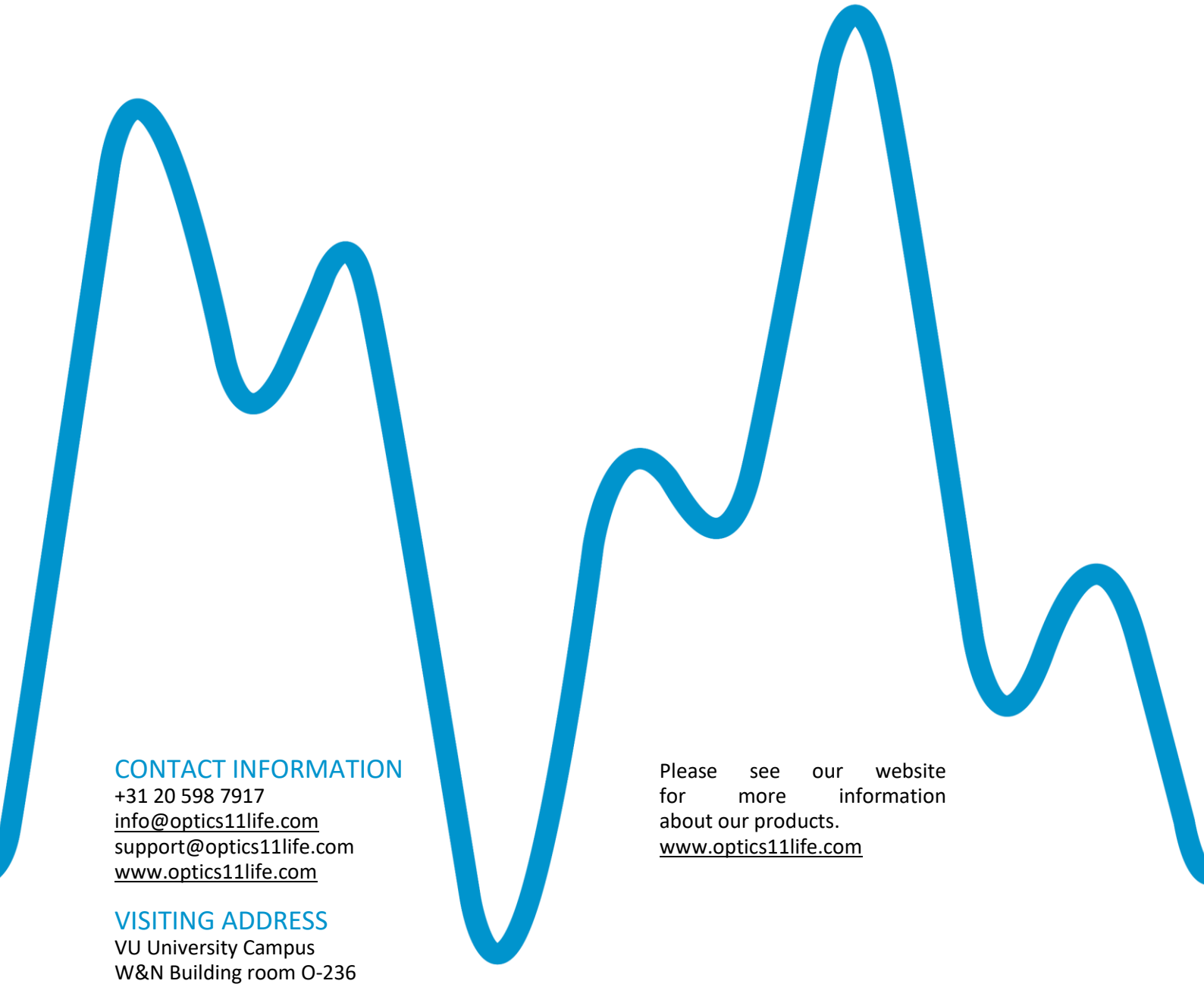
OPTICS life

USER MANUAL

PAVONE





CONTACT INFORMATION

+31 20 598 7917
info@optics11life.com
support@optics11life.com
www.optics11life.com

VISITING ADDRESS

VU University Campus
W&N Building room O-236
De Boelelaan 1081
1081 HV Amsterdam
The Netherlands

SHIPPING ADDRESS

De Boelelaan 1081
1081 HV Amsterdam
The Netherlands

COMPANY INFORMATION

Optics11 B.V.
KvK/CC: 52469417
VAT: NL850459734B01
Amsterdam, NL

Please see our website for more information about our products.
www.optics11life.com

CONTENTS

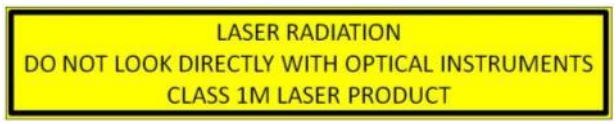
Revision history		4
Safety		5
Introduction		6
1.	Installation	7
2.	Preparing the setup for measurement	9
3.	Using the Pavone	25
4.	Additional system calibration and settings	48
5.	Optimizing instrument stability and sample mounting	54
6.	The OP1550 interferometer	58

REVISION HISTORY

Version 1.0	New document V1.	March 4, 2019	Ernst Breel
Version 1.5	Update for V1.5.2	February 18, 2021	Nelda Antonovaite
Version 1.6	Update for V1.6.0	October 20, 2021	Ali Paknahad

SAFETY

The OP1550 interferometer, part of the Pavone instrument, is equipped with a class 1M laser. The laser light is coupled out via a fiber connector on the front panel and has a terminator on the back panel. Do not view directly into the beam with optical instruments.



The OP1550 is equipped with a 220V/110V plug. Disconnect the instrument before changing the fuse or before switching from 220V to 110V (or vice versa). Do not open the box, as this might result in serious injuries.



Water damage to the Pavone components is not covered by the warranty. Please make sure that no liquids are spilled on the piezo, objective or other components.



PC of the Pavone is provided by the external supplier which includes operating system. Optics11 Life is not responsible for the functioning of it. Please back up your data in case of malfunctioning of the PC or operating system.



INTRODUCTION

This document describes the installation and operation of the Pavone instrument, as developed by Optics11 Life (see Figure 1). Details of the installation of the Pavone are provided in Chapter 1. Chapter 2 describes the preparation of the Pavone and probes for experiments. How to perform the measurements and operate the instrument is described in detail in Chapter 3. Chapter 4 describes how to calibrate camera and microplates. Chapter 5 describes the optimization of sample preparation and measurement conditions. Chapter 6 describes how to operate the OP1550 interferometer.

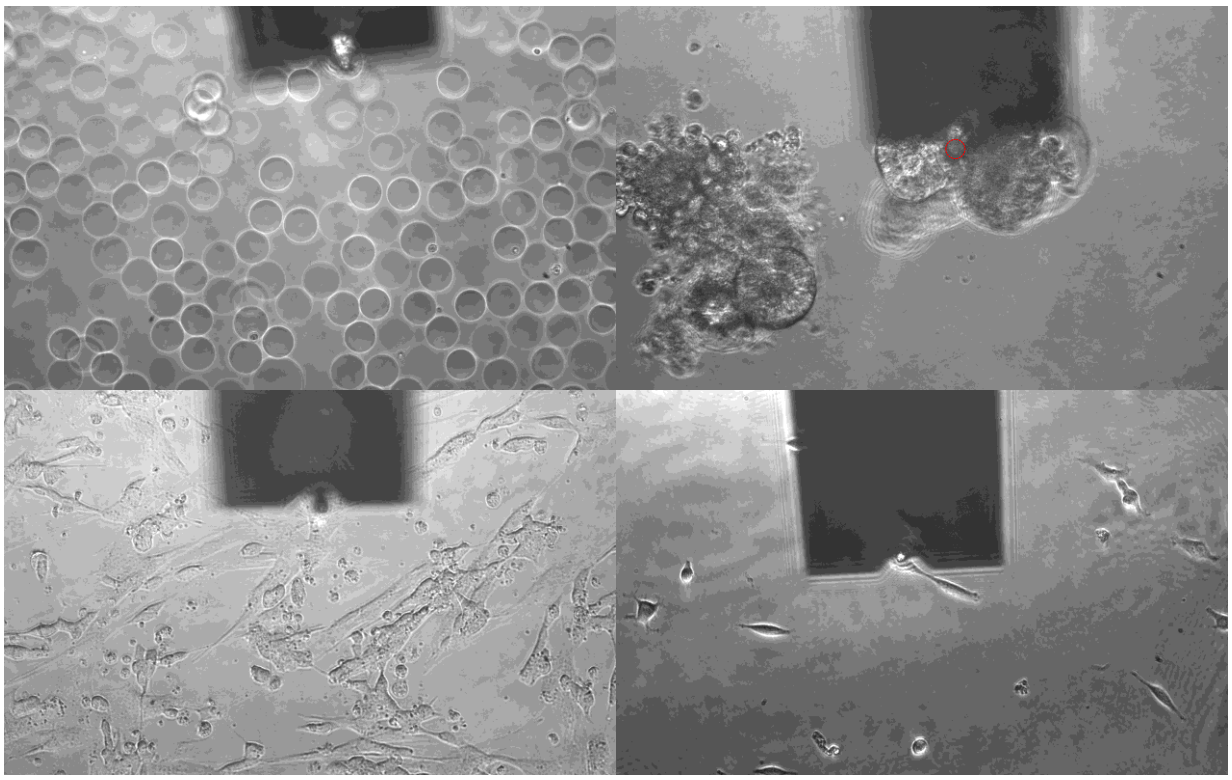


Figure 1: The Pavone in action: measuring microgels, organoids, cell cultures.

1. INSTALLATION

This section describes the general installation of the Pavone by providing an overview of its components, the considerations for finding a location to set up the system, the wiring scheme for connecting the individual components, and the actions required to start up the Pavone instrument. Please use a detailed **installation manual** that was delivered with the system as there might be some system version-dependent differences.

1.1 The Pavone and its components

The Pavone consists of (see Figure 2):

1. Pavone instrument body which also includes:
    a. CCD Camera with the mount
    b. Objective
2. Power supply unit
3. Interferometer OP1550
4. PC which also includes:
    a. Keyboard and mouse
    b. USB splitter
    c. USB camera for remote training
5. Optics11 Life probes

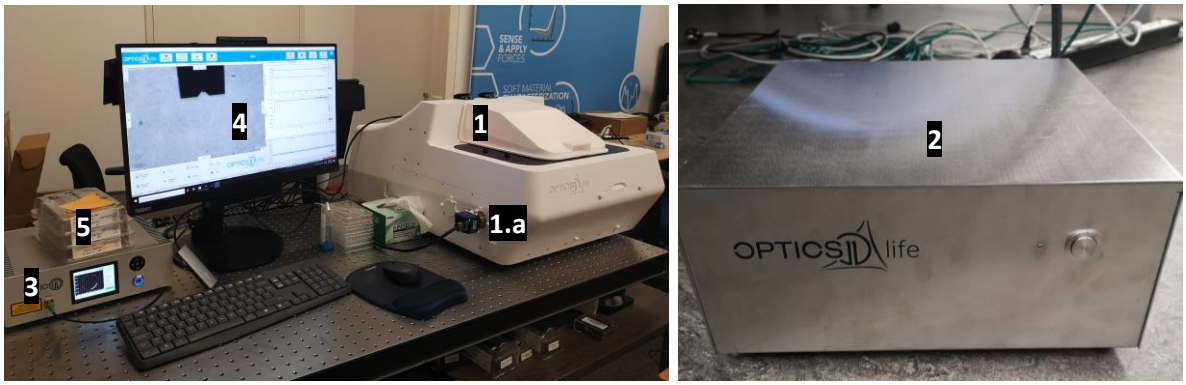


Figure 2: Pavone main components.

For the best user experience, it is recommended to first power on all separate components before running the software.

1.2 Connecting components

Together with the Pavone instrument, the following cables are provided (some cables might be different depending on the system configuration):

1. 2x power cables (for controller and interferometer)
2. 1x PC power cable including adapter
3. 3x USB 3.0 (A-B-type) cables
4. 1x USB 3.0 B – micro USB 3.0 B (camera type) cable

5. 1x BNC connector cable
6. 1x Pavone cable 26pin
7. 1x Intranet cable (maintenance)
8. 1x Patch cord cable (optical fiber)

Additional parts:

1. Piezo calibration probe and mirror
2. Screwdriver set
3. 24 empty wellplate
4. 6 wellplate Softwell
5. 50ml isopropanol and 3 pipettes

When installing the system, please carefully follow the scheme below (see Figure 3).

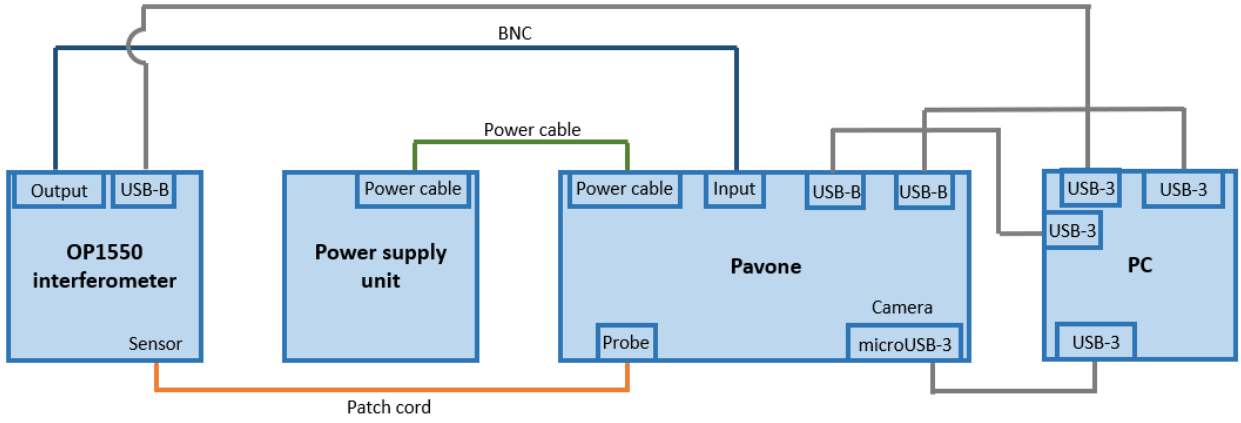


Figure 3: Schematic drawing of the connections that are required to set-up the Pavone instrument.

The power supply unit is connected to the Pavone using a special multi-pin power cable with a screw-on type connector (see Figure 4). The Interferometer is connected using a BNC cable, to transmit the outgoing optical signal to the acquisition electronics in the Pavone, and the USB-B cable to the PC, allowing for communication with the Pavone through the PC. Pavone is connected to a PC with two USB-B cables. The Camera, being a flexible component, connects to the PC through a separate micro USB cable. Please note that all USB cables and connections should be of the USB 3.0 type: only connect to the PC's USB ports that have a blue-color USB port and use USB 3.0 certified cables. Failing to do so might cause erratic system behavior. Connect patch cord fiber between OP1550 interferometer and Pavone which shortens the cable length of the fiber of the probe for easier probe handling.

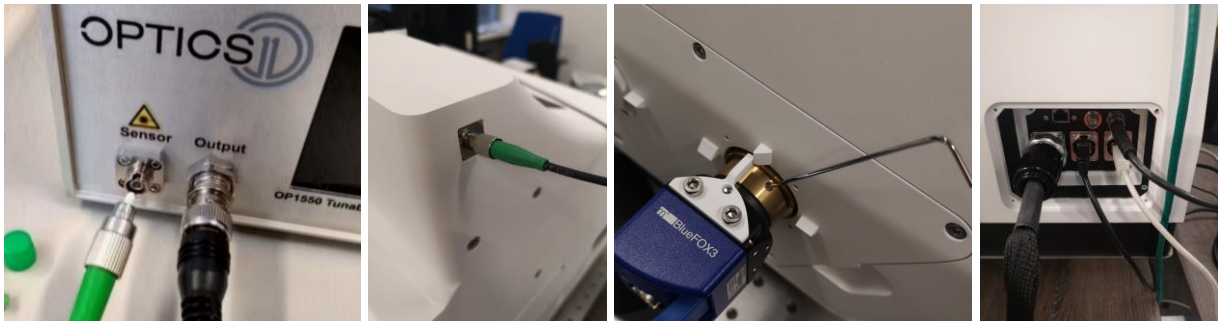


Figure 4: From left to right: patch cord and BNC cable connected to the interferometer, patch cord connection to Pavone, mounted CCD camera and Pavone body connections.

