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## Automated 3D optical coherence tomography to elucidate biofilm morphogenesis over large spatial scales --Manuscript Draft--

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**TITLE:**

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

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**KEYWORDS:**

biofilm, optical coherence tomography (OCT), in situ visualization, automation, monitoring, biofilm surface topology, 3D structure, hydrodynamics

**SUMMARY:**

Microbial biofilms form complex architectures at interphases and develop into highly scale-dependent spatial patterns. Here, we introduce an experimental system (hard- and software) for the automated acquisition of 3D optical coherence tomography (OCT) datasets. This toolset allows the non-invasive and multi-scale characterization of biofilm morphogenesis in space and time.

**ABSTRACT:**

Biofilms are a most successful microbial lifestyle and prevail in a multitude of environmental and engineered settings. Understanding biofilm morphogenesis, that is the structural diversification of biofilms during community assembly, represents a remarkable challenge across spatial and temporal scales. Here, we present an automated biofilm imaging system based on optical coherence tomography (OCT). OCT is an emerging imaging technique in biofilm research. However, the amount of data that currently can be acquired and processed hampers the statistical inference of large scale patterns in biofilm research. The automated OCT imaging system allows covering large spatial and extended temporal scales of biofilm growth. It combines a commercially available OCT system with a robotic positioning platform and a suite of software solutions to control the positioning of the OCT scanning probe, as well as the acquisition and processing of 3D biofilm imaging datasets. This setup allows the in situ and non-invasive autonomous monitoring of biofilm development and may be further developed to couple OCT imaging with macrophotography and microsensor profiling.

**INTRODUCTION:**

Biofilms are a highly successful microbial lifestyle adaptation and these interphase-associated and matrix-enclosed communities of microorganisms dominate microbial life in natural and

industrial settings<sup>1,2</sup>. There, biofilms form complex architectures, such as elongated streamers<sup>3</sup>, ripples<sup>4</sup> or mushroom-like caps<sup>5</sup> with important consequences for biofilm growth, structural stability and resistance to stress<sup>6</sup>. While much about biofilm structural differentiation has been learned from work on mono-species cultures grown in miniature flow chambers, most biofilms are highly complex communities often including members of all domains of life<sup>6</sup>. Appreciating these complex biofilms as microbial landscapes<sup>7</sup> and understanding how biofilm structure and function interact in complex communities is thus at the forefront of biofilm research.

A mechanistic understanding of the morphogenesis of complex biofilms in response to environmental cues requires carefully designed experiments in conjunction with spatially and temporally resolved observations of biofilm physical structure across relevant scales<sup>8</sup>. However, the non-destructive observation of biofilm growth in experimental systems has been severely limited by logistic constraints such as the need to move samples (e.g., to a microscope) often damaging the delicate biofilm structure.

The protocol presented here introduces a fully automated system based on optical coherence tomography (OCT), which allows the in situ, non-invasive monitoring of biofilm morphogenesis at the mesoscale (mm range). OCT is an emerging imaging technique in biofilm research with applications in water treatment and biofouling research, medicine<sup>9</sup> and stream ecology<sup>10</sup>. In OCT, a low coherence light source is split into a sample and reference arm; the interference of the light reflected and scattered by the biofilm (sample arm) and the light of the reference arm is analyzed. A series of axial intensity profiles (A-scans) which contains depth-resolved structural information is acquired and merged into a B-scan (a cross section). A series of adjacent B-scans composes the final 3D volume scan<sup>10</sup>. OCT provides a lateral optical resolution in the range of approximately 10  $\mu\text{m}$  and is therefore well suited to study mesoscopic structural differentiation of biofilms<sup>10,12</sup>. For a more detailed description of OCT, refer to Drexler and Fujimoto<sup>13</sup> and Fercher and colleagues<sup>14</sup>. Although the field-of-view of a single OCT xy-scan reaches up to hundreds of square micrometers, larger-scale patterns cannot be quantified by means of OCT in a single scan. With respect to biofilms in natural habitats such as streams and rivers, this currently limits our ability to assess biofilm morphogenesis at scales matching the physical and hydraulic template of the habitat.

In order to surpass these spatial limits and to acquire OCT scans automatically, a spectral-domain OCT imaging probe was mounted on a 3-axis positioning system. The installation permits the automated acquisition of several OCT scans in an overlapping mosaic pattern (tile scan), effectively achieving the tomographic imaging of surface areas up to 100  $\text{cm}^2$ . Furthermore, the high positioning precision of this system enables to reliably monitor the growth and development of biofilm features in specific sites during long-term experiments. The system is modular and individual components (i.e., positioning device and OCT) of the installation can be used as standalone solutions or flexibly combined. **Figure 1** provides an overview of the hard- and software components of the installation.

The system was tested with a commercially available GRBL-controlled CNC positioning device (**Table of Materials**). The operating distances of this specific positioning platform are 600×840×140 mm, with a manufacturer-indicated accuracy of +/- 0.05 mm and a programmable

resolution of 0.005 mm. GRBL is an open-source (GPLv3 License), high-performance motion control for CNC devices. Therefore, every GRBL-based (version > 1.1) positioning device should be compatible with the guidelines and software packages presented here. Moreover, the software could be adapted to other stepper motor controllers with STEP-DIR input type with few modifications.

