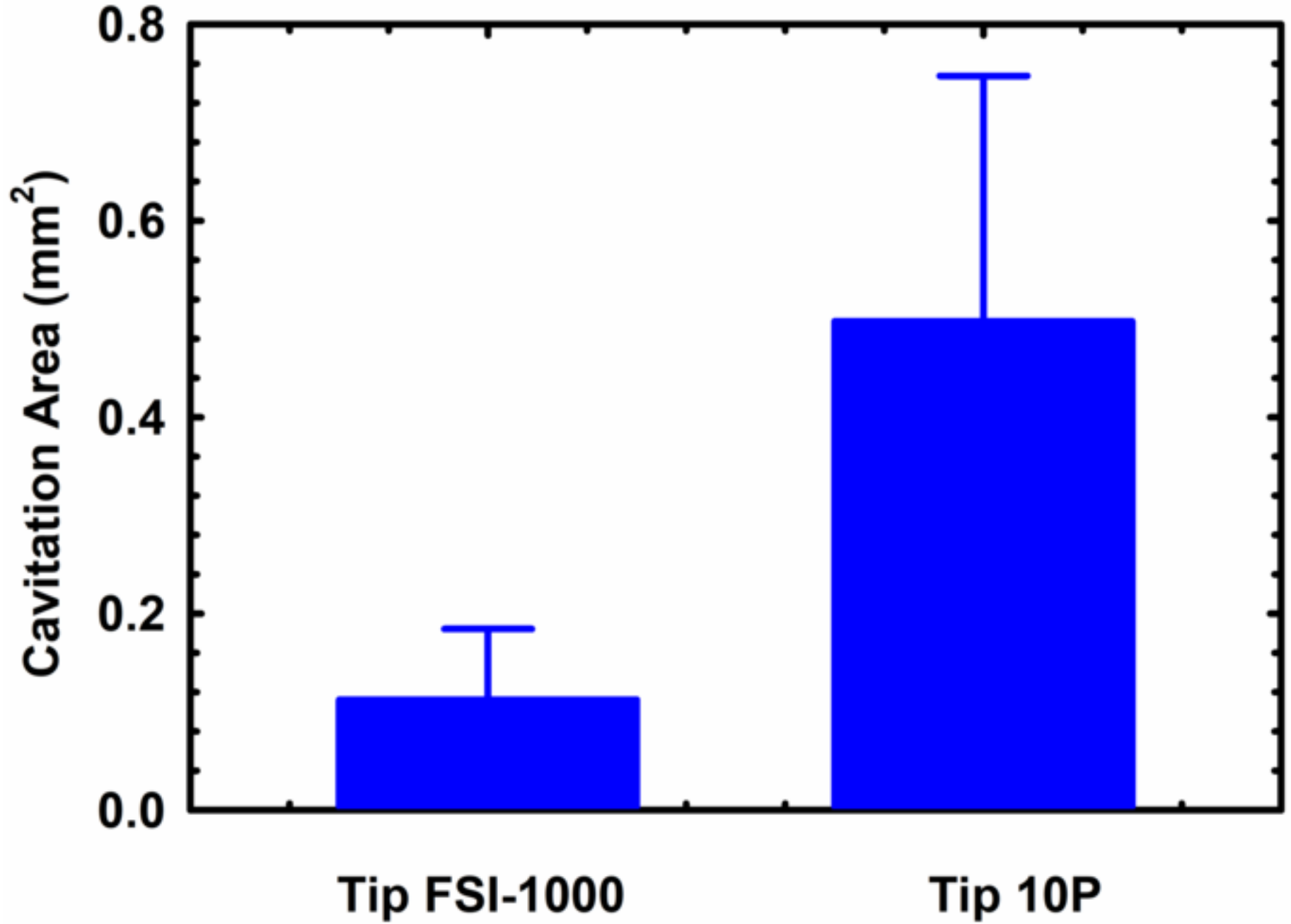


Table of Materials

Click here to access/download;Table of Materials;List of Materials Template.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.25x attachment	Navitar	1-50011	
12x with 12mm fine focus Long distance microscope zoom lens	Navitar	1-50486	
2x adaptor with f mount	Navitar	1-62922	
Cavitron Plus Ultrasonic Scaler	Dentsply Sirona	8184003	
Cavitron Ultrasonic Insert FSI 1000FSI 1000	Dentsply Sirona	UCAFTHD	
Fibre light guide. 8mm fibre bundle 1500mm length. Focussing lens assembly for Hayashi light, 1/4"-20 tripod thread for mounting.	Hayashi	LGC1-8L1500	
Geared head	Manfrotto	MN405	7.5kg load capacity
HDF7010 High-Power LED Endoscope light source. 150W LED provides cold output equivalent to 250W Xenon.	Hayashi	LA-HDF710	
Heavy weight Tripod	Manfrotto	MN475B	Geared centre column, 12kg load capacity
High Speed Camera	Photron	103526	FASTCAM Mini AX200 900K M3 (16GB memory)
High-Precision Rotation Stage	Thorlabs	PR01/M	
Laboratory jacks	Camlab	1194083	
Micropositioning sliding plate	Manfrotto	SKU 454	
Micropositioning stage 3D	Thorlabs	PT3/M	
Micropositioning stage rotation	Thorlabs	OCT-XYR1/M	OCT-XYR1/M - XY Stage with Solid Top Plate
NEWTRON P5 XS Ultrasonic Scaler	Acteon	F62118	
Ultrasonic Insert 10P	Acteon	F00253	

Changes to be made by the Author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have proofread the manuscript.

2. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

We have updated the materials table accordingly

3. Please specify all experimental parameters used. We need specific values and numbers instead of references to the desired settings. This is to help others replicate the protocol exactly.

All experimental parameters have been specified
4. Please move the macro code to a separate supplemental file.

We have moved the macro code to a supplemental macro file which can be opened in Fiji/ImageJ.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

Commercial names have been removed from the manuscript

Changes to be made by the Author(s) regarding the video:
1. Video & Audio Quality:
• There is a persistent green block at the top of the screen throughout. Perhaps this is some kind of video rendering glitch? It will need to be fixed before publishing. Try a different video codec such as h264.