2. PREPARING THE SETUP FOR MEASUREMENT

2.1 Starting the system

Powering up the instrument

Before starting up the instrument for the first time, make sure that the stage lock is removed (follow the installation manual provided with the system), all cables are connected correctly and the power switches on the back of the boxes are turned on. Switch on the devices:

1. Interferometer
2. Power supply
3. PC

The interferometer will show a live measurement signal on the LCD screen after the initialization of the laser is completed. The power supply unit automatically powers the Pavone unit.

Starting the software

Ensure that stage locks are removed, nothing can obstruct sample stage movement. Start the Pavone measurement software by double-clicking the Pavone software icon on the desktop. The software is loading all devices while checking the hardware connection status, which should all be 'Idle' (see Figure 5) after a maximum waiting time of 60 seconds. The software will then proceed to the main user interface.

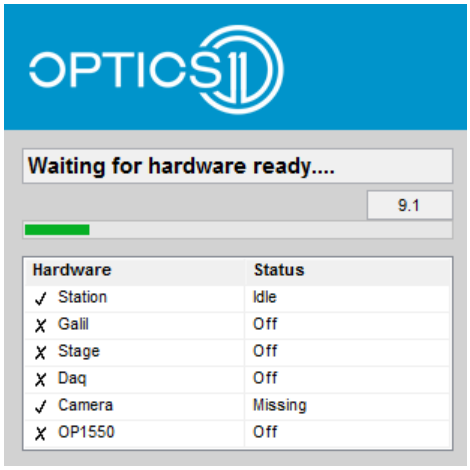


Figure 5: Hardware status check.

After starting the instrument the system will prompt homing of the XY sample stages: this is required to 'zero' the absolute position of the sample stages and ensure accurate positioning during use. After homing the stages the system state will switch to 'idle'.

2.2 Mounting a probe to the Pavone

Optics11 Life force sensors, also called probes, consist of optical fiber and cantilever (MEMS) that are glued to glass ferrule perpendicularly. At the end of the cantilever, there is a spherical tip mounted either directly on the cantilever or through an extended rod (see Figure 6). The spherical tip is used to deform

the sample while optical fiber reads out cantilever bending during indentation. The cantilever acts as a spring to measure the stiffness of the deformed material.

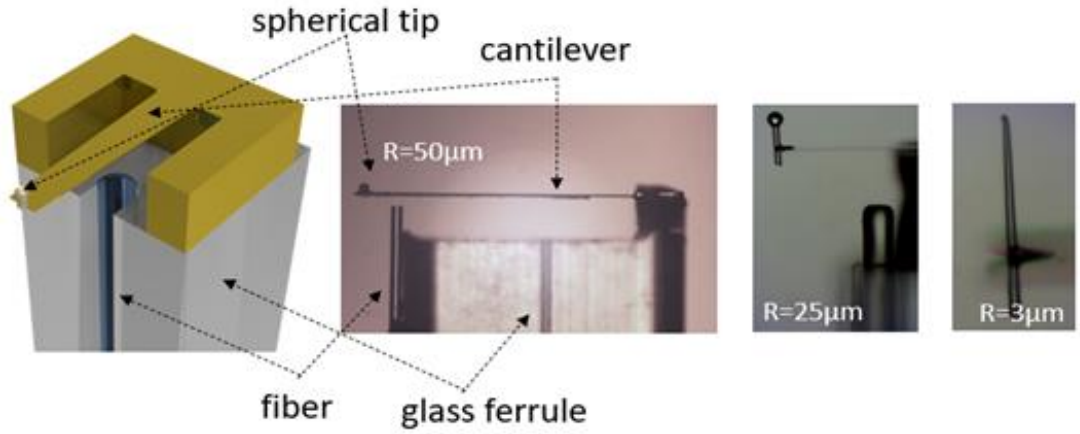


Figure 6: Force sensor (probe) design.

Selecting the right probe

Although a specific probe can measure a wide range of Young's moduli, selecting a probe with suitable parameters that match with the specific sample properties can enhance the quality of the measurement. For a softer sample, a probe with a less stiff cantilever is needed, and vice-versa, to ensure that significant cantilever bending as well as significant sample indentation is achieved during measurements. If the probe is either too stiff or too soft, there will be minimal cantilever bending or indentation respectively, resulting in a less optimal signal-to-noise ratio.

Advised cantilever stiffness is provided for ranges of sample Young's moduli in the table below. These are estimated for a tip radius of 25 µm, indentation depth of 2 µm. The full range is given considering minimum cantilever bending of 0.01 µm and a maximum of 30 µm. The optimal range is given for cantilever bending of 0.05 µm and a maximum of 20 µm.

Table 1: Young's modulus and advised cantilever stiffness range (tip radius of 25 µm and indentation depth of 2 µm)

Full range of Young's modulus	Optimal range of Young's modulus	Advised cantilever stiffness range
10 Pa – 30 kPa	50 Pa – 20 kPa	0.025 N/m
100 Pa – 298 kPa	500 Pa – 199 kPa	0.25 N/m
2 kPa – 6 MPa	10 kPa – 4 MPa	5 N/m
20 kPa – 59 MPa	99 kPa – 39 MPa	50 N/m
60 kPa – 179 MPa	298 kPa – 119 MPa	150 N/m

For a tip radius of 3 µm and indentation-depth of 0.5 µm:

Table 2: Young's modulus and advised cantilever stiffness range (tip radius of 3 µm and indentation depth of 0.5 µm)

Full range of Young's modulus	Optimal range of Young's modulus	Advised cantilever stiffness range
230 Pa – 688 kPa	1.1 kPa – 459 kPa	0.025 N/m
2.3 kPa – 6.9 MPa	11 kPa – 4.6 MPa	0.25 N/m
46 kPa – 137 MPa	229 kPa – 92 MPa	5 N/m

With a larger tip size, one can measure the lower stiffness range and vice versa. By indenting to larger indentation depths, one can measure lower stiffness values. Use an excel sheet called “probe selection calculator” to estimate the range of stiffness probe can measure for the given spring constant, tip size and indentation-depth. In case of doubt, please do not hesitate to contact Optics11 Life for advice (support@optics11life.com). For the exact availability of probe stiffness and tip radius combinations, check Optics11 Life webshop.

Installing a probe

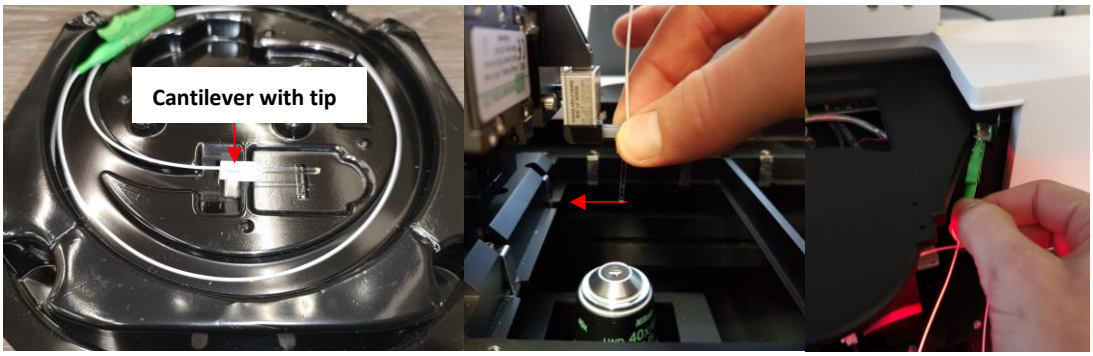


Figure 7: Probe and fiber inside the probe box (left), probe mounting to the Pavone, fiber is connected to the adapter (right).

After powering the Pavone instrument, starting the software and homing the stages, a probe can be mounted. To mount a probe, carefully open the probe box by ‘unclicking’ the four tabs holding the transparent cover. Ensure any sticky tape is removed. Carefully take the probe out of the box in such a way that the probe can be clicked in the probe holder in one go.

The probe is mounted in the Pavone instrument by compressing the probe’s spring by thumb and index finger and gently pushing the probe with the holder in the probe mounting bay (see Figure 7). Make sure the cantilever points downwards and the probe is mounted to the most back-right position. When handling a probe, take special care to avoid any contact with the cantilever as the glass cantilever at the end of the glass ferrule is extremely fragile and almost any unintended contact will cause permanent damage. Therefore, when handling the probe, always hold it by the plastic adapter, keep the optical fiber away from the cantilever and avoid touching the glass part. Given the size of the probe, it is advised to hold the probe between ones’ index finger and thumb. Hold your other fingers straight so that they are away from the probe. Next, connect the fiber with the patch cord adapter. Make sure to hear the click sound when connecting the fiber to the adapter.

Caution: Avoid bending the optical fiber sharply: this can break the glass fiber.

If a previous probe is present in the Pavone, remove it before inserting the new probe. To avoid the fiber from touching the probe when packing the fiber and optical connector, always place the probe first and then the fiber. Secure the optical connector in the box by placing a piece of tape on top of the connector. You can remove the probe from the Pavone by compressing the plastic connector and pulling it gently out of the holder. Carefully place the probe in the probe box to its designated location.

Caution: When installing the probe, always mount first the probe and then connect the fiber connector to the Pavone connector. When putting the probe back into the box, always place the probe first in and then the fiber.

2.3 Main software window and controls

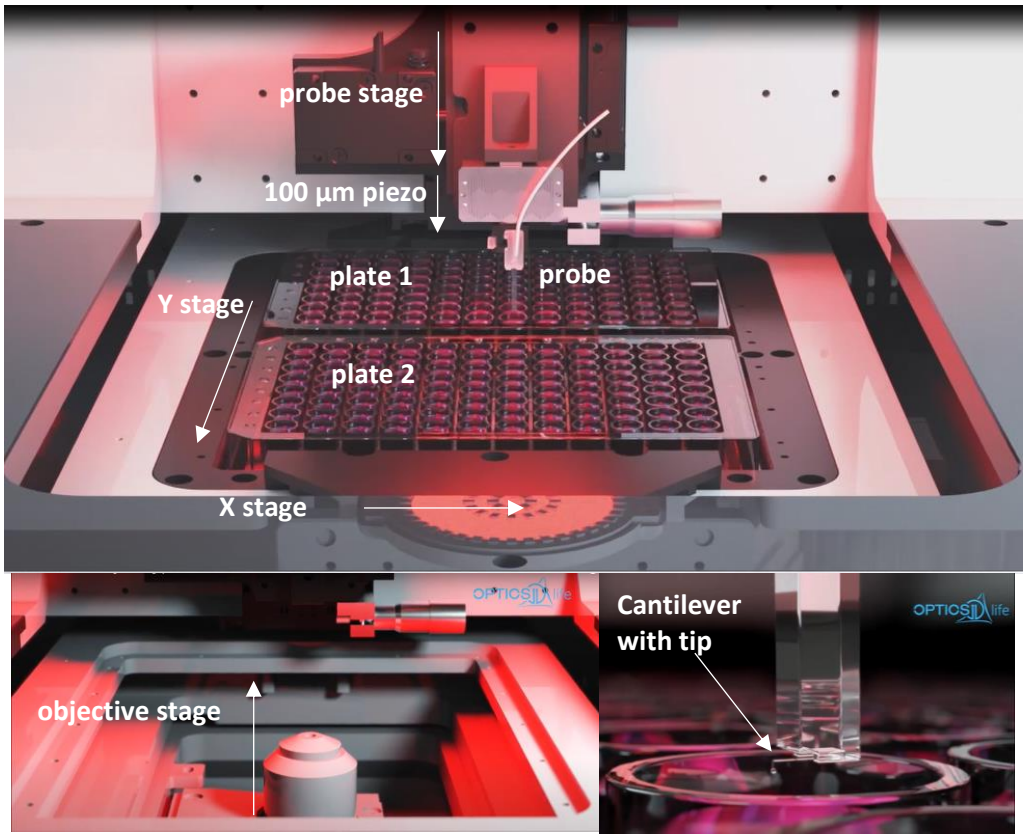


Figure 8: Main Pavone components.

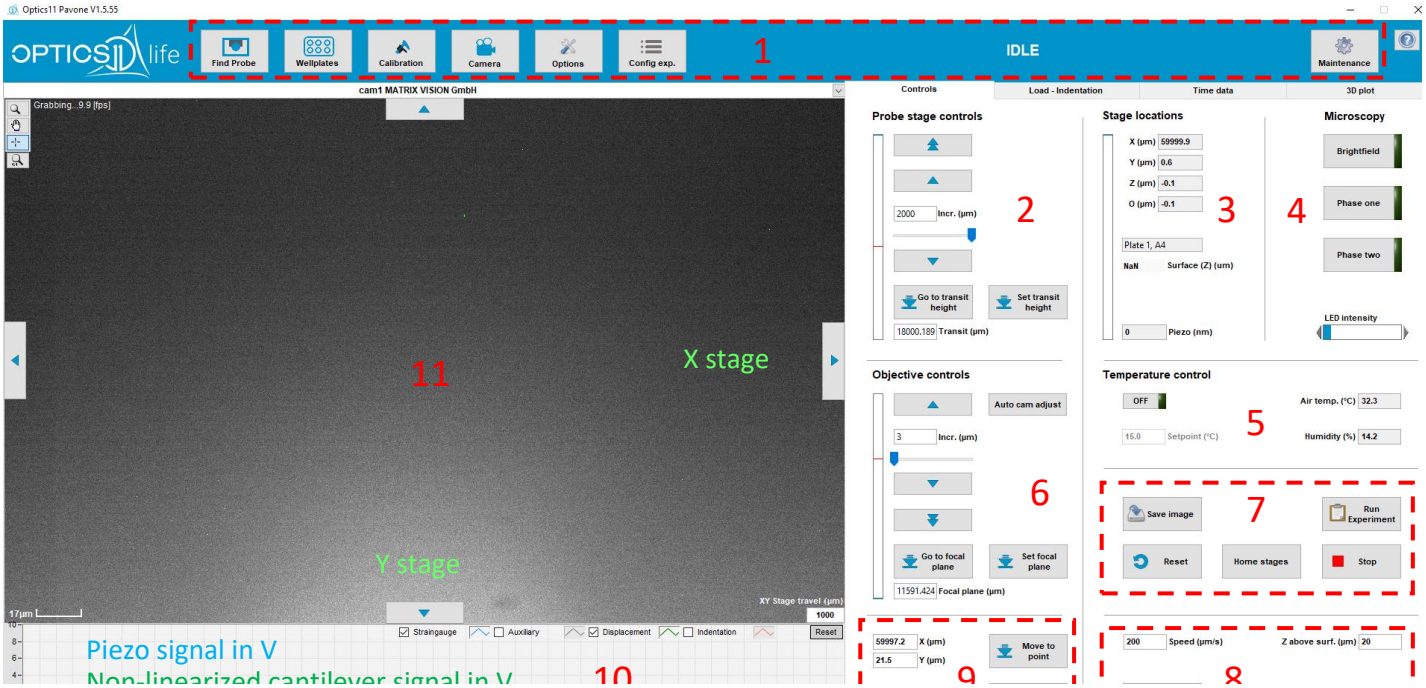


Figure 9: Main software window.

Short description of main window controls (see Figure 8 and Figure *9*):

1. **Top window selection:**
   - **Find Probe** – allows calibration of probe tip position in respect to the camera image.
   - **Wellplates** – allows moving to selected well in the plate.
   - **Calibration** – calibration of the probe.
   - **Camera** – camera settings and automatic image saving options.
   - **Options** – settings for Find Surface and fitting of data.
   - **Config. exp.** – set up indentation protocol and other steps.
   - **Maintenance** – additional settings.