The OCT device used to assess the performance of the system (**Table of Materials**) features a low coherence light source with a center wavelength of 930 nm (bandwidth = 160 nm) and adjustable reference arm length and intensity. In the example presented here, an immersion adapter for dipping the OCT probe into flowing water was also used (**Table of Materials**). The software package developed here for automated OCT scan acquisition critically depends on the SDK provided together with the specific OCT system, however, OCT systems from the same manufacturer with different scan lenses and central wavelengths should be readily compatible.

The GRBL device is controlled by a web server installed on a single-board computer (**Figure 1**). This grants remote control of the device from any computer with local network or internet access. The OCT device is controlled by a separate computer, allowing the operation of the OCT system aside the automated experimental setup. Finally, the software packages include libraries to synchronize OCT probe positioning and OCT scan acquisition (i.e., to automatically acquire 3D imaging datasets in a mosaic pattern or in a set of defined positions). Defining the position of the OCT probe in 3D effectively allows to adjust the focal plane specifically for (regional) sets of scans. Specifically, on uneven surfaces, different focal planes (i.e., different positions in z direction) can be specified for each OCT scan.

A set of software packages was developed to process raw OCT scans (**Table 1**). Navigation of the positioning device, OCT scan acquisition and dataset processing are performed with Python-coded Jupyter notebooks, which allow remarkable flexibility in the development and optimization of the software. Two worked and annotated examples of such notebooks (for image acquisition and processing, respectively) are available from <https://gitlab.com/FlumeAutomation/automated-oct-scans-acquisition.git>. They are intended as starting points for customization of the method. A Jupyter notebook is a web browser based application which contains cells with annotated Python code. Each step is contained in a cell of the notebook, which can be executed separately (e.g., by pressing **Cell | Run**, or with the key sequence: **Ctrl + Enter** or **Shift + Enter**). Due to the different length of the light path through the scan lens (spherical aberration)<sup>15</sup>, the raw OCT scans appear distorted (**Figure 2A**). We developed an algorithm to automatically correct for this distortion in acquired OCT scans (contained in *ImageProcessing.ipynb*, **Supplementary File 1**). Furthermore, biofilm morphology can be visualized as a 2D elevation map, as was previously used in membrane systems<sup>16</sup>, and we illustrate how elevation maps obtained from scans taken in a tiling array can be stitched.

Finally, the functionality of the described laboratory installation is illustrated using a flume experiment in which phototrophic stream biofilm is exposed to a gradient of flow velocity.

## PROTOCOL:



## 1. Setup of the Positioning Device

1.1 Wire the positioning device to a microcontroller board, following the instruction in <https://github.com/grbl/grbl/wiki/Connecting-Grbl>.

1.2 Connect the microcontroller to a single-board computer with internet connection via a USB cable and install the GRBL server as described in [https://gitlab.com/FlumeAutomation/GRBL\\_Server.git](https://gitlab.com/FlumeAutomation/GRBL_Server.git). Now the positioning device should be navigable from a webpage hosted at <http://IP:80/>. Alternatively, the positioning device can be navigated with a Python script, as demonstrated in the first part of the worked example *ImagesAcquisition.ipynb* (**Supplementary File 2**).

## 2. OCT Setup

2.1 Mount the OCT probe to the positioning device using a compatible dove-tail holder. If required, install an immersion adapter on the objective lens.

2.2 Position the computer and OCT base unit on a bench next to the experiment (e.g., microfluidic devices, flow chambers, flumes, filtration systems). Make sure that the optical cord (maximum length of approx. 1.8 m) is freely moving, long enough to reach all intended locations and not interfering with the experimental setup.

2.3 Install the OCT system together with the available software as described by the manufacturer.

2.4 Install the software packages for automated OCT scan acquisition as described in <https://gitlab.com/FlumeAutomation/automated-oct-scans-acquisition.git>.

## 3. Image Acquisition

3.1 Power on the OCT system and the positioning device. Make sure the device can move freely.

3.2 Open the file *config.json* in a text editor. Edit the *config.json* file to adjust image acquisition parameter (**Table 2**), such as the refractive index (1.33 for water at 20 °C, 1.00 for air), scan speed, as well as the destination folder for acquired data and metadata.

3.3 Define the size of the field-of-view (FOV) and the number of A-scans per B-scan in *config.json*.

NOTE: 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 probe (x-y voxel size should not be

smaller than half of the optical resolution). The number of A- and B-scans affects the spatial extent to be covered which trades-off against available disk space and processing power.

3.4 Define the signal boundaries of the output OCT scan in *config.json*. These depend on the type of sample. It is thus recommended to determine these parameters based on intensity histograms of a set of preliminary scans. Save the changes in *config.json*.

3.5 Navigate the OCT probe to a site of interest. Focus the sample and adjust the reference arm and light source intensity for optimal image quality. Repeat this procedure for a number of positions and note the coordinates.

NOTE: This will allow the subsequent automatic OCT scan acquisition around these reference points. Note that the reference arm length and intensity cannot be changed during automated image acquisition.

3.6 Open the *ImageAcquisition.ipynb* file (**Supplementary File 2**) in Jupyter Notebook. Each cell contains code to perform specific tasks and can be run separately via pressing **Cell | Run**, or **Ctrl + Enter** or **Shift + Enter**.

