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**Title:** **Automated 3D Optical Coherence Tomography to Elucidate Biofilm Morphogenesis over Large Spatial Scales**

**Authors and Affiliations: Anna Depetris1, Antoine Wiedmer2, Michael Wagner3, Sebastian Schäfer4, Tom J. Battin1, Hannes Peter1**

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**Author Questionnaire:**

**1.** Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **(Y/N) No. But we present a system which acquires “microscopy” images (optical coherence tomography).**

Can you record movies/images using your own microscope camera? **(Y/N) No**

**2.** Does your protocol include software usage? **(Y/N) Yes**

If yes, we will need you to record using [screen recording software](https://obsproject.com/) to capture the steps. If you use a Mac, [QuickTime X](https://www.apple.com/support/mac-apps/quicktime/) also has the ability to record the steps.

**3.** Which steps from the protocol section below are the most important for viewers to see? Please list 4-6 individual steps using the step numbers listed in this document. This information is important to prepare your Videographer for your shoot.

*Authors, please answer this question with the steps listed here in the Protocol section below for use by the videographer.*

**4.** What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1-2 individual steps using the step numbers listed in this document.

*Authors, please answer this question with the steps listed here in the Protocol section below for use by the videographer.*

**5.** Will the filming need to take place in multiple locations? **(Y/N) No.**

Section - Introduction

***Videographer: Interviewee Headshots are required. Take a headshot for each interviewee.***

1. **REQUIRED Interview Statements:**

Why is your protocol significant? *OR* What key questions can this method help answer?

* 1. **Hannes Peter:** Here we present an automated system based on optical coherence tomography – OCT - that allows us to monitor biofilm structure over large spatial scales and extended periods of time.
  2. **Hannes Peter:** OCT imaging is well suited to resolve structures in the micron range, but is currently limited to a maximum area of approximately 250 square millimeters. Biofilm structures often exceed this scale, especially when the differentiation is driven by large scale environmental gradients.

What is the main advantage of this technique?

* 1. **Anna Depetris**: The experimental setup allows us to monitor the 3-dimensional morphogenesis of biofilms over large spatial scales and extended periods of time. The system is precise, fast and works autonomously.

**OPTIONAL Interview Statements:**

Can this method be applied to any other systems?

* 1. **Hannes Peter:** We study the morphogenesis of biofilms in streams where they drive important ecosystem processes, however, this system may be used to study biofilms in engineered environments or in other natural environments.

Do you have any advice to offer to somebody who is trying this technique for the first time?

* 1. **Antoine Wiedmer:** The software for positioning, image processing, and analysis is written in Python and is available through Jupyter notebooks. These are user-friendly, freely available and flexible solutions.

Why is visual demonstration of this method critical?

* 1. **Hannes Peter**: We believe that the visual demonstration of this setup will help potential users to reproduce the installation and to better understand the software. We hope that it inspires other researchers to adopt similar approaches.