2. **Probe stage controls:**
   - **Single arrow** – controls the step size of the probe stage and allows to move it up and down.
   - **Double arrow (Z up)** – moves up the probe stage to 0 position.
   - **Incr. (µm)** – step size.
   - **Set transit height** – saves current probe stage position Z to "Transit(µm)".
   - **Go to transit height** – moves down the probe stage to the selected height in "Transit(µm)".

3. **Stage location:**
   - **X(µm)** – X stage coordinate.
   - **Y(µm)** – Y stage coordinate.
   - **Z(µm)** – probe stage coordinate.
   - **O(µm)** – objective stage coordinate.
   - **"plate 1, A4"** – current probe position within wellplate.
   - **Piezo (µm)** – current position of the piezo together with the bar.

4. **Microscopy:**
   - **Bright field** – turns on bright-field LED.
   - **Phase one** – turns on phase-contrast LED suited for x20 objective.
   - **Phase two** – turns on phase-contrast LED suited for x40 objective.
   - **LED intensity** – adjusts LED intensity.

5. **Temperature control:**
   - **ON/OFF** – turn on and off the temperature control.
   - **Set point (°C)** – set temperature from 15 to 50°C.
   - **Air temp. (°C)** – air temperature measured at the sensor (inside the frame).
   - **Humidity (%)** – relative humidity.

6. **Objective control:**
   - **Auto cam adjust** – automatically adjusts camera settings in "Camera" based on the current image.
   - **Single arrow** – controls the step size of the objective stage and allows to move it up and down.
   - **Double arrow (objective down)** – moves down the objective stage to 0 position.
   - **Incr. (µm)** – step size.
   - **Set focal plane** – saves current objective stage position Z to "Focal plane(µm)".
   - **Go to focal plane** – moves up the objective stage to the selected height in "Focal plane (µm)".

7. **Run experiment:**
   - **Save image** – saves the current camera image.
   - **Reset** – resets all the hardware.
   - **Home stages** – moves all stages to the initial position.

- **Stop** – stops experiment or any other function.
- **Run experiment** – runs experiment set in " Config. exp"

8. **Move to point:**
   - **Speed (µm/s)** – approach speed that Z stage moves down during "Find Surface".
   - **Z above surf. (µm)** – the distance that the probe is retracted after the surface is found.
   - **Find Surface** – moves Z-stage down until the surface is found.
   - **Threshold** – defines the threshold value of cantilever deflection when the surface is detected.

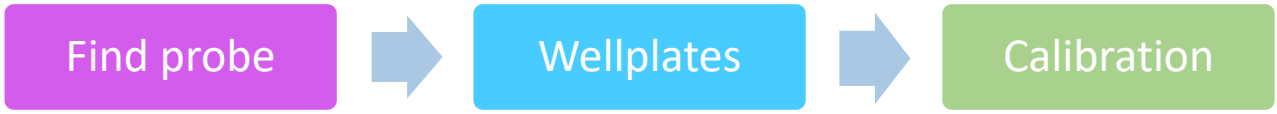
9. **Find surface:**
   - **X(µm)** – move to point X coordinate.
   - **Y(µm)** – move to point Y coordinate.
   - **Move to point** – executes the function.
   - **Speed (µm/s)** – the speed at which XY stages move when running "Move to point" function.
   - **Coord.list** – allows to set up a list of coordinates to be measured.

10. **Live signals**
    - **In blue** – piezo position signal in Volts.
    - **In green** – non-linearized cantilever position in Volts (the same as in interferometer "Measure" window).

11. **Camera feed and manual stage control:**
    - **XY stage travel (µm)** – step size when pressing arrows to move X and Y stages.
    - **Grabbing** – frame rate of the camera
    - **Scale bar**

2.4 Calibration sequence

When starting to work with the Pavone, three steps are required to calibrate the system:



In the software these three steps are indicated in the top menu:



Each button opens a menu enabling specific actions. In general, the sequence to follow is from left to right. In the paragraphs below each step is explained in more detail.

2.5 Find probe: in air



To couple indentation location with imaging data, the reference point of the probe tip is used. Follow the steps:



1. To start the procedure, mount the probe, leave plate 1 empty.
2. Move Y-stage by 30 000 µm so that objective is easily accessible and can move up without obstructions.
3. Move probe stage Z down by ~20 000 µm. Move objective stage O up in steps of 500 or 1000 µm.
4. Rotate a metal screw above the probe stage to loosen up manual XY stage knobs (seeFigure 10, left image). Using the knobs (see Figure 10, second image from the left), align the probe first by eye until the cantilever tip is hovering above the center section of the objective (see Figure 10, images on the right).
5. Turn on “Brightfield” and “Phase 2” and press “Auto cam adjust” in the software. If the image of the camera is dark, increase LED intensity or go to “Camera” settings. You should see an image or shadow of the probe. If necessary, adjust the height of the objective stage so that the probe is in the focal plane (check the working distance of your objective e.g. Nikon WD 3.1 mm for x20 objective, WD 2.1 mm for x40 objective).
6. Continue alignment of the probe with manual knobs until you see the end of the cantilever as in Figure 11. Use small steps of the objective stage to get the tip of the probe in focus. Position the tip at the top of the field of view of the camera.
7. Tighten back the top screw.
8. Press “Find Probe” in software, use the circle tool to mark the circumference of the tip (see Figure 11). Tip: press and hold SHIFT while doing so to keep a perfect circle during resizing. After

confirming the tip location, press save. Keep in mind that tip position in the camera view will deviate depending on the Z-stage position (>10µm along 2000 µm change in Z-stage)

9. Before commencing with the next steps, click ‘Home stages’ in the ‘Controls’ tab once, to move all stages to a safe position for sample plate mounting.

Caution: When using high magnification objectives please ensure that the objective is not touching the probe.

Caution: Please ensure to untighten the black knob at the top of the condenser before adjusting the probe position and tighten again after adjustment.

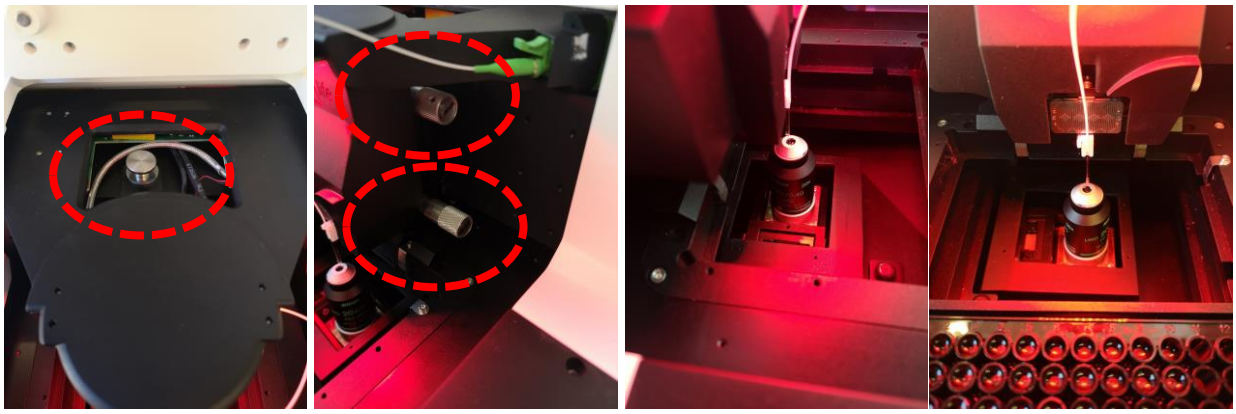


Figure 10: From left to right: knob to release manual stages circled in red; manual micromanipulators for probe adjustment circled in red: top one moves horizontally (along X axis) and bottom one moves vertically (along Y axis); checking probe position by eye along X and Y axis.

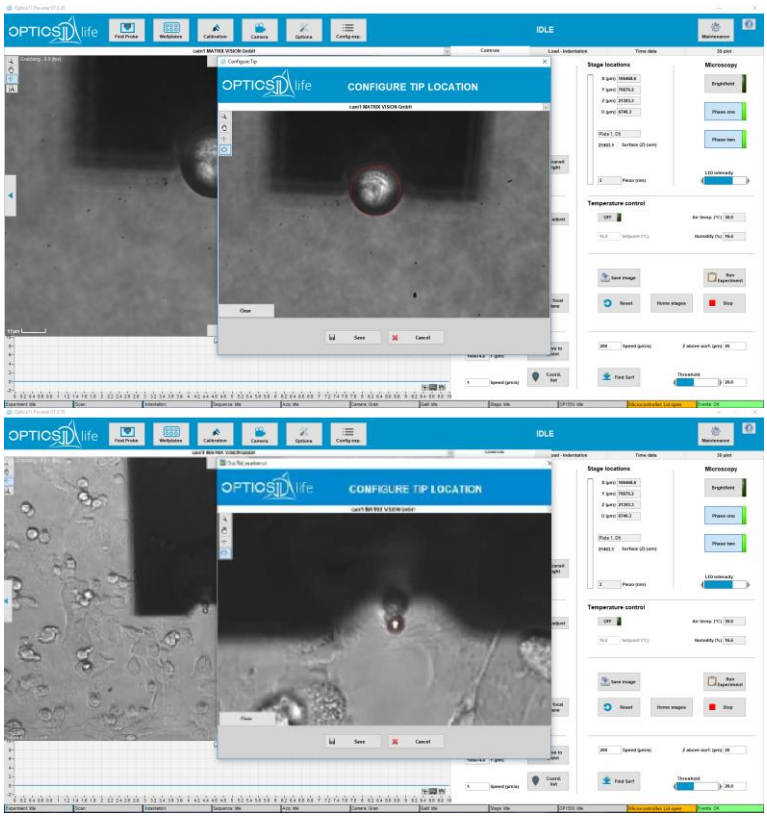


Figure 11: Locate tip menu. The red circle marks the tip position (top 25 µm radius tip and bottom 3 µm radius tip). You can use the zoom function for smaller tips.

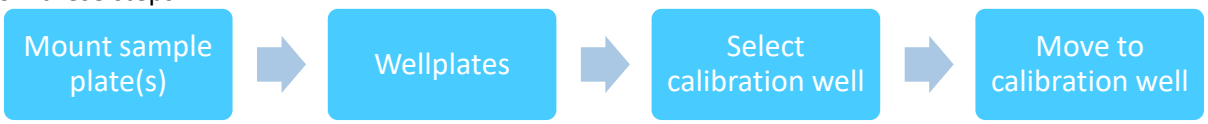
2.6 Configuring sample plates



After the probe is aligned, the sample plates can be mounted and configured in the software. The software allows loading different well plate formats (for more information see Section 4.1). With the delivery of the system, an empty “costar 24 well plate” is provided which dimensions are already calibrated. Please use it until you get more familiar with the system. Other calibrated well plates are:

- 96 and 24 microplates from Greiner (LINK).
- 96 and 6 Softwell plates from Matrigen (LINK).

Follow these steps:



1. First, mount the microplate by pushing it down until all sides touch the bottom of the plate holder and the plate feels firm in the X direction (there are springs on the right side that keep the plate firm). If the microplate can move in the Y direction, push it to the top. Choose the well that will act as the calibration well, it should be loaded without any sample, only filled with the same liquid as used for the samples such as phosphate-buffer saline (PBS), water, culture medium, or just air, and has a clean and hard surface at the bottom, without any coating which would make it sticky.

Caution: The probe is sensitive to the refractive index of the medium and temperature, thus, the probe needs to be calibrated in the same medium as the sample and at a stable temperature.

2. Open the “Wellplates” menu which will show the two sample docks (see **Figure 12**). To configure your sample plates, select the right sample plate format from the drop-down menu. Select the calibration well by pressing on the well (e.g. A1).
3. Press “Move to well” and the stage will adjust the position so that the probe is above the selected well. Then, move down the probe by pressing the probe stage down at steps of 1000 µm and check that the probe is going to enter the well at the top left corner without hitting the edge of the well (see **Figure 13**, left image). If the probe position with respect to the well is not correct, calibrate the wellplate (see Section 4.1).
4. Prewet the probe by pipetting the medium (the same solution as used in the calibration well) over the front face of the probe until you can clearly observe a droplet hanging underneath the probe, see **Figure 13**, right image. This will minimize the risk of trapping air bubbles underneath the cantilever. During prewetting droplets of medium or water may fall in the underlying well.

Caution: It is recommended to clean the probe with isopropanol followed by water before calibration to remove electrostatic charges, residue and decrease surface tension.

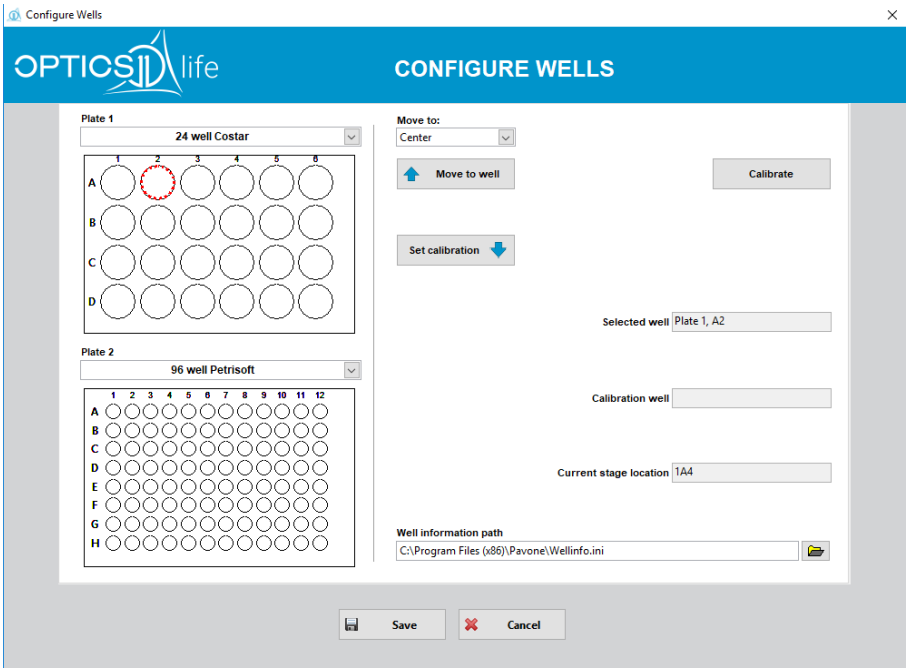


Figure 12: “Config. wells” menu which allows bringing the probe above the selected well. Wells need to be calibrated for their dimensions (see Section 4.1).