3.6.1 Set the path to the required library configuration parameter.

3.6.2 Connect to the positioning device and initialize the OCT.

3.6.3 Calibrate the positioning device (i.e., perform a “homing”).

3.6.4 Acquire the datasets covering the positions of interests in single-scan or mosaic pattern, specifying the number and the overlap (e.g., 30%) of neighboring tiles.

NOTE: The memory is allocated prior to the scan, which optimizes computer resource use. Data is saved in 8 bits *\*.raw* format to save storage space, into the destination folder defined in *config.json*, using the time stamp and the position as naming convention (i.e., %Y%m%d\_%H%M%S\_<position>). Metadata including the OCT settings and coordinates are saved in the same folder in a *\*.srm* 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.

3.7 To avoid abortion of data acquisition, make sure that there is sufficient free disk space or continuously move OCT datasets to an external hard drive.

## 4. Image Correction and Display

4.1 Open the Jupyter notebook *ImageProcessing.ipynb* (**Supplementary File 1**) for a worked example of OCT image processing (correction of distortion, background subtraction, calculation of elevation maps, elevation map stitching).

4.2 If required, crop OCT scans in order to exclude spurious signals and reoriented the dataset (biofilm should appear above the substratum).

4.3 Correct for spherical aberration. This is accomplished by an algorithm which identifies a highly reflective reference surface known to be flat (e.g., bottom of the flume, substratum). First, define a grid of 20×20 vertical lines regularly spaced across the xy-plane of the OCT scan. Select a circular area around each point and average along the vertical profile (**Figure 2B**). Apply a modified Gaussian filter to each vertical profile, in order to identify local maxima in signal intensity (i.e., the position of the highly reflective reference surface). The vertical profiles are filtered as follows:

$$\omega(x) = e^{\frac{1}{2}(\frac{x}{\sigma})^2} + C$$

where  $x$  is the input signal, and  $\sigma$  its standard deviation, while  $C$  is determined such as:

$$\int_{-a}^a \omega(x) dx = 0$$

Misidentified points are filtered based on the positions of their neighbors in three dimensions (**Figure 2C**). Finally, a 2<sup>nd</sup> order polynomial surface reflecting the distortion introduced by the scan lens is fitted across these points (**Figure 2C**). The fitted surface is then used to shift each pixel in z-direction, thus obtaining a flattened image. The parameter of this algorithm should be adjusted to the characteristics of the OCT scan.

4.4 Correct for background noise. Identify an empty area of the image (typically above the biofilm) and calculate the average background intensity, which is then subtracted from the intensity values of the image to produce a final corrected OCT image (**Figure 2D**).

4.5 Define a reference surface (e.g., the substratum) and compute an elevation map from the 3D OCT dataset. In this step, the corrected images are thresholded and the thickness of the biofilm is calculated for each coordinate (x,y) of the binary mask (**Figure 3A**). Thickness values are then assigned to a 2D matrix of the size of the original image in x and y directions. An image is rendered in which the elevation of the surface is reported as grayscale value (**Figure 3B**).

4.6 In case several OCT scans are taken in a mosaic pattern, define the number of rows and columns and stitch the elevation maps. **Figure 5** presents examples of stitched elevation maps, covering the broad range of spatial scales and resolutions achievable with the described setup.

#### REPRESENTATIVE RESULTS:

We demonstrate the functionality of the automated OCT imaging system using a flume experiment designed to study the spatio-temporal morphogenesis of phototrophic stream biofilms. A gradually narrowing geometry of the flumes induced gradients in flow velocity along

the center of the flume (see reference<sup>17</sup>). The temporal development and structural differentiation of biofilm was monitored over 10 days with the aim to better understand the effects of hydrodynamic conditions on biofilm morphogenesis. Surface morphology of the biofilm was quantified using the toolset described above (**Figure 4A**). Biovolume was calculated (see worked example *ImageProcessing.ipynb*, **Supplementary File 1**) for a square moving window with 3.6 mm edge length (**Figure 4B**) for each position along the flow velocity gradient (**Figure 4C**). Biofilm accumulation significantly decreased with increasing flow velocity (indicated as the distance from the widest part of the flume; **Figure 4**). Importantly, this experimental setup allows a continuous measurement of structural parameters (e.g., biovolume, thickness, roughness) along large spatial gradients. Hence, this new tool provides the means to gain insights into relationships between biofilm structure and environmental cues.

#### **FIGURE AND TABLE LEGENDS:**

**Table 1.** Software components.

**Table 2.** OCT parameter settings.

**Figure 1. Overview of hard- and software components.** The steppermotors of a GRBL-controlled positioning device are wired to a microcontroller, connected via USB to a single-board computer. The GRBL server is installed on the latter, and motion of the positioning device can be controlled from any web browser via TCP/IP connection. Alternatively, navigation of the positioning device can be performed from a Python-encoded Jupyter notebook (*ImagesAcquisition.ipynb*, **Supplementary File 2**) using the GRBLServer.py library. The OCT system is connected to a separate computer from which automated OCT scan acquisition can be performed via a Python script.