This has been changed.
• There is a regular beep tone in the background. This should be removed. If it's an audio watermark (copyright protection in a music track), then purchase the license to the track so that the watermark is removed.

This has been changed.

2. JoVE Video Format:
• The video has no chapter title cards. Please add chapter title cards for the beginning of the protocol, any individual sections of the protocol, the results (03:45), and the conclusion (04:07).

Title cards have been added.
• 03:08-03:26 Place this statement by Nina near the end with the concluding statements by Damien

(currently starting around 04:07).
3.
This has been changed.

Please upload a revised high-resolution video here:
https://www.dropbox.com/request/cVdPEoUGhYjJeE2vt3rP?oref=e

We have made the changes to the video.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The experimental protocol presented in the manuscript "Imaging and Quantification of the Area of Fast Moving Microbubbles Using a High Speed Camera and Image Analysis" and the corresponding video by N. Vyas et al. includes a method to image fast cavitation bubble phenomena occurring around dental ultrasonic scalers as well as an image processing technique to extract the projected area of theses cavitation bubbles. It describes how to set up the high-speed imaging experiment with the camera and a zoom lens, translation stages, appropriate calibration and experimental procedure. The image analysis method describes how to calculate the area of the cavitation bubbles from a binarised image sequence obtained in the experiments, using the open-source ImageJ software and appropriate thresholding and reference subtraction.

Despite the experimental protocol and especially the image analysis method being very simple, the measurement instruments used (high-speed camera, zoom lens, and illumination) are sophisticated and not trivial to use for non-experts. I think the video was very well made and the explanations it contained were clear, and therefore has a potential to be useful as an introduction for other researchers to replicate such an experiment.