Section - Protocol

1. **Setup of the Positioning Device**
   1. Begin by connecting the positioning device to a microcontroller board by following along with instructions posted on github.**[1-TXT]**
      1. MED: Talent demonstrates the connected devices **TEXT: https://github.com/ grbl/grbl/wiki/Connecting-Grbl**
   2. Then, connect the microcontroller to a single-board computer with internet connection using a USB cable. **[1]** Once connected, install the GRBL server as described in a separate github page.**[2-TXT]**
      1. MED: Talent uses USB cable to connect the described device
      2. SCREEN: To be provided by the authors – Screen capture video as talent performs the above steps in the order listed *Authors, please upload this screen capture to* [*your project page.*](http://www.jove.com/files_upload.php?src=18103628)**TEXT:** **https://gitlab.com/ FlumeAutomation/ GRBL\_Server.git**
   3. **Antoine Wiedmer:** “The positioning device should now be navigable from the webpage shown here. Alternatively, the positioning device can be navigated with a Python script*,* as demonstrated in the first part of the worked example supplied in this article’s supplementary files.**[1-TXT]**
      1. INTERVIEW: Author says the above statement interview style **TEXT: http://IP:5020/**
2. **Optical Coherence Tomography Setup**
   1. Using a compatible dove-tail holder, mount the optical coherence tomography probe to the positioning device.**[1]** If required, install an immersion adapter on the objective lens.**[2-TXT]**
      1. CU: Talent demonstrates the assembled OCT probe as described
      2. CU: Talent installs an immersion adapter **TEXT: Additional immersion adapters are available for individual setups**
   2. Next, position the computer and the OCT base unit on a bench next to the experimental setup containing microfluidic devices, flow chambers, flumes, and filtration systems. **[1]** Make sure that the optical cord is long enough to reach all intended locations and that it does not interfere with the experimental setup.**[2]**
      1. MED: Show the computer and OCT base unit next to the experimental setup
      2. MED: Talent moves the device to all likely locations to test cord length
   3. If not already installed, install the OCT system together with the available software as described by the manufacturer.**[1]** Then, install the software packages for automated OCT scan acquisition as described in the gitlab documents linked to here. **[2-TXT]**
      1. MED Over the Shoulder: Talent opens OCT software at the computer
      2. SCREEN: To be provided by the authors – Screen capture video as talent performs the above steps in the order listed *Authors, please upload this screen capture to your* [*project page*](http://www.jove.com/files_upload.php?src=18103628)*.* **TEXT: https://gitlab.com/FlumeAutomation/automated-oct-scans-acquisition.git**
3. **Image Acquisition**
   1. To begin image acquisition, power on the OCT system and the positioning device… Make sure that the device can move freely. **[1]**
      1. MED: Talent powers on the system and moves it around
   2. Next, open the file *config.json* in a text editor. Edit the *config.json* file to adjust the default acquisition parameters including the refractive index, scan speed, and the destination folder for acquired data and metadata, the size of the field-of-view and the number of A-scans per B-scan, the signal boundaries of the OCT scan based on intensity histograms of preliminary scans.**[1]**
      1. SCREEN: To be provided by the authors – Screen capture video as talent performs the above steps in the order listed *Authors, please upload this screen capture to your* [*project page*](http://www.jove.com/files_upload.php?src=18103628)*.*
   3. Open the commercial OCT software, locate a site of interest, focus on the sample, and adjust the reference arm and light source intensity for optimal image quality. Note the coordinates and repeat this procedure for a number of positions while maintaining the same reference arm length and intensity. **[1]**
      1. SCREEN: To be provided by the authors – Screen capture video as talent finds site, adjusts light intensity and records the coordinates. *Authors, please upload this screen capture to your* [*project page*](http://www.jove.com/files_upload.php?src=18103628)*.(please gray out the IP address of our computer)*
   4. Next, open the *ImageAcquisition.ipynb* file found in this article’s **supplementary file 2** in Juypter Notebook. Each cell contains code to perform specific tasks and can be run separately via pressing **Cell** and then **Run**, or **Control and Enter** or **Shift and Enter. [1]**
      1. SCREEN: To be provided by the authors – Screen capture video as talent opens the file and performs one of the specific tasks listed. *Authors, please upload this screen capture to your* [*project page*](http://www.jove.com/files_upload.php?src=18103628)*.*
   5. Follow the worked example to set the path to the required library configuration parameter… connect to the positioning device… initialize the OCT scanner… and then calibrate the positioning device. **[1]**
      1. SCREEN: To be provided by the authors – Screen capture video as talent performs the above steps in the order listed *Authors, please upload this screen capture to your* [*project page*](http://www.jove.com/files_upload.php?src=18103628)*.*
   6. Acquire a single OCT scan following the worked example. Note that you can specify the FOV, number of A scans and B scan directly before scan acquisition. Data is saved in 8 bits \**.raw* format to save storage space. Metadata including the OCT settings and coordinates are saved in the same folder in a \**.**json* file with the same naming convention. Depending on settings such as FOV and resolution, file size may reach up to 1.5 GB per OCT scan.**[1-TXT]**
      1. *SCREEN: To be provided by the authors – Screen capture video as talent takes images with different # of A scans per B , opens the folder with saved files and points out the size of the files using the cursor and the metadata. Authors, please upload this screen capture to your* [*project page*](http://www.jove.com/files_upload.php?src=18103628)*.* ***TEXT: Ensure sufficient free disc space***
   7. **Anna Depetris:** “These two parameters determine the size of the voxels of the final dataset and the size of the output file and should match the optical resolution of the OCT probe. These parameters trade-off against the available disk space and computer processing power.”**[1]**
      1. ***INTERVIEW: Author says the above statement in interview style.***
   8. In oder to acquire a tiled OCT scan, specify the number of images and the overlap of neighboring tiles and acquire the datasets covering the positions of interests in a mosaic pattern, as outlined in the worked example. **[1]**
   9. Disconnect the OCT device once scans acquisition is finished
      1. SCREEN: To be provided by the authors – Screen capture video as talent performs the above steps in the order listed *Authors, please upload this screen capture to your* [*project page*](http://www.jove.com/files_upload.php?src=18103628)*.*
4. **Image Correction and Display**
   1. **Anna Depetris**: The raw OCT images appear distorted due to differences in pathlength through the optical system. We have developed a set of algorithms that performs these corrections. A worked example for image correction is supplied with this article’s supplementary files. **[1]**
      1. INTERVIEW: Author says the above statement interview style
   2. To begin image correction, open the Jupyter notebook *ImageProcessing.ipynb* , load and visualize the raw images.**[1]**
      1. SCREEN: To be provided by the authors – Screen capture video as talent opens the described file *Authors, please upload this screen capture to your* [*project page*](http://www.jove.com/files_upload.php?src=18103628)*.*
   3. Following this example, first crop the OCT scans in order to exclude spurious signals and reoriented the dataset so that the biofilm appears above the substratum.**[1]**
      1. SCREEN: To be provided by the authors – Screen capture video as talent performs the above steps in the order listed *Authors, please upload this screen capture to your* [*project page*](http://www.jove.com/files_upload.php?src=18103628)*.*
   4. Next, correct for spherical aberration. To accomplish this, the algorithm defines a grid of 20 by 20 vertical lines regularly spaced across the xy-plane of the scan. Then, a circular area around each point is selected and averaged along the vertical profile. A modified Gaussian filter is then applied to each vertical profile. This will identify local maxima in the signal intensity and showthe position of the highly reflective reference surface. Misidentified points are filtered based on the positions of their neighbors in three dimensions. (yellow points are kept, while purple points in quality check images are discarded). Next, fit a 2nd order polynomial surface reflecting the distortion introduced by the scan lens across these points… Use this fitted surface to shift each pixel in the z-direction, thus obtaining a flattened image. The parameter of this algorithm should be adjusted to the characteristics of the OCT probe. Once the image has been flattened, mean background signal intensity (measured in an empty area of the image above the biofilm) is subtracted.[1]
      1. SCREEN: To be provided by the authors – Screen capture video as talent performs the above steps in the order listed *Authors, please upload this screen capture to your* [*project page*](http://www.jove.com/files_upload.php?src=18103628)*. (the algorithm takes a while, the video can be cropped so to show just the last corrected slice)(the above is just one step, as it is all performed automatically)*
   5. Next, a reference surface, such as the substratum, is defined and an elevation map is computed from the 3D OCT dataset. **Specifically, the corrected images are thresholded and the height of the biofilm for each coordinate of the binary mask is calculated. Biofilm height is then assigned to a 2D matrix of the size of the original image in x and y directions. Then, an image is rendered in which the elevation of the surface is reported as grayscale value. [1]**
      1. SCREEN: To be provided by the authors – Screen capture video as talent performs the above steps in the order listed *Authors, please upload this screen capture to your* [*project page*](http://www.jove.com/files_upload.php?src=18103628)*. (the algorithm is a bit slow, the video may be cropped)(this is a unique step, as it is all done automatically)*
   6. *If images wereacquired in a tiled pattern, stitch the relative elevation maps in a unique image, by selecting the number of rows and column and applying the stitching algorithm as shown in the worked example.*
      1. *SCREEN: To be provided by the authors – Screen capture video as talent performs the above steps in the order listed*