**Figure 2. OCT scan correction workflow.** Panel A shows a non-processed B-scan of biofilm growing on a flat plexiglass surface. The image is distorted (bend) because of differences in path length of the low coherence light through the lens. OCT image distortion can be corrected by identifying a strongly reflecting, flat reference surface in the image. First, 20×20 reference points are evenly distributed across the entire stack of images. In each of these points, the image signal is averaged across a circular area (in x-y direction) for each depth (z plane), obtaining an averaged depth profile of signal intensity. Then, a modified Gaussian filter is applied to each of the 400 reference profiles. Panel B provides an example of the original signal along the depth profile indicated by the vertical red line in Panel A, the averaged depth profile, and the same profile after the modified Gaussian filter has been applied. The modified Gaussian filter allows the identification of local maxima in signal intensity, thus identifying the location of the strongly reflecting reference surface. Correctly identified reference points are then selected based on the coordinates of their neighbors in three dimensions. In the example in panel C, the yellow points were kept for subsequent image correction whereas purple ones were discarded. A 2<sup>nd</sup> order polynomial surface is then fit to the correctly placed reference points and used to correct the distortion in the original OCT image by shifting pixels in z direction. Average background intensity is estimated from an empty area of the image and subtracted from the corrected images. Panel D shows the same B-scan after correction and background subtraction.

**Figure 3 Elevation maps.** Biofilm topology can be visualized as 2D elevation maps in which thickness of the biomass is color coded. For this, a 3D OCT image is thresholded and biofilm thickness calculated as the distance of the uppermost signal to the substrate. Panel A shows the binary mask of a B-scan obtained after thresholding. The blue line indicates the uppermost signal while the red line shows the reference surface. Panel B shows an example of the obtained elevation map, scaled according to the axial resolution of the OCT probe. The red line indicates the position of the B-scan in Panel A.

**Figure 4 Representative results showing the effect of flow velocity on biofilm growth.** We studied phototrophic stream biofilm morphogenesis along a gradient in flow velocity using flume experiments. Flow velocity increased with distance from the inlet of the flume. After 10 days of growth, biofilm morphology was characterized by automated OCT at different resolution and covering different spatial scales. Elevation maps (A, B and C) demonstrate the morphology of biofilm grown under low, medium and high flow velocity, respectively. These elevation maps are calculated from OCT scans with voxels size in x, y direction of 4  $\mu\text{m}$ . The scan surface area is a square of 3.6 mm edge length. Panels D, E and F show elevation maps (low, medium and high flow velocity, respectively) obtained by stitching 3 $\times$ 3 OCT scans with a voxel size in xy-direction of 11  $\mu\text{m}$ , scan area of 10 mm<sup>2</sup> and an overlap between neighboring scans of 30%. Panel G shows an elevation map of biofilm growing along the entire velocity gradient achieved in this flume experiment. It was obtained by stitching 3 $\times$ 51 OCT scans with a voxel size in xy-direction of 40  $\mu\text{m}$ , scan area of 10 mm<sup>2</sup> and an overlap between neighboring scans of 30%. The total scan area achieved is 24 $\times$ 353 mm. Panel H reports biovolume in a square moving window of 3.6 mm edge. Average biovolume significantly decreased as a function of distance from the inlet (I).

**Figure 5 Precision test for the positioning device.** The precision of the positioning device was assessed by mounting a 20.2 Megapixels camera equipped with a 35 mm macro lens on the positioning device, focused on a colored mark. The positioning device was moved in a random direction away from the mark and then positioned back for a total of 80 cycles. The position of the mark was then compared. The figure shows the shift in x and y direction with respect to the first picture. Note that the maximum shift is approximately 0.02  $\mu\text{m}$  in x-direction and even less in y-direction.

**Supplementary File 1. *ImageProcessing.ipynb*.**

**Supplementary File 2. *ImagesAcquisition.ipynb*.**

## **DISCUSSION:**

OCT imaging is well suited to resolve structures in the micrometer range with a FOV of several square millimeters. It is thus a powerful tool for biofilm research<sup>10,18</sup>. However, OCT is currently limited to a maximum scan area of 100 - 256 mm<sup>2</sup>, while biofilm structural patterns often exceed this spatial scale<sup>19</sup>, especially when morphological differentiation is driven by large scale environmental gradients<sup>20</sup>. The automated OCT imaging system described in this protocol extends the surface area characterized by OCT to several square centimeters, effectively enabling

us to monitor biofilm morphological differentiation over a relevant range of spatial scales (from few millimeters to several centimeters). The high positioning accuracy (within 0.02  $\mu\text{m}$ ; **Figure 5**) allows to accurately monitor the structural development of biofilms over extended periods of time, effectively boosting the opportunities for obtaining a mechanistic understanding of the drivers of biofilms morphological differentiation. At the same time, this in situ biofilm characterization technique is non-invasive and minimizes the interference with biofilm growth. The image processing solutions presented here build on previously employed analyses of biofilm OCT datasets<sup>16</sup>, yet the automation provides tools for unprecedented time- and space resolved OCT dataset analyses.