Minor Concerns:

1. The effect of the scaler oscillations on its subtraction from the images in the authors' experiments was not clear. They mentioned that a whole reference video, instead of just a single still picture (which would work if the object was stationary), was subtracted from the "cavitation" video. The authors mention that "The error can also be eliminated by synchronizing the oscillations of the scaler in both videos before subtracting" - so was this what the authors did in the presented results? Would it be accurate to assume that the oscillations correspond well to each other in these two measurements? And how about if the cavitation bubbles affected the motion of the object: would there be a reliable way to remove the scaler area in this case? Are there techniques to distinguish bubbles from objects that move in an unpredictable way?

With regards to this question: And how about if the cavitation bubbles affected the motion of the object: would there be a reliable way to remove the scaler area in this case? Are there techniques to distinguish bubbles from objects that move in an unpredictable way?

The cavitation bubbles would not affect the movement of the ultrasonic scaler tip. If in other studies they affected it, then it might not be possible to use this subtraction technique and therefore other image analysis methods such as machine learning could be used, we have mentioned some more techniques in the discussion section.

We have clarified the first point in the manuscript:

For studies where the moving object to be subtracted has a much higher variance, we recommend to synchronise the movements in both videos before subtracting for accurate results. In the current study we did not synchronise the oscillations but since the vibration was low, we can assume that the oscillations correspond well to each other in these two measurements.

2. "It is difficult to compare this method with other methods as they have different applications": extracting bubbles of images in multi-phase flows (bubble flows, droplet sprays, etc.) is a widely used process that can be generalised to a wide range of applications. This method is not so different

We thank the reviewer for this comment. We have changed the text to this:

Image analysis has been used for many other bubble imaging studies. These also use a similar method of backlighting to get optimum contrast between the bubbles and the background, and thresholding to segment the bubbles. The method shown in the current study can also be generalised to use for many different bubble imaging applications, which are not limited to only high speed imaging.

3. "Image analysis has been used in other studies to gather more information about bubbles although these studies have not used high speed imaging with a zoom lens." I do not quite believe this sentence. There are many studies applying various image analysis techniques to quantify cavitation bubble formation and behaviour at micro-scale.

We thank the reviewer for pointing this out. We have removed this sentence.

4. "If it is not possible to obtain a uniform background during imaging, the imaging and image analysis technique used in Vyas et al. [23] can be used." There are many more sophisticated image processing techniques beyond thresholding that can get rid of/smoothen out background noise or perturbations before having to rely on learning-based techniques. I recommend mentioning some.
5. Figure 5 should include 10P ultrasonic scaler as a label below the second bar

We thank the reviewer for this comment. We have changed this to the following:

If it is not possible to obtain a uniform background during imaging, other image processing techniques can be used such as the use of background subtraction using a rolling ball radius to correct for uneven illumination, filtering using median or gaussian filters to remove noise, or also using machine learning based techniques.

Reviewer #2:
Manuscript Summary:
This article presented a method for imaging the cavitation bubble around dental ultrasonic scaler tips using high speed camera and zoom lens. Also this article presented calculated cavitation bubble area using image analysis software.

Major Concerns:
1. Instrument Setup, High Speed Camera Setup, I think it would be hard to understand if you just read it, so I hope you have a diagram that you can understand at a glance.

We thank the reviewer for this suggestion. We have added a new figure of the experimental setup and labelled it to help the reader understand it at a glance.

2. Image Processing part will eventually be the part where you calculate the area by dividing the filming part into two white/black colors, and it will be good to understand if you show the still cut process in the image picture.

We were not sure exactly what the reviewer meant by showing the still cut process, we assume they would like to show the image analysis steps in more detail. We have therefore redrawn and labelled the image analysis diagram to clarify.

3. Describe the detailed characteristics of Cavitron FSI tip and Satelec 10 P tip, which are different alternative scaler tip, and describe the reason for the difference in the culture area in discussion.

We have added the following in the discussion:

In this study we demonstrated the technique by imaging cavitation around two different dental ultrasonic scaler tips. The amount of cavitation varies between the two tips tested in this study, with more cavitation clouds observed around the free end of tip 10P. This has previously been linked to vibration amplitude19. The high speed videos show that the FSI 1000 tip has less vibration, which is likely to be why there is less cavitation around this tip.