Section – Results

1. **Results: Effect of Flow Velocity on Biofilm Growth**
   1. Using automated OCT imaging, the spatio-temporal morphogenesis of phototrophic stream biofilms was examined using flume experiments. The flumes are made of plexiglass and gradually widen from the in- to the outflow. This results in a gradient in flow velocity.**[1]**
      1. Video provided by authors showing the described Flume experiment (~20 sec in length) *Authors, please upload this video to your* [*project page*](http://www.jove.com/files_upload.php?src=18103628)*.*
   2. Here is an elevation map of a biofilm growing along the entire flow velocity gradient.**[1]** 
      1. LABMEDIA: Figure 4G
   3. Importantly, the automated OCT imaging system allows a continuous measurement of structural parameters such as biofilm thickness, roughness, and biovolume under differing flow conditions ranging from low flow velocity … to high flow velocity conditions.**[1]**
      1. LABMEDIA: Figure 4G, 4A, 4B, and 4C **- Video Editor: Label Figure 4g “Biofilm Morphogenesis”Start by showing Figure 4G. With the word “low** **flow velocity”, show Figure 4a coming from the left side of Figure 4G and growing to take up a significant part of the screen before disappearing. Then, with the words “high flow velocity conditions” show Figure 4c coming from the right side of Figure 4G and growing to take up a significant part of the screen before disappearing.**
   4. Along with morphological changes, average biovolume significantly decreased as a function of distance from the inlet in the flume. **[1]**
      1. LABMEDIA: Figure 4h/i **- Video Editor: Show Figure 4h above 4i as currently shown in the figure. Highlight the x-axis label of both figures with the words “distance from the inlet”**

Section - Conclusion

1. **Conclusion Interview Statements:**

What is most important thing to remember when attempting this procedure? Please indicate the steps (*e.g.*, 2.4., 2.5.) in the Protocol section this advice correlates to.

* 1. **Anna Depetris**: The quality of the OCT scans critically depends on the focus distance and reference arm length. You may need to re-adjust these settings during experiments.
  2. **Anna Depetris**: (step 3.6.3) To ensure the accuracy of the positioning device, remember to regularly perform homing operations.

Following this procedure, what other methods can be performed? What questions would these additional methods answer?

* 1. **Anna Depetris**: In order to answer additional questions, this automated imaging device can be readily coupled with microsensors to profile functional parameter of biofilms.

After its development, did this technique pave the way for researchers to explore new questions within a specific scientific field? If so, how?

* 1. **Hannes Peter**: OCT is an emerging imaging technique and we anticipate that the system presented here stimulates research on biofilm structural properties. This maybe be relevant for technologies such as drinking water treatment or bioprocessing technologies.