This system was conceived and benchmarked with a specific OCT device, as described in the protocol. Critical steps in the protocol mainly concern the setting of OCT resolution and focusing, which are both critical for high image quality. A limitation of the correction of spherical aberrations routine is that it depends on the presence of a highly reflective flat surface. Alternatively, a standard correction surface could be measured, and then used to correct OCT scans. Furthermore, the stitching of OCT scans depends on sufficient structural features to align neighboring scans. In case of uniform biofilm distribution or low biofilm coverage, stitching may be achieved relying solely on the precision of the positioning device. Finally, as in any other image processing pipeline, when setting up these tools, it is critical to carefully assess the performance of the processing algorithm on a set of representative scans before handling batches of images. However, both hard- and software were designed to provide full modularity of the individual parts. More specifically, this system can be easily adapted to work with other tools for biofilms characterization such as macro-photography imaging using hyperspectral cameras or microsensor profiling. The coupling of structural information with localized gradients in resources around and within biofilms will provide novel and pivotal insights into the way how biofilms are adapted to optimize resource allocation. The flexibility is also implemented through the use of Jupyter notebooks, an open-access, fast and versatile software developing tool.

A critical limitation of OCT imaging in general remains the disability to resolve rapidly moving objects. For instance, streamers elongating into and moving with the flow are not accurately depicted. The applicability of this tool is thus limited to relatively fixed, non-moving biofilm structures. The system is optimized to work autonomously, however, initial settings and if necessary, the adjustment of focus and illumination, are still required. This represents a significant limitation if samples differ significantly in density and reflective properties. Full automation, including software-guided focusing and adjustment of illumination may however be achieved using similar principles (e.g., stepper motors and software-hardware feedbacks) as described here.

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#### **DISCLOSURES:**

Sebastian Schäfer is employed at Thorlabs Inc.

