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

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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. Overview of the experimental system
   1. Here is an overview of the installation. The system is composed of a precision positioning device, the OCT probe and the OCT base unit. The system is assembled around a plexiglass flume.
      1. PHOTO 2-1-1: found in folder **“overview of experimental setup (photo 2.1.1) “** . Please add arrows appearing in sync with audio (OCT probe, precision positioning device etc) as shown in the annotated picture (for reference only, same folder).
2. **Setup of the Positioning Device**
   1. Begin by wiring the positioning device by following along with instructions posted on github.**[1-TXT]**
      1. SCREEN: 3-1-1\_GRBLpage.flv

**TEXT:** **https://github.com/ grbl/grbl/wiki/Connecting-Grbl**

* 1. Once connected, install the GRBL server as described in a separate gitlab page.**[2-TXT]** 
     1. SCREEN: 3-2-1\_GRBLsrv\_installation.flv

**TEXT:** **https://gitlab.com/ FlumeAutomation/ GRBL\_Server.git**

* 1. **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/**](http://IP:5020/)
     2. MED: insert here MED 1:26-1:30 of the first video version. In this MED you see a computer on a desk with a blue/greenish box close to it and a motor that turns. Of this media, please remove the part where a hand connects a cable and show just the following part, where you see the hand on the mouse and the motor rotating. **TEXT:** [**http://IP:5020/**](http://IP:5020/)

1. **Optical Coherence Tomography Setup**
   1. ~~P~~osition the computer and the OCT base unit on a bench next to the experimental setup containing microfluidic devices, flow chambers, flumes, or filtration systems. **[1]** 
      1. MED: Show the computer and OCT base unit next to the experimental setup
   2. If not already installed, install the OCT system together with the available software as described by the manufacturer.**[1]**
      1. MED Over the Shoulder: Talent opens OCT software at the computer
   3. Then, install the software packages for automated OCT scan acquisition as described in the gitlab documents linked to here. **[2-TXT]**
      1. SCREEN: 4-3-1\_octautomated\_installation.flv