Reviewer #3:
Manuscript Summary:
The topic is highly relevant and is well explained.
I do have a couple of suggestions before it is published.

Major Concerns:
In the abstract,
The authors say: "Cavitation occurring around dental ultrasonic scalers could be used as a novel method of dental plaque removal, which would be more effective and cause less damage than current periodontal therapy techniques"

I think there are several examples that have demonstrated it, in the scientific literature and commercially available equipment: so the "coulds" and "woulds" can be changed to "can" etc.
For example, a very similar study:
Macedo, R. G., et al. "Sonochemical and high-speed optical characterization of cavitation generated by an ultrasonically oscillating dental file in root canal models." Ultrasonics sonochemistry 21.1 (2014): 324-335.

We thank the reviewer for this point and we have cited this paper and changed this in the manuscript.

This statement could be improved in line with Macedo's work:
"Therefore, it is not able to accurately determine exactly where it happens on the instrument, and also no information can be gained on the bubble dynamics."

We have added the following to this statement, citing Macedo's work:

Therefore, it is not able to accurately determine exactly where it happens on the instrument, and also no information can be gained on the bubble dynamics, unless it is combined with other imaging techniques.

Introduction:
"The purpose of imaging bubbles is to understand more about the bubble dynamics."
This statement needs clarification in line with what is described in this report.
Their method cannot really say much about the dynamic of individual bubbles; it is more about a cloud of bubbles, and they tend to overlap.

Thank you for this point. Our method could be applied to individual bubble experiments, so we have changed this to:

The purpose of imaging bubbles is to understand more about the bubble dynamics in the case of individual bubbles, or about the direction and motion of a cloud of bubbles.

In line with the previous paragraph, the authors should also make a distinction with bright and dark field illumination, and provide other experimental method examples to contrast the differences of each method, advantages-disadvantages.
They can use for example:
Rivas, D.F., et al., 2013. Ultrasound artificially nucleated bubbles and their sonochemical radical production. Ultrasonics sonochemistry, 20(1), pp.510-524.

We thank the reviewer for this point. We have added the following:

Similar experimental approaches have been used in other studies for imaging bubbles with a high speed camera, for example Rivas et al and Macedo et al used a high speed camera attached to a microscope, with illumination provided by a cold light source, to image cleaning with cavitation, and to image cavitation around an endodontic file. Bright field illumination provides more contrast between the background and the bubbles, making it possible to use simple segmentation techniques such as thresholding, as demonstrated by Rivas et al. for imaging and quantifying cavitation erosion and cleaning over time. Dark field illumination makes thresholding more difficult due to the higher variation in grey scales.

Page 8, line 291: I think the comment that zoom lens is used for the first time is superfluous.
"Image analysis has been used in other studies to gather more information about bubbles although these studies have not used high speed imaging with a zoom lens1,2."

We thank the reviewers for pointing this out and we have removed this comment.

Image Analysis Macro

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT - UK

Title of Article:	Imaging and Quantification of the Area of Fast Moving Micfccrobubbles Using a High Speed Camera and Image Analysis
Author(s):	Nina Vyas, Mehdi Mahmud, Qianxi X Wang, A. Damien Walmsley

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access ☑ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution 3.0 Agreement (also known as CC-BY), the terms and conditions of which can be found at: http://creativecommons.org/licenses/by/3.0/us/legalcode ; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4 and 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video - Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video - Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties**. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT - UK

discretion andwithout giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

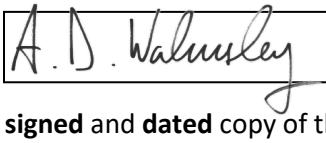
12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contaminationdue to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, Jo VE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Professor Damien Walsmley
Department:	School of Dentistry
Institution:	University of Birmingham
Title:	Professor
Signature:	
Date:	25th March 2020

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140