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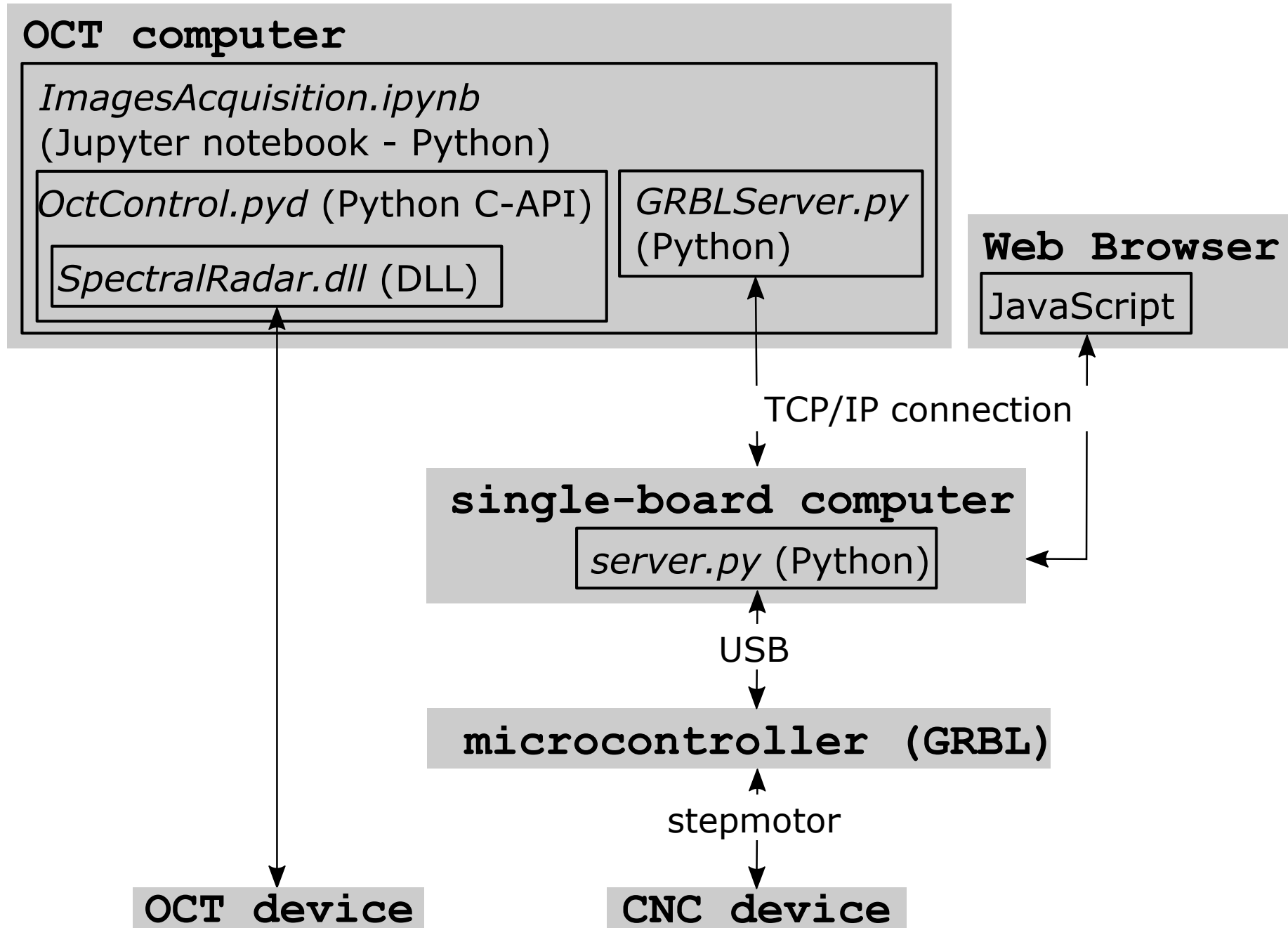
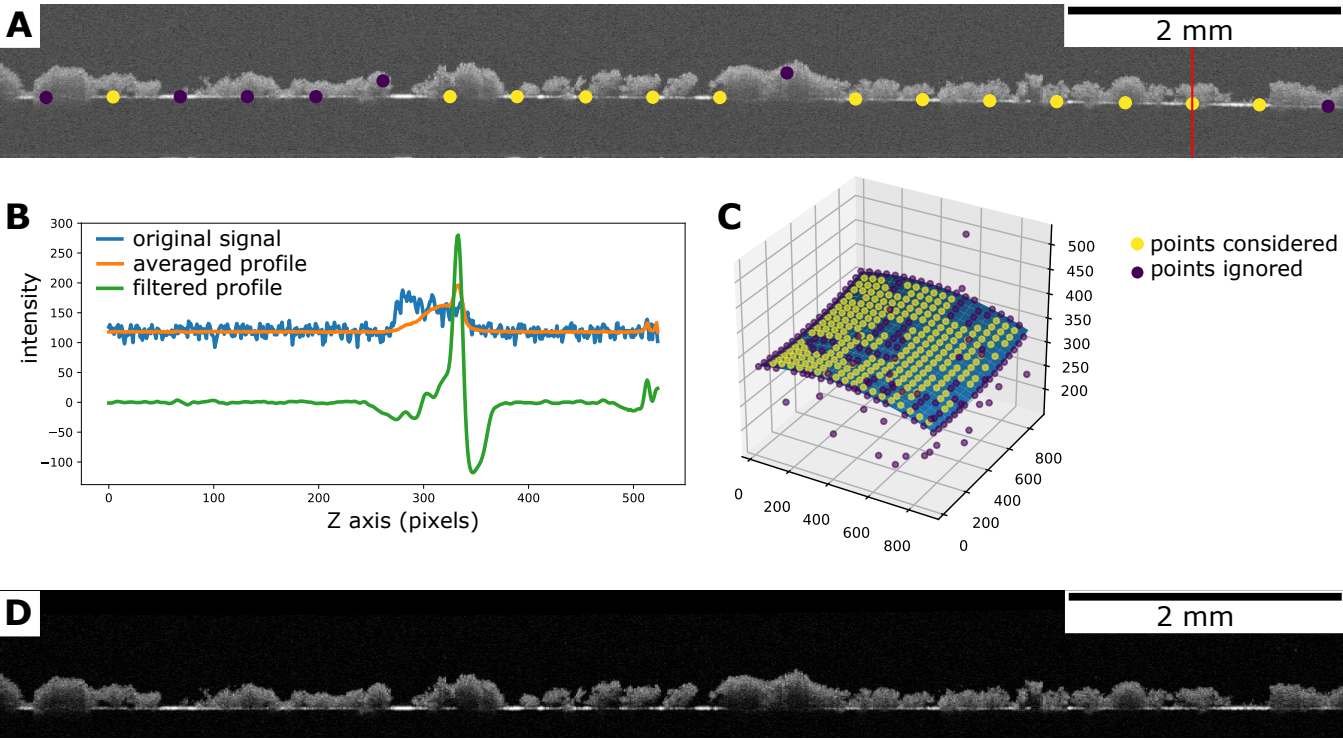
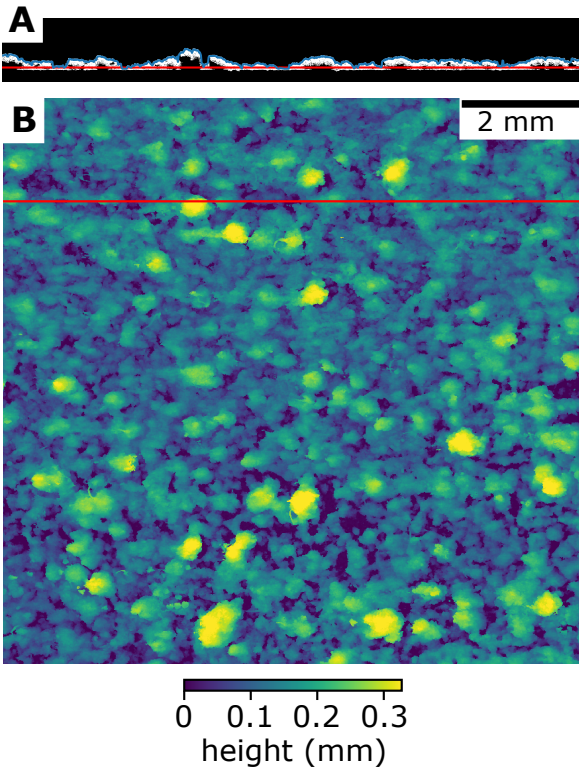