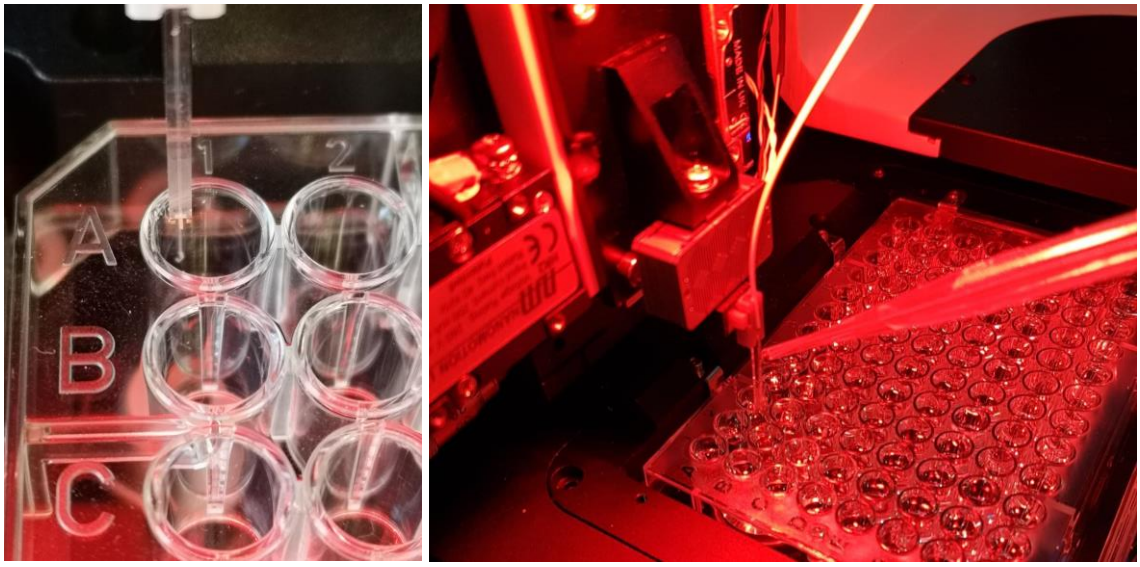
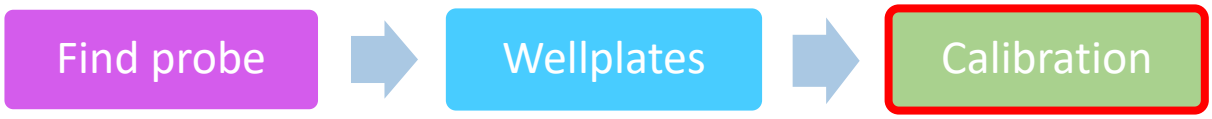
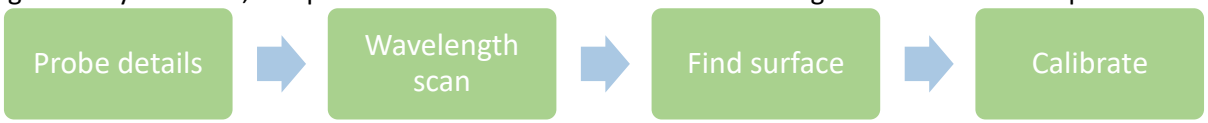


Figure 13: Probe is entering at the top left corner of the well (left image), and probe is being preweted by pipetting liquid down the probe front-face (right image).

2.7 Initialize probe



Before the use of the probe, the laser inside the interferometer and geometrical factor of the probe must be calibrated as it is unique for each probe and medium. This process must be performed for each new medium/probe combination: to measure multiple samples in a solution of which the refractive index is not significantly different, this procedure does not have to be executed again. Follow these steps:



1. Input the probe parameters in the software suite to obtain meaningful measurement results. Probe parameters that need to be set in the program are the cantilever spring constant k (N/m) and probe tip radius R (µm). These parameters can be entered in the 'Calibration' meniu, see **Figure 14**. The numbers can be found on the side of the probe packaging box and are unique for each probe. They are calibrated in the air by indenting on a scale[1]. If you accidentally forget to update the probe details after changing the probe, you can still change those in the DataViewer software while analyzing the obtained data.

[1] Beekmans, S. V., & Iannuzzi, D. (2015). A metrological approach for the calibration of force transducers with interferometric readout. *Surface Topography: Metrology and Properties*, *3*(2), 025004. https://doi.org/10.1088/2051-672X/3/2/025004

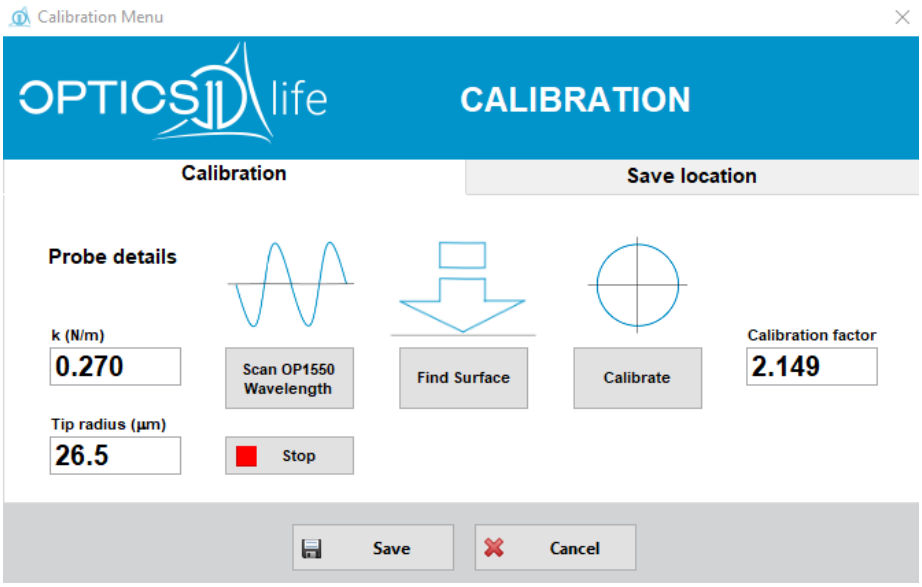


Figure 14: Probe calibration menu.

2. Move the probe to the calibration well using 'Wellplates' window.
3. Make sure that the probe is prewetted.
4. Now you should move down the probe in "Incr. (µm)" steps of 5000 µm until the probe is clearly below the liquid level "Z(µm)"~15 000 µm. If needed, fill the well with more medium so that the probe is clearly submerged but not too close to the bottom of the well or too close to the surface of the liquid.
5. Wait a few minutes so that the probe can adjust to the new environment. When performing experiments with temperature control, the calibration should be accomplished at the same temperature as the measurement temperature. This is because probe is sensitive to temperature variations.
6. Click the "Scan OP1550 Wavelength" button once. The interferometer screen will now show a progress bar. The live signal window on the screen show oscillations, see **Figure 15**-b (zoom in if needed). Wait until that is finished and check if no error (see **Figure 15**-d) is reported on the interferometer screen or software. You can also check the final result of the "Wavelength scan" in the corresponding interferometer window 15-c.

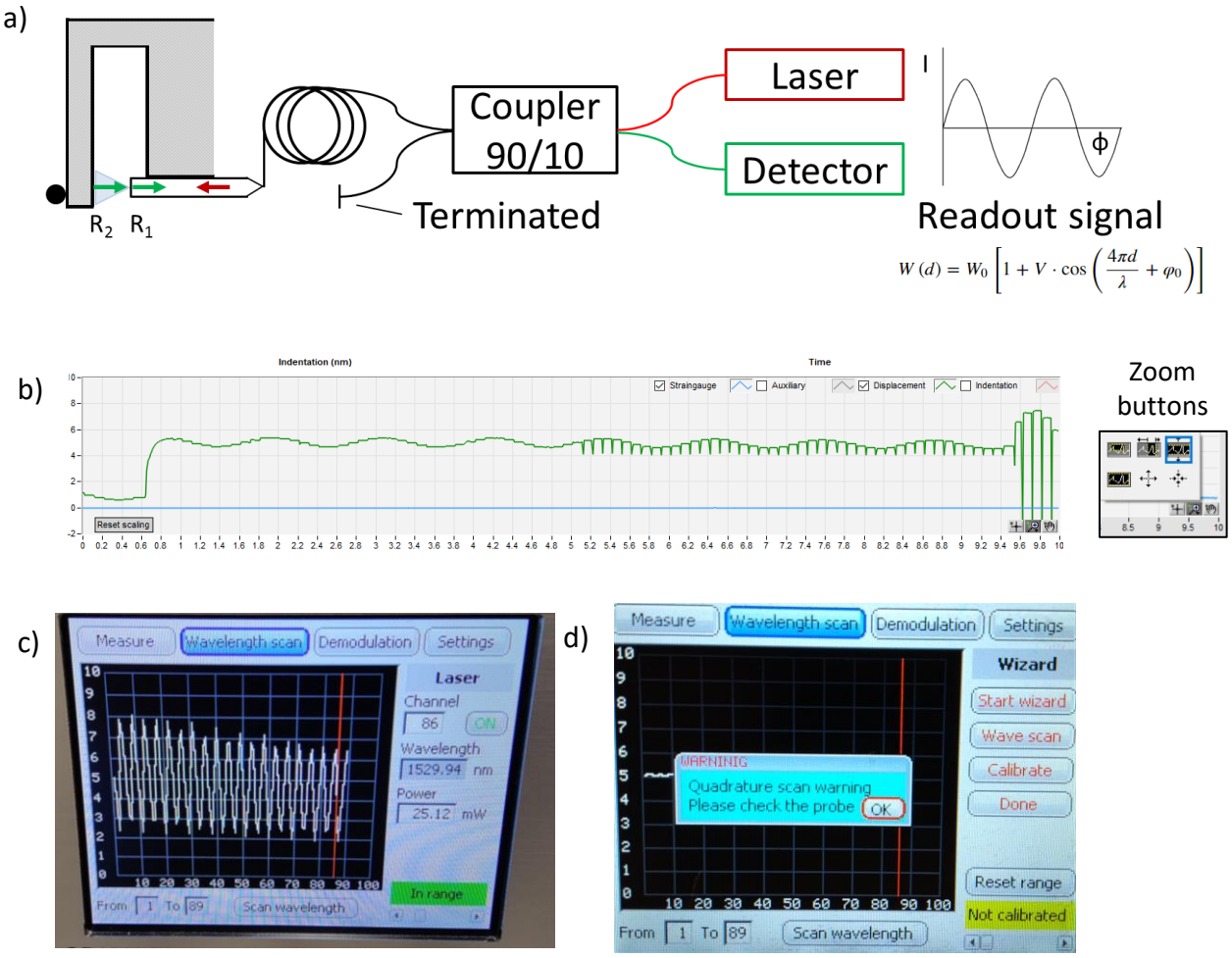


Figure 15: a) Working principle of interferometer. b) Live wavelength scan signal, use buttons on the right bottom corner to zoom in and out. c) Good wavelength scan and d) failed one.

Principle: Laser light (~1550nm) is coupled to the single-mode fiber. The light is reflected back from the two interfaces: the end of the fiber and the cantilever. The back-reflected light interferes and the signal is measured in the detector. The interference signal is described by the formula in **Figure 15**-a where the signal is a cosine function of the gap size (between the cantilever and the fiber) and wavelength. It means that whenever the cantilever is bending or the wavelength of the laser is changing over time, interference fringes (cosine function) will be observed.

During the wavelength scan, the laser will sweep its wavelength rapidly from ~1565nm to ~1525nm and creates an interference pattern. As the photodiode's output voltage depends on the intensity of the interference pattern, an automated offset and gain setting will be set, which optimizes the photodiode output for the 0-10V range. When changing the medium or probe, the new wavelength scan needs to be performed in order to optimize the laser settings. [2]

[2] Chavan, D. C., Watering, T. C. van de, Gruca, G. L., Rector, J. H., Heeck, K., Slaman, M. J., & Iannuzzi, D. (2012). Ferrule-top nanoindenter: An optomechanical fiber sensor for nanoindentation. *Review of Scientific Instruments*, *83*(115110). https://doi.org/10.1063/1.4766959

Reasons for an error during wavelength scan:

- Noise – get rid of any noise sources close to the instrument;
- No connection – check the fiber connections (unplug-plug);
- No cantilever – exchange probe;
- Dirty cantilever – clean the probe with demi water & Isopropanol & water again;
- Air bubble between the cantilever and tip fiber – get rid of the air bubble by moving the probe out of the medium and running medium over it;
- Cantilever stuck at the tip fiber – clean the probe or get it unstuck by touching with the tissue.

Repeat wavelength scan until it's successful.

7. The next step is to press 'find surface'. The system will now start to continuously move down the probe stage while piezo is fully extended and operates in a closed-loop. Once the cantilever bends by a threshold value set in the main window (see **Figure 17**), the stage will stop and the piezo will retract so that the probe is in contact with the surface. You can check if the probe is in contact with the surface by pressing the probe stage up and down in 1 µm steps. The green signal in the live window of the software will change its baseline with each step when the cantilever is in contact with the sample (see **Figure 16**). If it does not, it means that the cantilever is not in contact and you should either increase the 'threshold' value in the main window and repeat the 'find surface' step or manually bring down the probe to contact in small 1 µm steps.

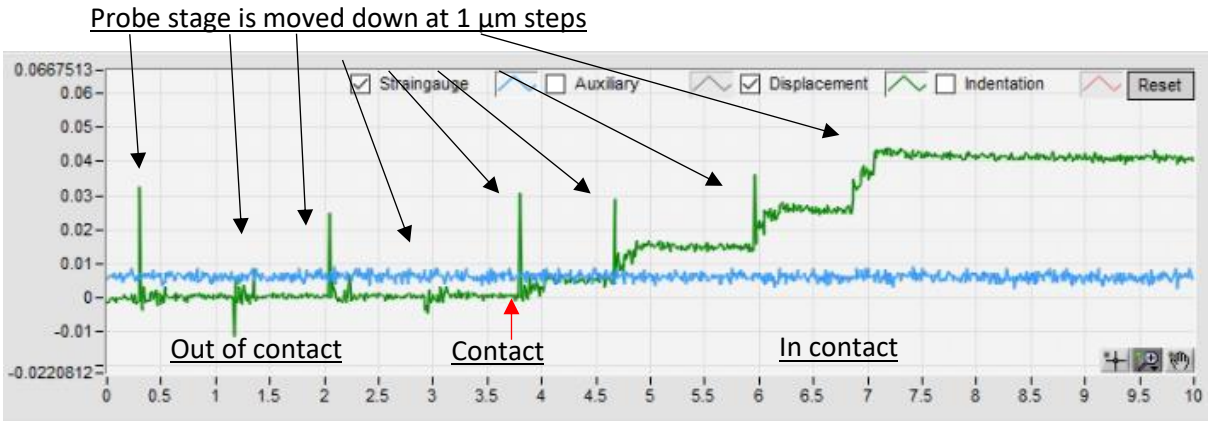


Figure 16: Manual finding of the surface position by pressing down the probe stage at 1 µm steps.

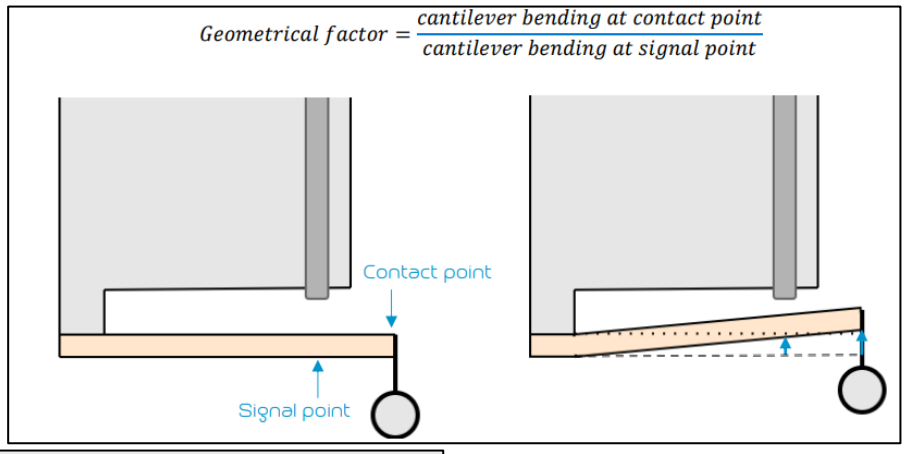
Caution: For the softest probes of k=0.025N/m the 'threshold' value most likely will have to be increased so that, during 'find surface' stage movement, the probe does not stop too early. This early triggering of the surface is caused by a noisier cantilever signal during the stage movement due to the high force sensitivity of these probes. Decreasing the' Speed (µm/s)' of 'find surface' will decrease the noise level as well. Settings can be found in the main window (Find-Surface panel). If Find-Surface still triggers too early, please contact support team.

Caution: When the probe is calibrated in liquid, the functions such as "find-surface" and "Run Experiment" do not work when the probe is not immersed in liquid.