**TEXT: https://gitlab.com/FlumeAutomation/automated-oct-scans-acquisition.git**

1. **Image Acquisition**
   1. To begin image acquisition, mount the optical coherence tomography probe to the positioning device using a compatible dove-tail holder.**[1]** If required, install an immersion adapter on the objective lens **TEXT: Additional immersion adapters are available for individual setups**.**[2-TXT]** Power on the OCT system and the positioning device. Make sure that the device can move freely. **[1]**
      1. MED: Talent mounts the OCT probe on the positioning device and turns on the instrument
      2. MED: Talent tests cord length
   2. 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 5-2-1\_Thorimage.flv *(please gray out the IP address of our computer)*
   3. 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 **5.3.1\_open\_image\_acquisition.fl**v (just from second 2 to second 21)
   4. Follow the worked example to set the path to the required libraries… to connect the positioning device… to calibrate the positioning device … to initialize the OCT scanner… .
      1. SCREEN **5-4-1\_new\_params.flv** (from second 1 to second 14), highlight cells in sync with voice (connect…calibrate…initialize), for instance by adding a red rectangle around the specific cell  *(please gray out the IP address of our computer)*
   5. Then, adjust the acquisition parameters including the refractive index, the size of the field-of-view and the number of A-scans per B-scan. Further set the signal boundaries of the OCT scan based on intensity histograms of preliminary scans and the destination folder for acquired data and metadata.**[1]** Depending on the Field of View and resolution, the file size may reach up to 1.5 GB per OCT scan.
      1. SCREEN **5-4-1\_new\_params.flv** (from second 14 on)
      2. **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]**
   6. As highlighted in the worked example, you may acquire a single OCT scan with default parameters …. or acquire a single scan specifying a different set of parameters….. You may also provide specific coordinates to move the positioning device and acquire a single OCT scan. This feature allows you to repeatedly return to the exact same position in the experiment with high spatial accuracy.
      1. SCREEN **5-6-1\_makeScan\_metadata.flv** Freeze at second 5, and highlight cells in sync with audio (makeScan...makeScan(params…)...makeMoveScan)
      2. MED: OCT probes moves and dips into the water
   7. 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.
      1. SCREEN **5-6-1\_makeScan\_metadata.flv** Continue from second 5 to about second 120, please crop the part of the video where nothing is happening and accelerate or crop the part where the image is acquired. The final video should show the black box coming out and the numbers close to “Current image xxx/900” starting to increase, then the cursor opening the destination folder and then opening the metadata MicrosoftWord file.
   8. You can also specify a list of positions of interest …… and acquire the respective scans automatically.
      1. SCREEN – **5-8-1\_listpositions.flv (**from second 1 to second 5) highlight: positions = {…} …. ‘for p positions…’ in sync with audio.
      2. MED: OCT moves and dips into the water in different locations that are far apart from each others (in contrast with following MED where the locations are close to each other)
      3. SCREEN – **5-8-1\_listpositions.flv (**from second 33 on) Accelerate/crop as best but show the last part of the video where the destination folder is open and the two folders named “2019-06-07...pos1” “2019-06-07….pos2” are visible.
   9. In order to characterize biofilm morphological structures across large environmental gradients you can acquire scans in a mosaic pattern. For this, specify the number of neighboring tiles with a default overlap of 30%.
      1. SCREEN – **5-9-1\_multiplescan.flv** from second 1 to second 5,highlight cell multipleScan
      2. MED: OCT moves and dips into the water in locations that are close to each others (in contrast with previous MED)
      3. SCREEN – **5-9-1\_multiplescan.flv** from second 70 on, crop/accelerate as needed but folders named “…...pos1-0” to “…...pos1-9” at the end of the video should be visible.
2. **Image Correction and Display**
   1. **Antoine Wiedmer**: 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*.
      1. **SCREEN 6-2-1\_correctionpart1.flv (**from second 1 to about second 20)
   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 6-3-1\_correctionpart2.flv** (accelerate/crop as needed)
   4. Next, correct for spherical aberration. To accomplish this, we provide an algorithm which localizes a highly reflective flat surface in the OCT scan and uses this reference surface to flatten the scans. Across a 20 by 20 grid, the algorithm identifies local maxima in signal intensity to localize the reference surface. Then a 2nd order polynomial surface is fitted across these points and used to shift each pixel of the OCT scan in z-direction. The parameters of this algorithm should be adjusted to the characteristics of the OCT scan. This correction enable a homogeneous reference surface across multiple images and thus facilitates stitching of large-scale images.

Once the image has been flattened, the images are corrected for background noise by identifying an empty area of the image above the biofilm and subtracting average background intensity.**[1]**

* + 1. SCREEN: **6-4-1\_correctionpart3.flv** (accelerate/crop as needed)
  1. Next, compute an elevation map from the 3D OCT dataset. In order to do so, define a reference surface such as the substratum and choose an appropriate intensity threshold. Then, an elevation map is rendered with the height of the biofilm reported as grayscale value. [1]
     1. SCREEN: **6-5-1\_elevmap.flv** (...)
  2. If images were acquired in a mosaic pattern, stitch the respective elevation maps by applying the stitching algorithm.
     1. *SCREEN:* ***6-6-1\_stitching.flv*** *(accelerate/crop as needed, but make sure the final image is displayed, eg second 17)*

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) (videos uploaded in folder **“missing media 7.1.1”)**
   2. The high spatial accuracy allows us to follow the morphogenesis of biofilm features over time.
      1. LABMEDIA: **Figure 4,** please show one panel at a time in a time-lapse fashion, rather than showing them in column one below the other
   3. Here is an elevation map of a biofilm growing along the entire flow velocity gradient.**[1]** 
      1. LABMEDIA: Figure 5G
   4. 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 5G, 5A, 5B, and 5C **- Video Editor: Label Figure 5g “Biofilm Morphogenesis”Start by showing Figure 5G. With the word “low** **flow velocity”, show Figure 5a coming from the left side of Figure 5G and growing to take up a significant part of the screen before disappearing. Then, with the words “high flow velocity conditions” show Figure 5c coming from the right side of Figure 5G and growing to take up a significant part of the screen before disappearing.**
   5. Along with morphological changes, average biovolume significantly decreased as a function of distance from the inlet in the flume. **[1]**
      1. LABMEDIA: Figure 5h/i **- Video Editor: Show Figure 5h above 5i 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.