Figure 2





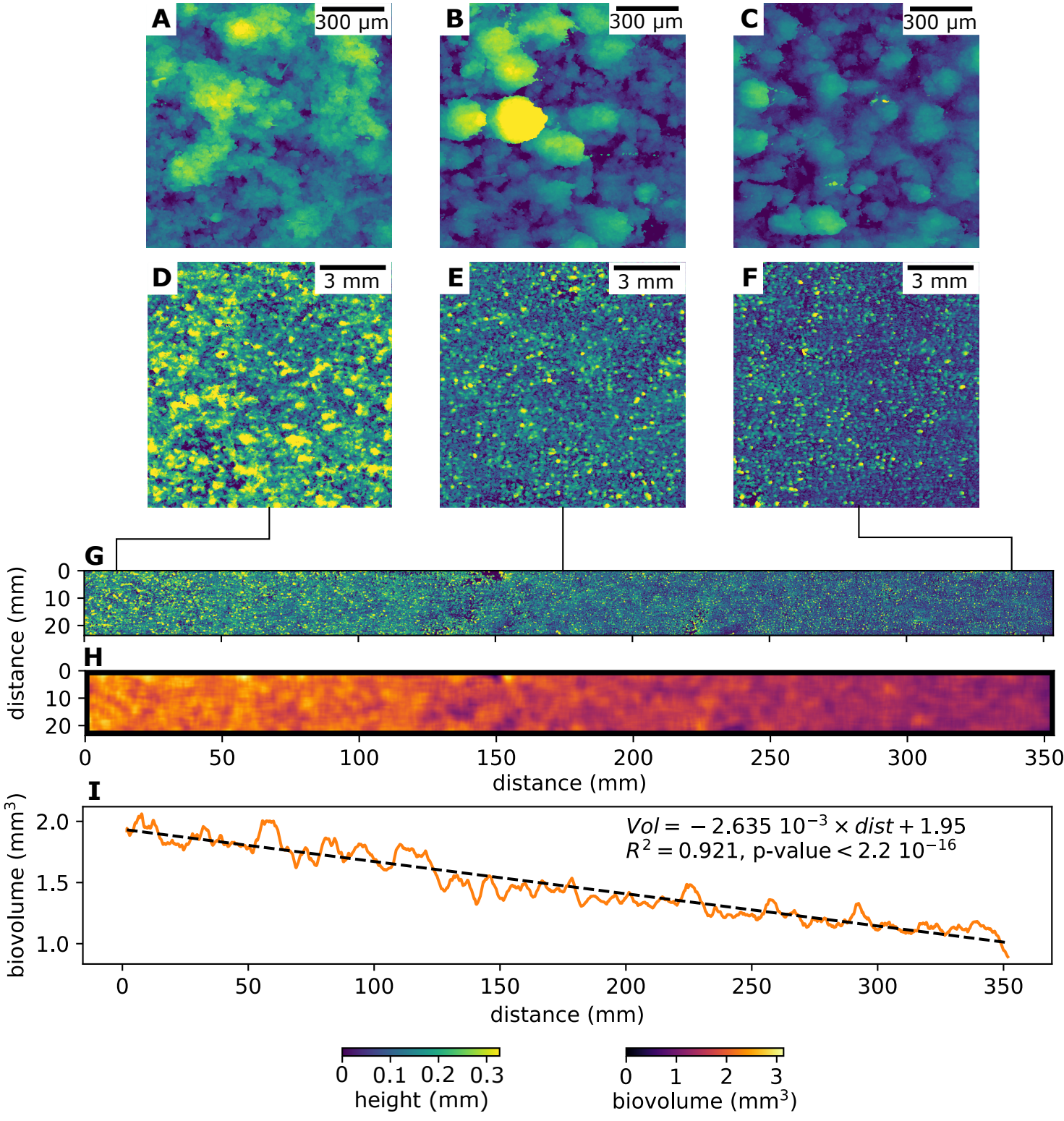
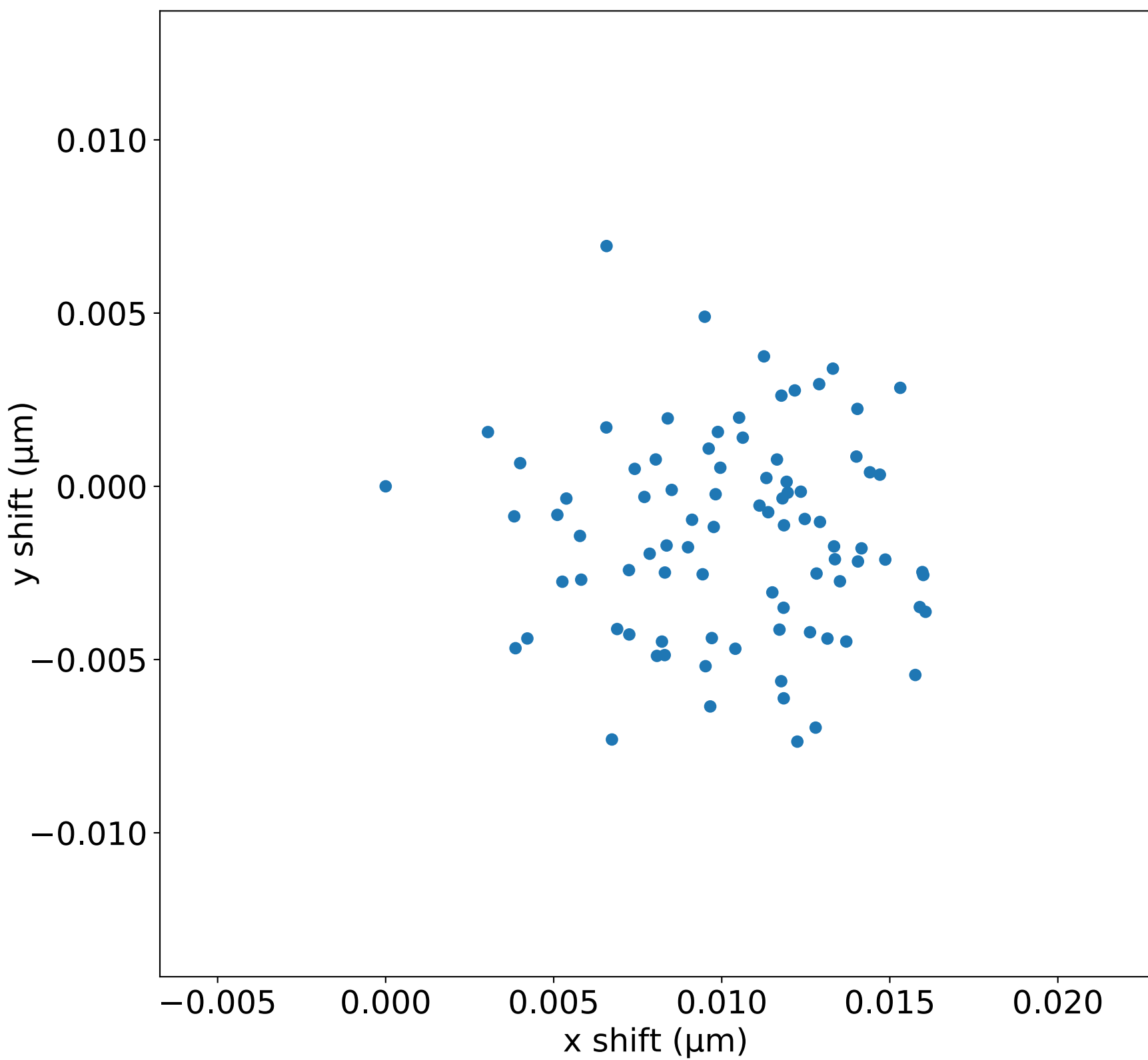