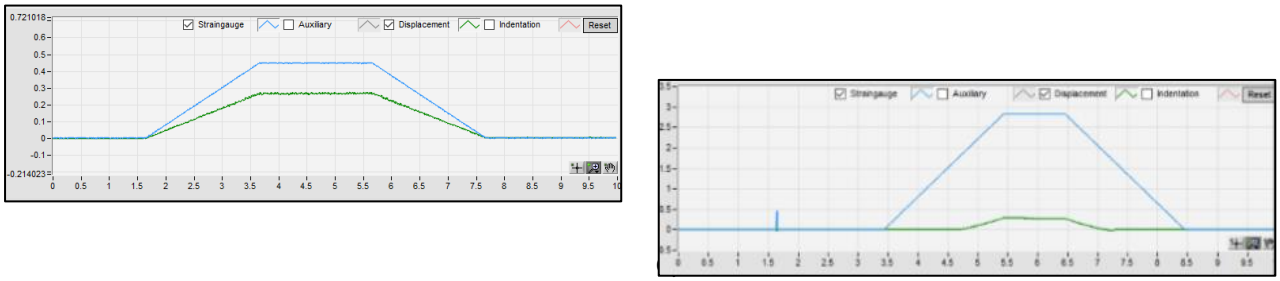
8. Next, the linearization of the interferometer signal (demodulation circle) and calibration of the cantilever arm, also called the geometrical (calibration) factor, needs to be performed. Those two steps are accomplished in one calibration procedure. To perform this step, press the final button 'Calibrate' in the calibration menu.

a)

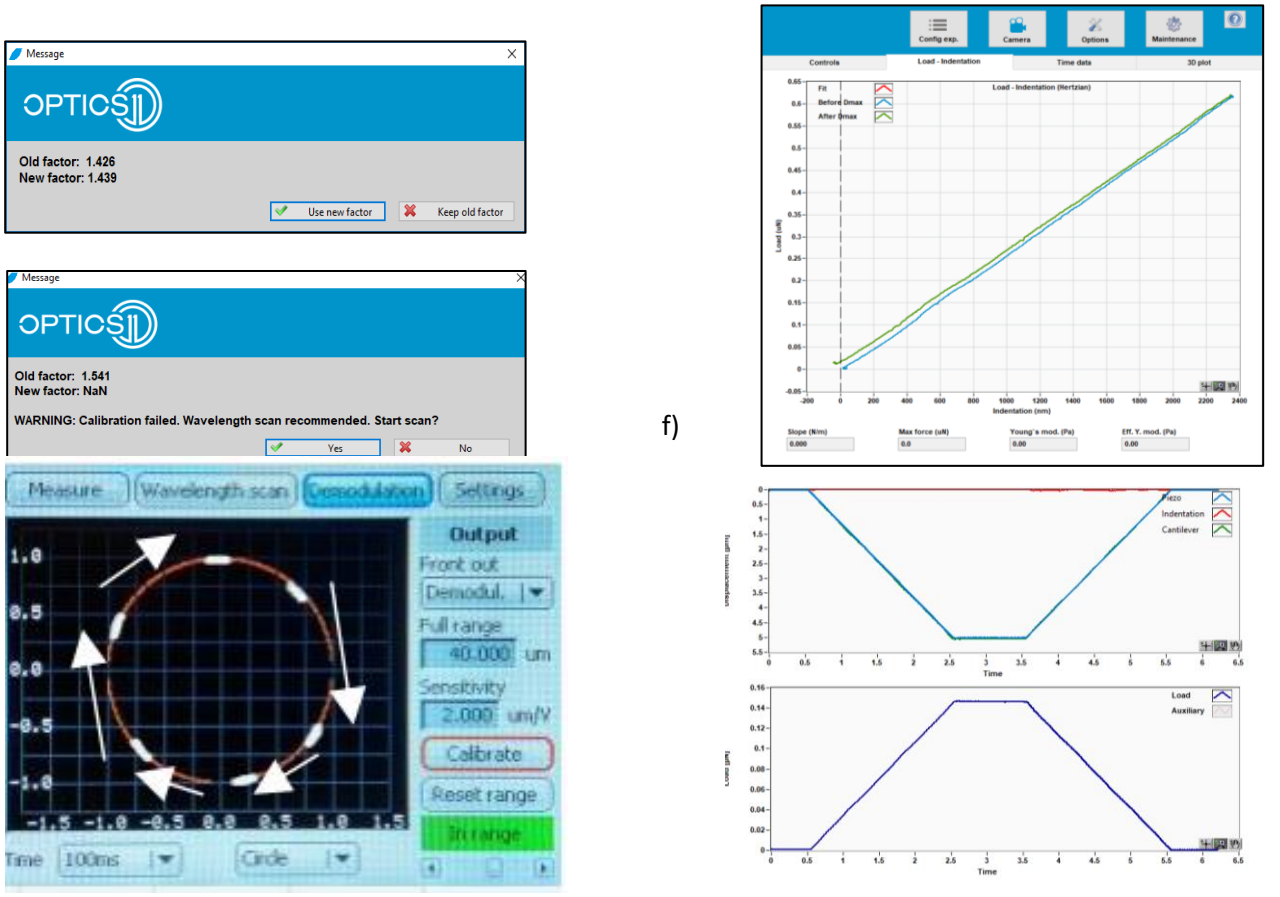
$$Geometrical\ factor = \frac{cantilever\ bending\ at\ contact\ point}{cantilever\ bending\ at\ signal\ point}$$



b)



c)



e)

f)

Figure 17: a) Schematic drawing of the meaning of geometrical factor. b) Live piezo and cantilever signals during calibration when probe is in contact with the hard surface (left) and when it is out of contact (right). c) Successful (top) and failed (bottom) calibration messages. d) Calibration curve. e) Signal movement in time on demodulation circle after it is calibrated. f) There is no indentation on a hard surface after calibration (red line is flat).

9. During the signal linearization, the interference signal measured at the detector (Volts over time) is transformed into a linear signal of cantilever bending (µm over time), using the unit circle as a linearization tool. You can find the unit circle or so-called demodulation circle in one of the tabs of the LCD screen of the interferometer (see **Figure 17**-e). Sensitivity is already set in the interferometer (µm/V) and used to translate Volts to µm.
10. The geometrical factor called 'calibration factor' originates from the mismatch in the spherical tip position and the readout fiber position (see **Figure 17**-a). By indenting on a stiff surface, the distance is measured by the fiber can be compared with the distance that piezo displaced. Taking the ratio between them gives a geometrical factor that is used to correct the cantilever signal by multiplying it.
11. During the calibration step, the piezo will move down twice by the amount set in the 'Options' menu 'Calibration depth (nm)'. We recommend using the value between 5000 and 10000 nm. If 'live calibration' is used (you can check it in maintenance), decrease 'Calibration distance' to 3000 nm (explained in Section 6.2). Check that in the live signal window both piezo and cantilever signals move up at the same time as shown in **Figure 17**-b, left. If there is a mismatch in time (**Figure 17**-b, right), it means that the probe is close to contact but not fully in contact when piezo starts to move. Manually move down the probe with steps of 1um until you see that the baseline of the cantilever signal has changed. If the cantilever signal does not change at all during calibration, it means that the probe is far away from the sample. Repeat calibration procedure from the wavelength scan step.

Caution: During the calibration step, ensure the probe is in contact with a stiff surface.

12. When calibration is completed, 'New factor' is given. By pressing 'Use new factor' the software automatically saves the new 'Calibration factor' in the probe configuration menu (see **Figure 17**-c). The calibration factor should be ~1.33 times lower in the medium than it is in the air which is given on the box of the probe, e.g. if the number on the box is 3.2, then the geometrical factor in the medium should be 3.2÷1.33≈2.4. When repeating geometrical factor calibration, only a small variation is expected <5%. If calibration has failed, see below the possible reasons. You can also check in Load-Indentation data that loading and unloading data overlap and are straight slopes (see **Figure 17**-d). The small mismatch is expected due to drift and hysteresis in piezo movement.

Reasons for failed calibration:

- The tip is not in contact with the surface during calibration (go to step 7).
- Attractive forces between tip and surface – snap-on behavior results in the calibration of the over-bended cantilever – clean probe and the surface or use Teflon surface for calibration (shite substrate).
- Dirty tip or surface – clean the probe with demi water & Isopropanol & water again.
- Air bubble between the cantilever and tip fiber – Get rid of the air bubble (see Figure 18-d). Cantilever stuck at the tip fiber – dry it and unstuck with a piece of paper (seeFigure 18-a to c).
- Repeat wavelength scan before calibrating again.

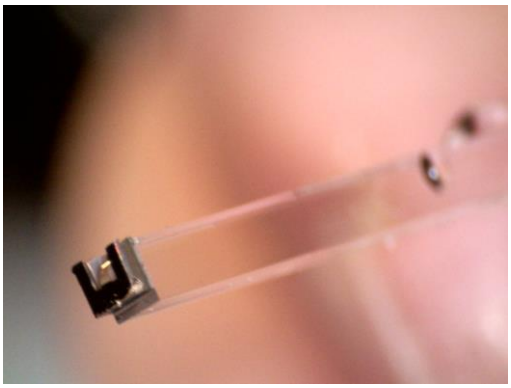
13. Next, check whether the demodulation circle was calibrated correctly. Either tap on the body of Pavone to induce a sufficient amount of noise to see the whole white signal around the red demodulation circle or move down the probe at small steps e.g. 1 µm. In both cases, the white signal should overlap with the red circle.

Caution: If during the operation of the instrument, you observe that the white signal is not on the red demodulation circle or you get a warning from the system about it, you need to recalibrate the demodulation circle. This can be done in two ways: 1)

continuously tap on the body of Pavone to induce one circle of noise and press the "calibrate" button on the interferometer, 2) go in contact with the sample and press calibrate from the "Initialize" menu, do not save calibration factor. However, if the signal is not just slightly displaced from the demodulation circle but rather became very small, it means that the cantilever got stuck to the fiber. To fix it, you can try one of these methods: 1) lift the probe up out of the well, prewet the probe and move down, 2) perform cleaning procedure, 3) dry the probe with the tissue, 4) get the cantilever unstuck by gently touching it from below with the tissue.

14. Finally, calibration can be verified by performing an indentation directly after the calibration. Load or make an experiment file ("Config.experiment"), using the default displacement mode settings and changing to a calibration distance to displace the probe again on the stiff substrate. When 'Run experiment', check the demodulation circle in the interferometer window. The white signal should be on top of the red circle during indentation. The results in 'Time data' should show that the piezo displacement (blue line) is equal to the cantilever bending (green line) because the indentation starts in contact and no material deformation is expected (see **Figure 17**-f). If both lines do not superimpose, check the reasons above for failed calibration.

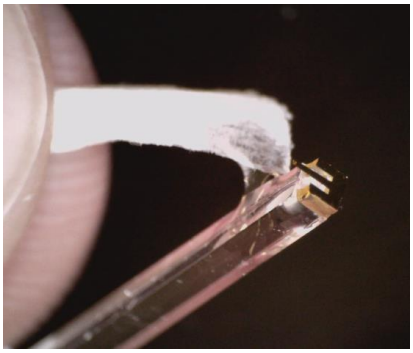
a)



b)



c)



d)

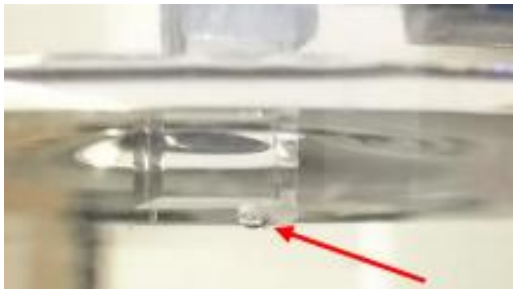


Figure 18: a) Cantilever stuck to fiber. b) Releasing cantilever with the tissue. c) Drying the probe with the tissue. d) Air bubble stuck to cantilever.

3. USING THE PAVONE TO MEASURE SAMPLES

This section describes the use of the Pavone instrument after having prepared the instrument.

3.1 The Find Surface procedure

The Pavone instrument allows bringing the probe into contact with the sample surface. The automated find-surface function can be used by clicking the 'Find Surface' button on the main screen once the probe is submerged in the medium. In the main window (see Figure 9 -panel number 8) and also 'Options' menu (see **Figure 19**), one can set the settings of the find-surface procedure which are explained here:

Threshold defines the threshold value of cantilever deflection when the surface is detected. Units are arbitrary. The higher the noise level, the higher the value will be needed.
Speed (µm/s) is approach speed that stage moves down during Find Surface. The speed of 200 µm/s is recommended for stiffer probes and <100 µm/s for softest probes of 0.025N/m because higher speed causes higher noise.
Z above surf. (µm) is the distance that the probe is retracted after the surface is found. If this distance is too small, the measurements will start in-contact with the sample as shown in **Figure 20**-e and f which could be due to 1) not ideal match between the probe and sample stiffness (probe is too stiff), 2) threshold is too high, 3) sample is adhesive. For load-indentation curves that start in-contact, the contact point between the sample and the probe is not known and is placed at the beginning of the load-indentation curve. For the correct estimation of the contact point, approach distance where the probe is out-of-contact with the sample is needed as shown in **Figure 20**-d to fit the Hertz model (red line) from which contact point is given as one of the fitting parameters.

> **Caution: The maximum distance that piezo can extend is ~100 µm thus the distance to the sample's surface after the Find-Surface procedure cannot be higher than that.**

Adhesion mode is used when the sample is sticky/adhesive which results in in-contact measurements. One can choose "**height(µm)**" to which probe is retracted to get out of contact with the sample and time "**wait(s)**" after which the probe is brought back to Z-above surface position. For example, if the sample is found at stage position 23 500 µm and height is set to 1000 µm, then after the Find Surface step, the probe will be retracted to 22 500 µm position and then brought back to 23 480 µm when Z-above surface is set to 20 µm.

Even though we can use adhesion mode to start the measurements from out of contact for adhesive samples, during indentation, adhesion increases the contact area between the sample and the probe resulting in the overestimation of Young's modulus. Therefore, decreasing adhesion might be beneficial for the precision of your measurements. Here are some tips:

- measure the sample in liquid rather than the air (if possible);
- clean the probe regularly to remove residue from the sample/probe interaction;
- make the sample as flat as possible e.g. by using vibratome to slice it;
- coat the sample with hydrophobic coating for example bovine serum albumin (BSA);
- coat the sphere with 1% pluronic solution to make it hydrophobic.

Exact protocols and more tips can be found in the Sample Preparation document at support@optics11life.com.

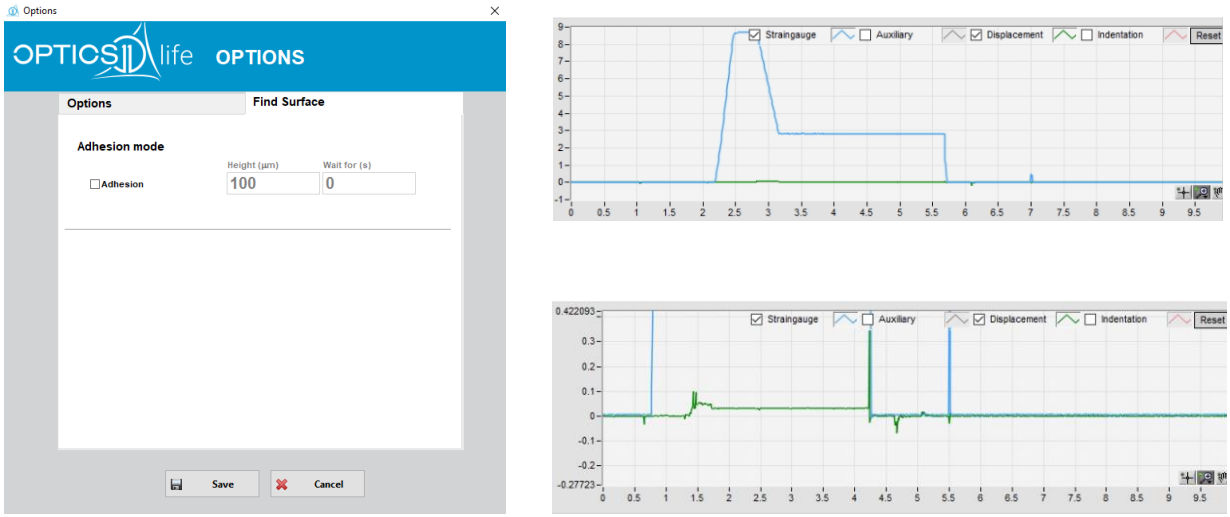


Figure 19: Find Surface options window. Piezo and cantilever signals during the "Find Surface" step.

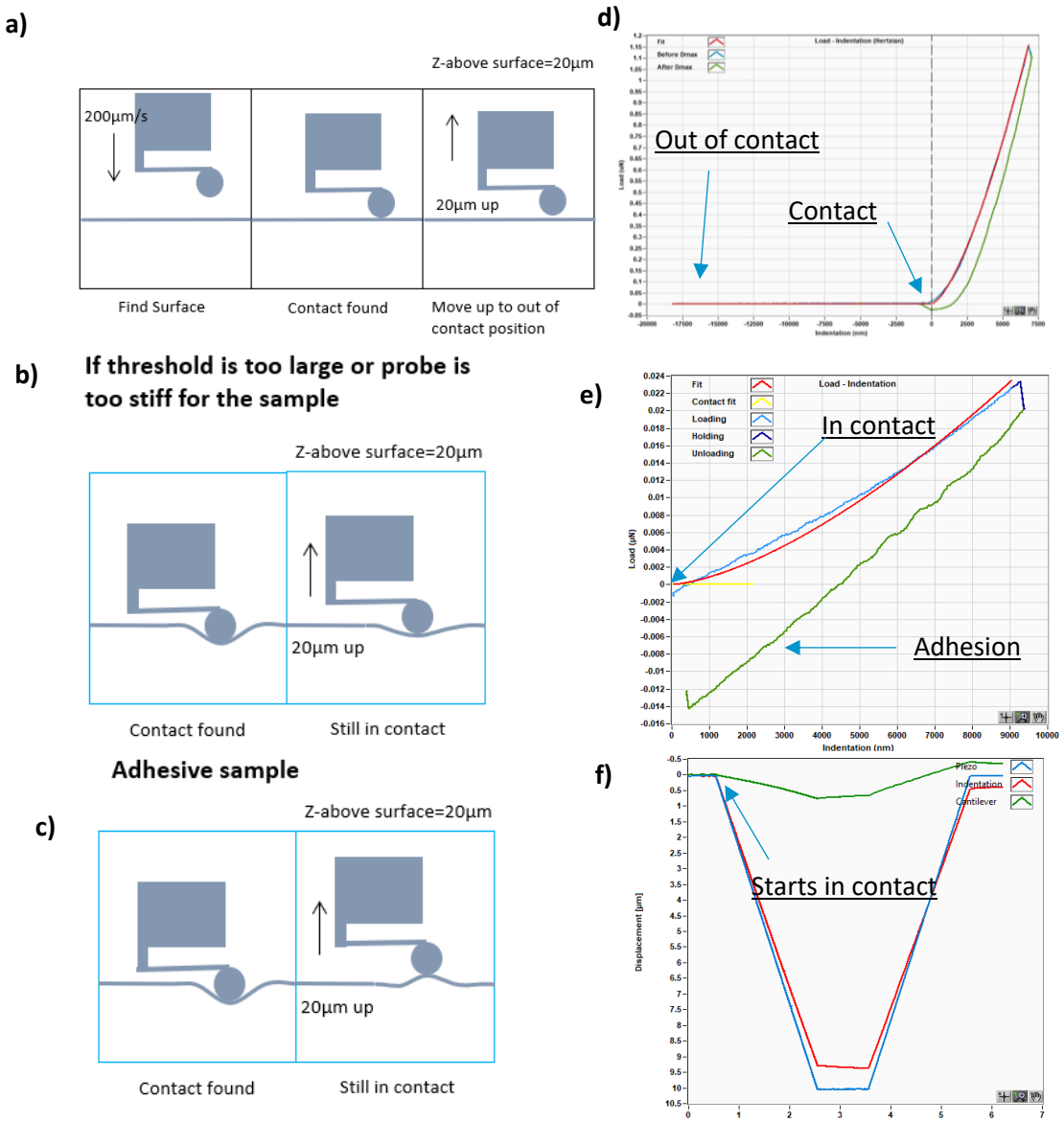


Figure 20: Find Surface procedure. a) Successful measurement resulting in d) load-indentation curve with out of contact part of the curve needed for contact point determination. b) Find Surface procedure when Z-above surface is too low due not ideal probe and sample stiffness combination resulting in in-contact measurement shown in e. c) Z-above surface is too low due to the adhesive sample resulting in in-contact measurements shown in e-f).

Find Surface settings need to be tuned for the specific sample and probe combination. For many samples, the standard settings of Find-Surface will be suitable and, thus, tuning won't be needed. However, for very soft or adhesive samples tuning will be required. When tuning Find Surface settings follow these steps:

1. Submerge the probe in the medium;
2. Press "Find Surface";
3. Set up "Config exp" with a single indentation in D-mode (explained in the next chapter);
4. "Run Experiment";
5. Look at the load-indentation curve, adjust Find Surface settings (increase 'Z above surface', activate adhesion mode) and indentation profile accordingly (increase displacement) and repeat the procedure until out-of-contact measurements are obtained.

Caution: If the same probe is used to measure the range of samples e.g. from very soft to stiff or very adhesive to non-adhesive in a high throughput manner, tune the settings for the softest and most adhesive sample, and then the same settings will automatically work for the stiff and least adhesive samples.

3.2 Setting transit height and focal plane

Next, one can set up the transit height of the probe stage and focal plane of the objective stage to be able to fasten up high-throughput measurements when moving from well to well. Follow these steps:

1. Pres "Find Surface".
2. Note the position of the probe stage Z(µm), e.g. 23 640 µm.
3. If all wells are filled with the same volume of material and, thus, has approximately the same surface position, fill in ~1000 µm lower value in Transit (µm), e.g. 22 000 µm or move up by 1000 µm and press "Set transit height".
4. If samples in different wells have different positions of the surface, then you will need to find the one with the highest surface position and set transit height based on that well. Otherwise, you can also set transit height much higher e.g. just below the level of the medium, however, this going to make the measurements longer.
5. Next, bring back the probe close to the surface of the sample by pressing Find-Surface again.
6. Move up the objective stage in steps of 500 µm until you start seeing the shadow of the probe. Then, decrease the step size and move up until you find the probe in focus. Probe tip position in the medium will differ from its position in the air, thus, adjust the "Find probe" position as described in Section 2.5.
7. Now move down the objective stage until you see the surface of the sample in focus and press "Set focal plane". Keep in mind that the probe tip is only in focus when touching the sample during indentation.

Now, when moving from well to well, you can quickly go to transit height and focal plane, and use these positions to set up high-throughput experiments (see **Figure 21**).

Caution: keep in mind the working distance of your objective. If the sample's surface is high (thick sample), you might not be able to reach it with the objective due to the working distance of the objective. Use a long working distance objective or lower magnification objective. Compatible options from Nikon are x4, x10, x20, x40.

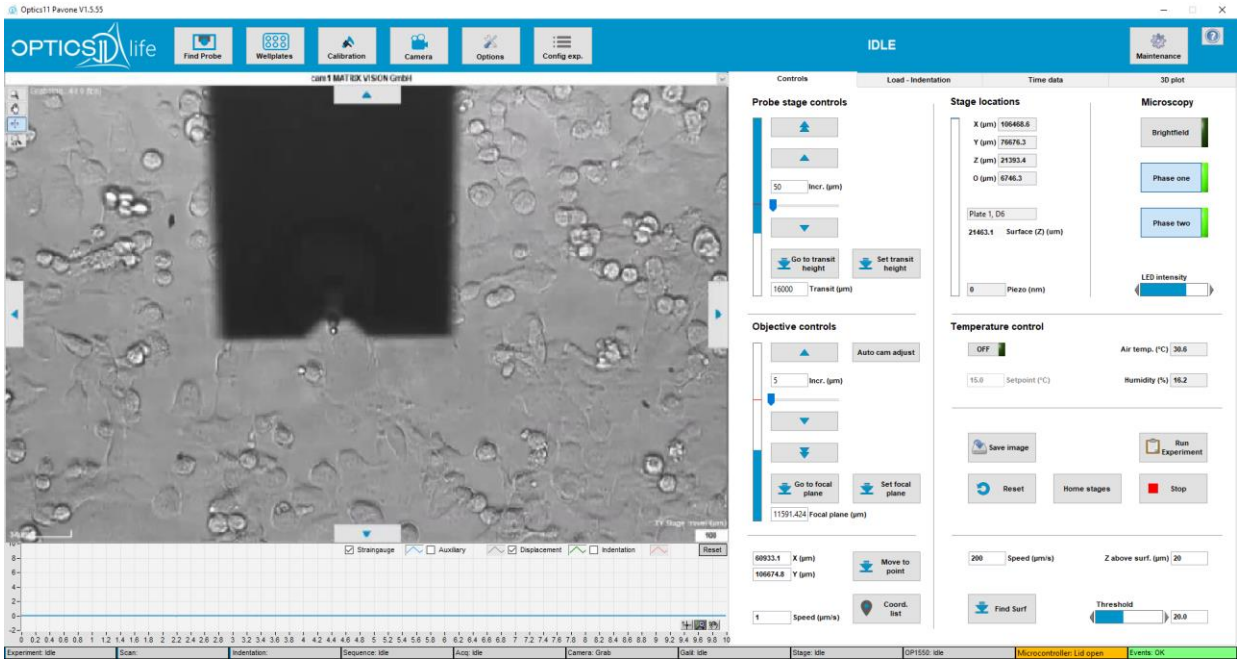


Figure 21: Probe tip and sample in focus.

3.3 Moving within the well

3.3.1 Stage movement

The X and Y stages allow one to move the sample stages within the well. The 'XY stage travel (µm)' field in the bottom-right of the camera feed allows the user to set a displacement (in µm) which can be used by pressing the arrows in the camera feed window. When the probe is in the well and the edge of the well is reached, stages won't move and indicate an error at the bottom right corner. This is called a safety zone which can be calibrated in the wellplate calibration window. Read Section 4.1 about wellplate calibration.

3.3.2 Move to point

The X-Y stage can also be moved using the camera: after clicking anywhere on the camera feed window, the green dot will appear and its coordinates will be recorded in "X(µm)" and "Y(µm)" (see **Figure 22**). Then, by clicking 'Move to point' in the 'Stage location' tab, the selected location will be brought under the center of the probe. The "find probe" position of paragraph 2.5 and objective pixel size which is set in "Camera" are used in this procedure. You can also choose the speed at which the stages will reach the selected point at "Speed (µm/s)".

> **Caution: if "move to point" is not precise, check camera alignment (Section 4.2), pixel size in "Camera" settings and "find probe" position.**

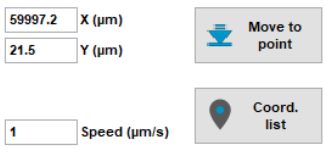


Figure 22: Move to point function settings.

3.4 Configure experiment

Configure experiment window can be opened from the main window. It allows to set up an indentation profile and automatize experimental procedure. Experimental procedures can be saved as .dat files and reloaded at a later time. The left side of the window in **Figure 23** shows steps in the experimental procedure which can be added, deleted, copied, cleared, or moved (arrows up and down). The right side of the window shows possible experimental steps described in the next sections.

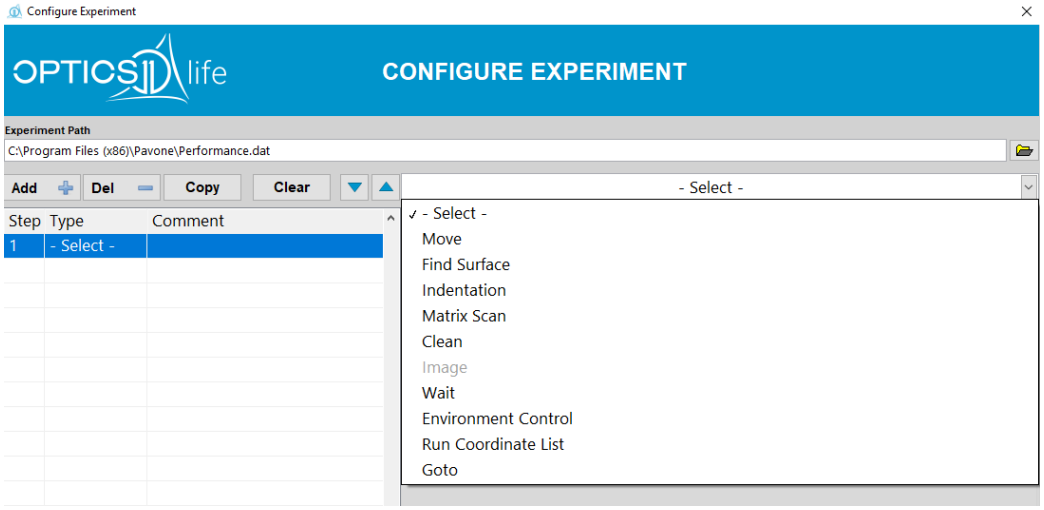


Figure 23: Configure experiment window with possible experimental steps used to automatize experiment.

3.4.1 Move

Experimental step Move allows to move X, Y stages to the selected well (either top left or center of the well) when “Move to Well” is selected (see **Figure 24**-a). Simply press on the well and a red circle will indicate the selected well. Plates can be changed in “Wellplates” meniu in the main software window. If “Move Absolute” is selected (see **Figure 24**-b), probe and objective stages can be moved to the absolute coordinates. One can either fill them in manually by typing in the coordinates or by checking “Transit Height” and “Focal Plane” boxes which will use corresponding coordinates selected in the main window. If “Move Relative” is selected (see **Figure 24**-c), the relative step of all the stages can be selected.

Caution: make sure that the probe won’t hit the sample during these steps.

When designing an experiment, one should first set the “Move to well” step to move to the desired well which should be followed by the “Move absolute” step to move objective and stage to transit and focus heights which should be determined before the experiment. Finally, if a different area needs to be measured within the well, add the “Move relative” or “Move absolute” step and insert step size or coordinates, respectively.

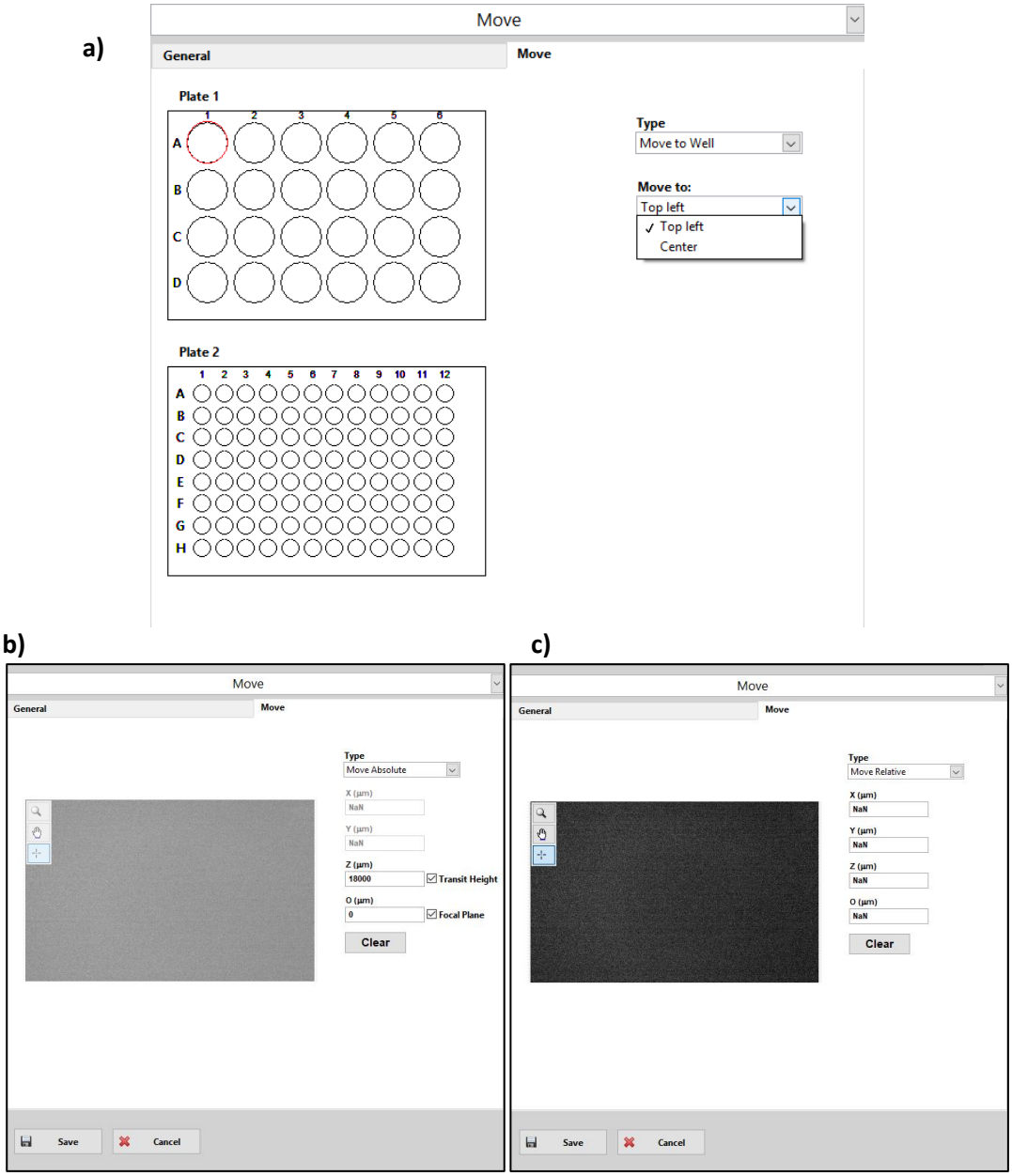


Figure 24: Move option windows: a) Move to well; b) Move absolute; c) Move relative.