Figure 5



Software component	Description
stepcraft.py	A python library to control the positioning device. It contains definitions for navigating and homing the device.
OctControl.cpp	C++ code derived from the Software Development Kit (SDK) distributed with the OCT system. This has to be compiled using VisualStudio 2017, PythonC/API and the SDK.
ImagesAcquisition.py	A Python library containing the commands for taking OCT scans in selected positions and defining the scan tiling pattern.
ImagesAcquisition.ipynb	Jupyter notebook used to navigate the positioning device, acquire OCT scans and for automated image acquisition.
OctCorrection.py	A Python library defining the functions used for the correction of the raw OCT images and background subtraction.
OctProcessing.py	A Python library containing the functions to calculate and stitch elevation maps.
OctProcessing.ipynb	Jupyter notebook to visualize, correct and process OCT scans. This also contains an example data analysis (calculation of biovolume).

Parameter	Value	Description
Ganymede	1, 2, 3	Choice of OCT system and version
Probe	1, 2	Choice of scan lens
nAscans	32-900	Number of A-scans per B-scan
nBscans	1-900	Number of B-scans
nCscans	128-1024	Number of depth pixels
X	0.1-10	Size of image in x-direction (mm)
Y	0.1-10	Size of image in y direction (mm)
refr	1-1.6	Refractive index (1 for air, 1.33 for water)
avg_Ascans		3 Number of A-scan averaging
scanspeed	1,2,3	A-scan Rate (5.5, 15 and 36 kHz)
path	"../ %Y-%m-%d_%H_%M_%S"	Destination folder for the acquired OCT scans, uses time stamp as naming confention
colorBoundaries	[0.0-256.0,0.0-256.0]	Color boundaries of the acquired scans

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
OCT Probe	Thorlabs	GAN210C1	OCT imaging device
OCT scan lens	Thorlabs	OCT-LK3-BB	
Immersion adapter	Thorlabs	OCT-IMM3-SP1	
Stepcraft 840 CK	STEPCRAFT	NA	
microcontroller	Arduino Uno R3	NA	
Single-board computer	Raspberry PI	NA	positioning device
camera	Canon EOS 7D Mark II	NA	
camera lens	Canon MACRO EFS 35 mm	NA	



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AUTOMATED 3D OPTICAL COHERENCE TOMOGRAPHY TO EVALUATE  
BIOPILH MORPHOGENESIS

Author(s):

ANNA DEPETRIS, ANTOINE WIGNER, MICHAEL WAGNER, SEBASTIAN  
SCHAFER, JON BATTIN, HANNEK PETER

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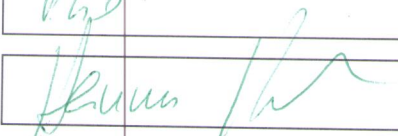
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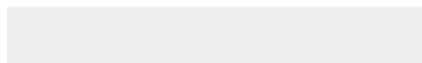
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