3.4.2 Find Surface

After the probe is positioned above the well and lowered to the transit height where it is submerged in the medium, the "Find Surface" step is used to bring the probe closer to the sample. Similarly to the settings in the main software window, one can select the speed at which the probe moves down "Speed (µm/s)", distance to which the probe is retracted after the surface is found "Z above surf(µm)" and "Adhesion mode" settings can be used when the sample is sticky (see Section 3.1 for more details) (see **Figure 25**).

> **Caution: make sure that the signal is on the demodulation circle after moving the probe from air to medium, ideally prewet the probe. Do not leave the probe to dry out in the air if you calibrated in liquid.**

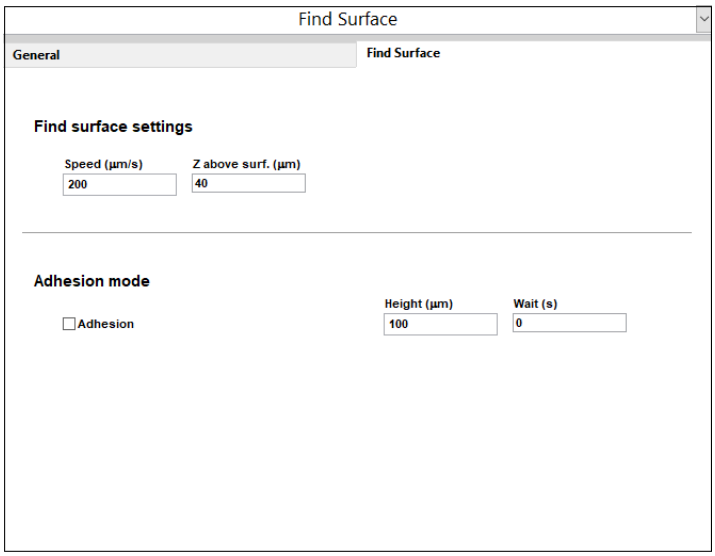


Figure 25: Find Surface settings.

3.4.3 Wait

When the probe is just submerged in the medium, the drift on the cantilever signal might be present when using soft probes to measure soft samples (0.025N/m, E<1kPa) or when the temperature of the medium is not stable, i.e. warming or cooling to room temperature or the temperature set in Pavone. If one would start immediately with the matrix scan, the initial several indentations might be failed or distorted. Therefore, one can add a waiting step, either relative or absolute to allow the probe to stabilize to the environment and temperature of the medium (see Figure 26).

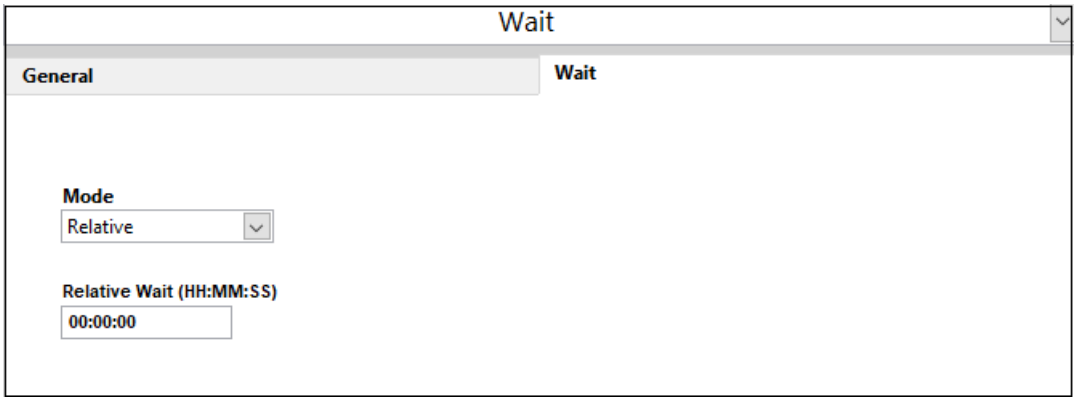


Figure 26: Settings of wait step.

3.4.4 Clean

When measuring biomaterials, some residue might get stuck to the probe tip, especially when samples are adhesive. Also, the residue might deposit on the gold coating of the cantilever from the salts in the medium which would decrease the signal-to-noise ratio. The cleaning step can be added to prevent tip and cantilever contamination. One can either clean probe manually by:

1) running a few ml of isopropanol over the probe;
2) running a few ml of demi water to remove the residue of isopropanol.

This can also be done automatically with two options as “Submerge” and “Oscillate”:

1) fill a well with isopropanol and another well with demi water.
2) This function is in development (not all functionality is available): Add cleaning sequence as shown in **Figure 27**. With choosing the option “Submerge”, the probe will submerge in the selected “Cleaning wells” for pre-defined duration time (“Duration (s)”) and for the option “Oscillate”, the probe will submerge in the selected “Cleaning wells” and oscillate according to the selected “Amplitude (µm)” and “Frequency (Hz)” for pre-defined duration time (“Duration (s)”).

Caution: Do not leave the probe submerged in isopropanol or any other solvent for a prolonged duration because it will dissolve the glue used to assemble the probe. 30 s is enough.

a) b)

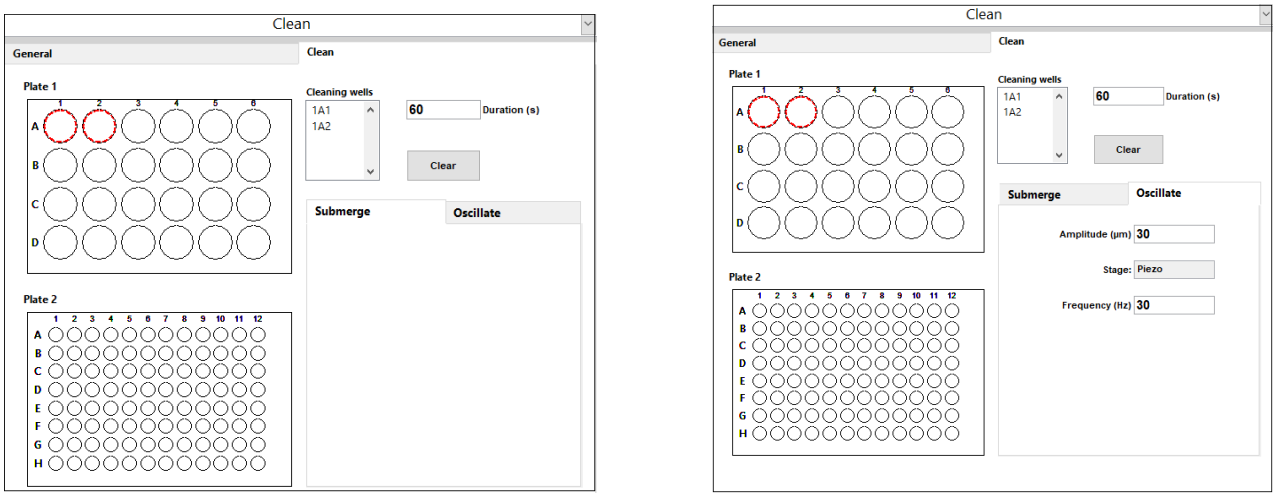


Figure 27: Settings of automatic cleaning step, a) Submerge, b) Oscillate.

3.4.5 Indentation

Single indentations

Single indentations can be configured in the ‘Indentation’ tab. The first subtab “General” allows to select the location of the data, the folder name, and write comments. The second tab “Profile” allows setting an indentation profile in one of the modes: displacement, load, or indentation. The third tab is dedicated to setting up oscillatory frequency sweep, also called dynamic mechanical analysis (DMA). Any profile configured here can be executed by pressing the “Run experiment” button on the home screen. Results are plotted in the ‘Load – indentation’ and ‘Time data’ tabs of the home screen.

Modes of operation

The Pavone can operate in four different indentation modes. The modes differ in the controlled parameter as shown in the following table[3].

[3] Hoorn, H. van, Kurniawan, N. A., Koenderink, G. H., & Iannuzzi, D. (2016). Local dynamic mechanical analysis for heterogeneous soft matter using ferrule-top indentation. *Soft Matter*, *12*(12), 3066–3073. https://doi.org/10.1039/C6SM00300A

Table 3: Comparison of the three modes of operation.

	D-mode	P-mode	I-mode	PLP-mode
Controlled parameter	*Piezo displacement*	*Load*	*Indentation depth*	*Piezo speed & max load*
Sample Circuit	*Open-loop*	*Closed-loop*	*Closed-loop*	*Open-loop*

- Displacement control operates in an open-loop, meaning that only piezo displacement is controlled but not load or indentation-depth. The actual load and indentation-depth will depend on the stiffness of the sample and the stiffness of the cantilever (**Figure 28**-a, c).
- Load control operates in a closed-loop, meaning that the load and time it takes to reach this load (load-speed) is selected by the user.
- Indentation-depth control works in closed-loop as well, but this time indentation-depth and time needed to reach it (indentation speed) are set by the user. The piezo is adjusted accordingly to induce a selected load profile on the sample (**Figure 28**-b).
- Peak load poking mode operates in an open-loop in which piezo speed is controlled to reach the maximum load that is selected by the user after which the probe is retracted at high speed. This mode can be considered as a mode between Displacement control and Load control modes. Operating in an open-loop makes this mode very fast and well suited for single cells in combination with Hertz model fitting.

During all three modes of operation, the instrument is recording piezo movement (blue) and cantilever bending (green) shown in **Figure 28**-a. **Indentation depth is calculated by subtracting cantilever bending from piezo movement while in contact with the sample. The load is calculated by multiplying cantilever bending with the spring constant of the cantilever.** The contact point with the sample (0 point in indentation graph) and effective Young's modulus is determined from the Hertz model to fit over the initial load-indentation curve (**Figure 28**-e, red line):

$$F = \frac{4}{3} E_{eff} \sqrt{R} \cdot h^{3/2}$$

$$E = E_{eff}(1 - \nu^2)$$

where *F* is load, *R* is the tip radius, *h* is indentation depth, ν is Poisson's ratio, E_{eff} is effective Young's modulus and *E* is Young's modulus which can be estimated with Poisson's ratio is known. Thus, if the fitting has failed, the wrong contact point will be drawn in the graph. This can be corrected in DataViewer. Changes to fitting in the main software can be done in the "Options" menu (**Figure 28**-b). Keep in mind that this fit is just a preliminary one and one can change fitting parameters and contact mechanics models in DataViewer.

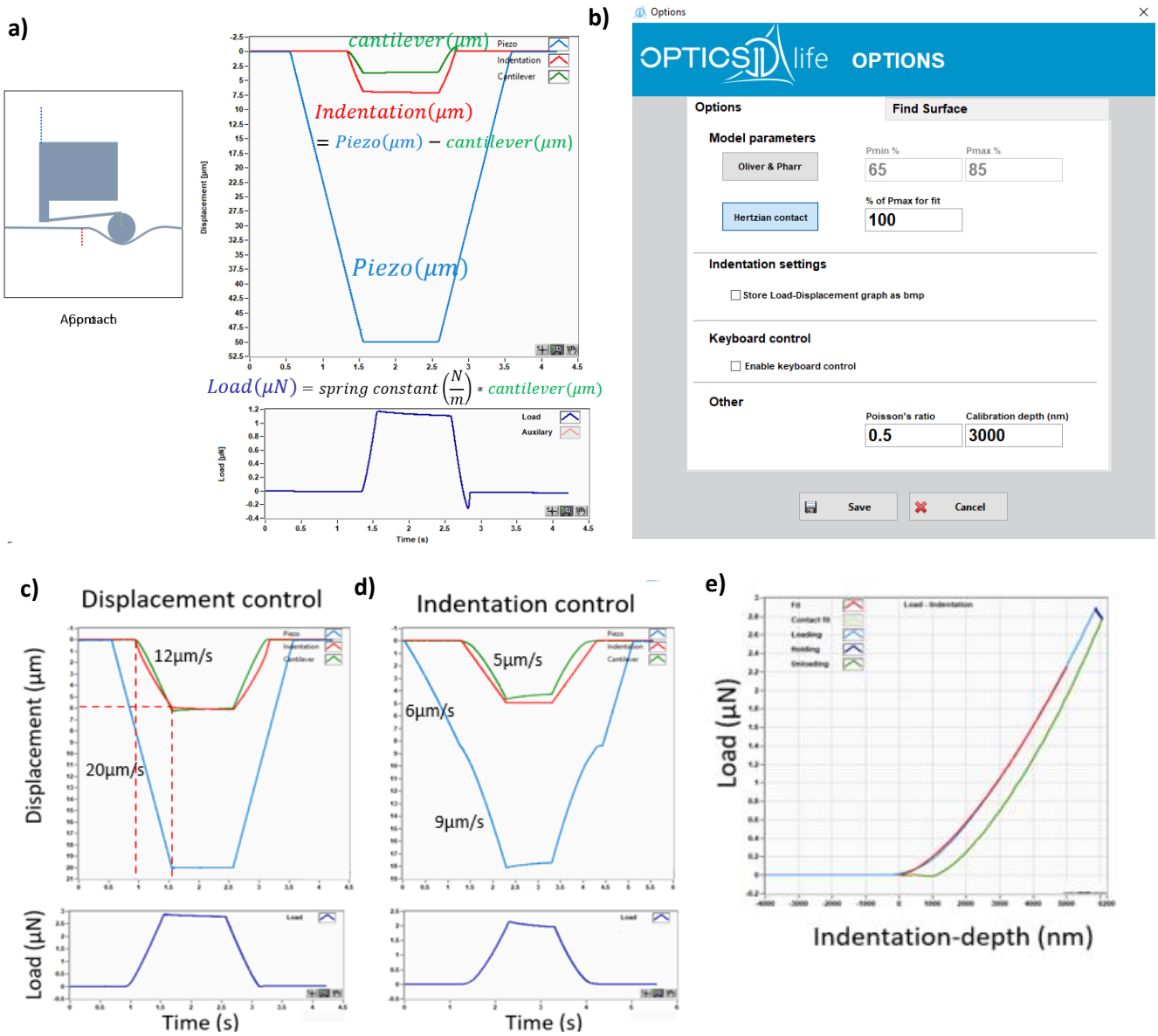


Figure 28 a) Measured signals of piezo movement and cantilever bending and calculated ones, indentation-depth and load, as a function of time. b) Options menu to set fitting parameters for the preliminary Young's modulus values. c) Displacement control measurement where the set piezo speed is 20 µm/s and resulting indentation speed is 12 µm/s. d) Indentation control measurement where set indentation speed is 5 µm/s, approach speed is 6 µm/s and resulting piezo speed is 9 µm/s e) Load-indentation curve and Hertz fit in red.

Displacement control – D-mode

In D-mode, a piezo displacement profile is defined by the user. The indentation depth depends on the distance from the sample surface and the ratio of cantilever and sample stiffness during piezo movement while in contact with the sample. Since the piezo is not receiving any feedback signal from the sample (cantilever bending), it is working in an open loop with the sample meaning one has no control over indentation-speed, depth or load. The displacement (µm) and time (s) variables can be customized. A maximum segment number of 12 can be defined, by default the segment number is set to 5 (**Figure 29**-a).

When measuring the sample for the first time, always start with Displacement control to assess sample and probe stiffness interaction. Keep in mind that displacement distance should be higher than the distance to the sample (Z-above surf.). Time(s) will define how fast the piezo moves and is usually

determined as piezo speed which is equal to the displacement divided by the time. See **Figure 28**-c where piezo speed is defined by the user and indentation-speed is a free variable.

Load control – P-mode

The P-mode of operation allows you to set a specific load and speed at which it is reached and also the load can be kept constant over a certain time interval for a creep experiment. The P-mode of operation is therefore in a closed-loop with the sample. In order to set a load profile, simply click "Load control" in the 'Mode Selection' part of the indentation configuration window and define the profile accordingly. The three segments are suggested. Note that the units change to µN.
Before the closed-loop starts, the piezo moves the probe down at the "Approach speed (µm/s)" until the "Contact threshold" value is reached which initiates the closed-loop. The contact threshold value can be assessed experimentally by running an experiment with the lowest value 0.0005 and checking whether a loop was triggered on the noise and thus no indentation was performed or whether the set profile was induced on the sample. Keep increasing the contact threshold value until the measurements are successful. Decreasing approach speed results in lower noise levels and thus lower contact threshold values can be used. Use the load values that were obtained from the Displacement control measurements to assess the loads that can be reached with a specific probe.

> **Caution: Do not set the load in P-mode without first checking whether the probe can induce it with displacement control measurements.**

If in doubt regarding the closed-loop operation, consult with support@optics11life.com.

Indentation-depth control – I-mode

Operating in I-mode gives you the possibility to set an exact indentation depth and indentation-speed and also, indentation-depth can be kept constant over time for a stress-relaxation experiment (see **Figure 29**-c). Before setting the indentation profile, simply click 'Indentation control' in the 'Mode Selection' part of the indentation configuration window and define the profile accordingly. Note that the units change to nm.
Figure 28-d shows an example of how indentation speed is set by the user to be 5 µm/s while approach speed is set to 6 µm/s. The piezo speed during close-loop operation will be determined by the system in order to achieve 5 µm/s indentation-speed.

> **Caution: Biological materials are usually viscoelastic meaning that mechanical properties depend on the indentation-speed. Biological materials are also nonlinear meaning that mechanical properties depend on indentation-depth, thus, measurements should be performed at the same indentation-speed and analyzed by fitting to the same indentation-depth.**

Peak load poking – PLP mode

The Poking-mode of operation allows you to set a maximum load and piezo-speed at which it is reached in an open-loop operation meaning that indentation-speed, load-speed or indentation-depth are not controlled. This mode can be described as a mode between the Displacement control (D-mode) and Load control (P-mode) modes. In fact, the main difference between Peak Load Poking mode (PLP) and Load control (P-mode) is the open-loop operation. Hence, this mode can be used when a large number of experiments need to be performed in a short amount of time and when Hertz model is sufficient for extracting Young's modulus. "Max load (µN)" shows the maximum load that needs to be reached with poking the sample and "Piezo speed (µm/s)" defines how fast the piezo moves down to reach the sample

(see **Figure 29**-d). In order to set a peak load profile, simply click on “Peak load poking” in the ‘Mode Selection’ part of the indentation configuration window and define the values accordingly.

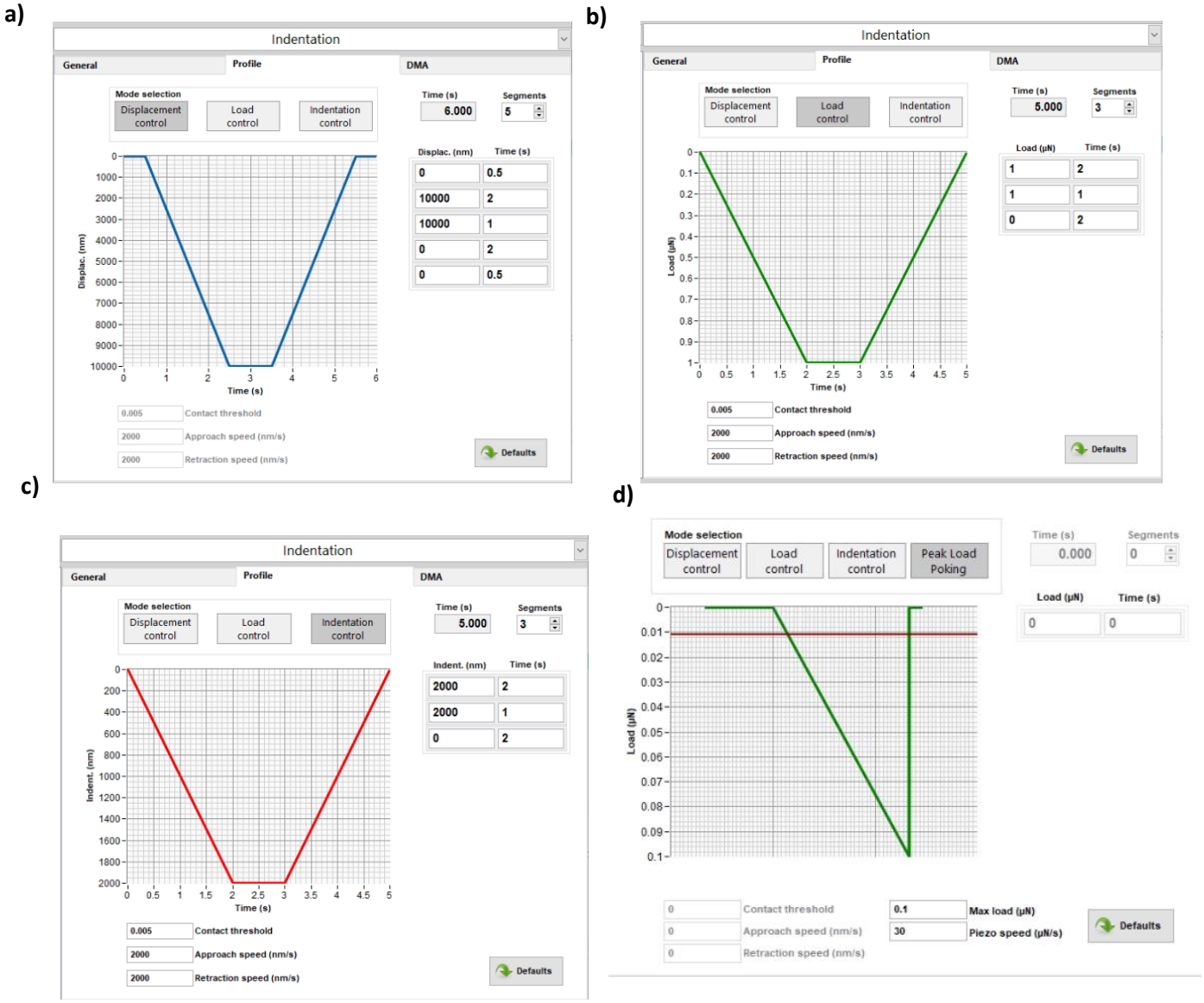


Figure 29: Four modes of control.

3.4.6. Dynamic mechanical analysis - DMA

The Pavone features, besides the quasi-static operation, oscillatory indentations also called dynamic mechanical analysis (DMA). DMA mode allows mechanical oscillations in all three modes of operation while indenting in a sample and can be switched ‘On’ in the DMA tab of the corresponding profile menu. Oscillation parameters like frequency, periods, amplitude, and relaxation time are freely adjustable to provide a flexible measurement design (see Figure 30Figure 31: DMA data analysis in DataViewer.

). The minimum initial relaxation time should be 5s. However, if the sample shows a long relaxation duration, the time should be chosen so that equilibrium is reached before DMA starts. Amplitude should be chosen as small as possible due to the assumption that the contact area does not change during oscillations and material nonlinearity is not sensed. The frequency range can be chosen between 0.05Hz up to 20Hz in closed-loop operation and 75Hz in open-loop operation. However, the performance might

differ depending on PID settings set in the 'Maintenance'. Therefore, initial tests need to be performed to evaluate system performance for the specific sample/probe combination. Get in contact with support@optics11life.com to get more information and training. The number of periods should be chosen so that oscillations take at least 1s, for instance when using 0.5 to 5Hz, the number of periods should be 5 while for higher frequencies, the number of periods should match the frequency, e.g., 75Hz - 75 periods.

Table 4: Parameters range for DMA profile.

Min. initial relaxation time	5s
Min. relaxation time	2s
Min. amplitude	2x noise level
Lowest frequency possible	0.01 Hz
Highest frequency possible	20 Hz closed loop, 75Hz open loop
Min. oscillation time	1s

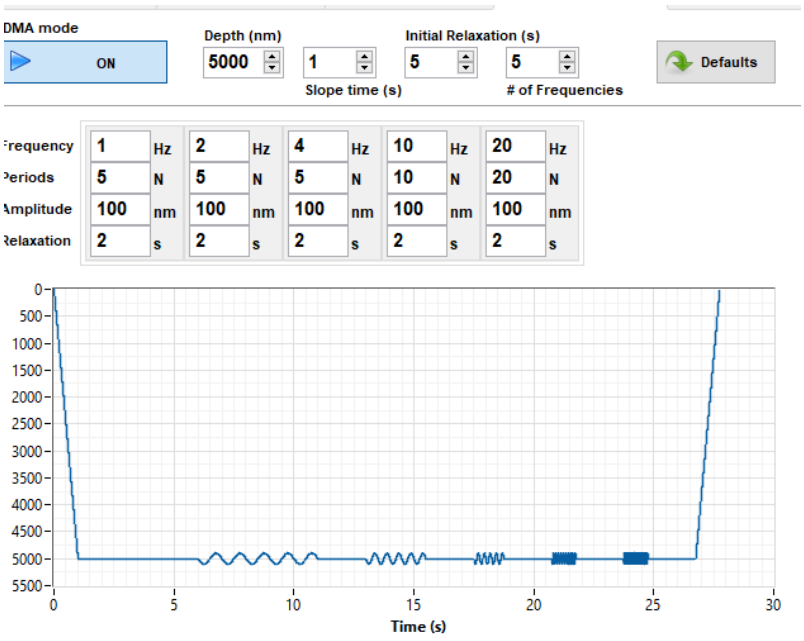


Figure 30: Configuration of DMA profile.

Dynamic Mechanical Analysis (DMA) uses a cyclic motion with frequency, f, while controlling indentation-depth or load with an amplitude of h_0 or F_0, respectively, if used in closed-loop. In an open-loop, only the displacement of piezo is controlled, thus, h_0 and F_0 will depend on the relationship between sample and probe stiffnesses. The frequency-dependent storage modulus, E', and loss modulus, E'', which represent elastic and viscous components respectively, are calculated with the following equations:

$$E'(f) = \frac{1}{2}\frac{F_0}{h_0}\cos(\delta)\frac{(1-\nu^2)}{\sqrt{hR}}$$

$$E''(f) = \frac{1}{2}\frac{F_0}{h_0}\sin(\delta)\frac{(1-\nu^2)}{\sqrt{hR}}$$

where δ is the phase lag between oscillations of indentation and load (see right side of **Figure 31**. tan(δ) is the dissipation (damping) factor which is the ratio between loss and storage modulus. Materials with a higher damping ratio than 1 are considered to be viscoelastic fluids and below 1, viscoelastic solids. Also, viscoelastic solids have a higher storage modulus than loss modulus at low frequencies which switches at higher frequencies while it is the other way around for viscoelastic fluids. Complex modulus can be calculated according to this formula:

$$E^* = \sqrt{E'^2 + E''^2}$$

If the amplitude of the input oscillation is small enough, the measurements can be considered within the material linear viscoelastic region (LVR). Within the LVR, the response is assumed to be independent of the input amplitude and sinusoidal. Furthermore, when selecting the amplitude of oscillations, the depth should be considered due to the curvature of the sphere, as it is assumed that the contact area does not change during oscillations, so that $\frac{h_0}{h} \ll 0.25$. The performance of the DMA can be evaluated in the DataViewer by comparing the amplitude which was measured with the amplitude which was set in the experiment and by looking at the R-squared values of cosine fits.

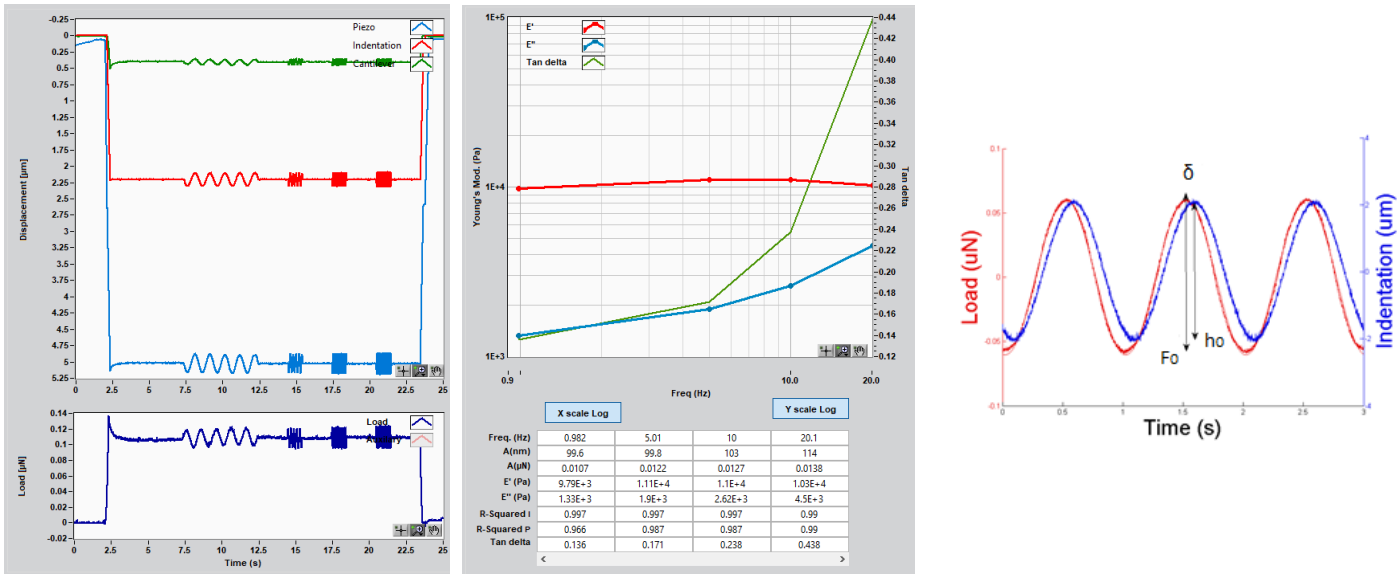


Figure 31: DMA data analysis in DataViewer.

3.4.7 Matrix scan

Instead of performing a single indentation, an automated grid scan can be configured to obtain the map of mechanical properties. Parameters that can be selected are these:

- the number of points in the X and Y directions ("# X", "# Y");
- distance between indentation points in the X and Y directions ("dX", "dY");
- distance by which the probe is lifted up between indentations "Z at XY move (µm)";
- automatic find surface before each indentation "Auto find surface";
- "Coarse within safe zone while maintaining the number of points" – if checked, it will decrease the distance between indentation locations to keep the number of points and if unchecked,

it will decrease the number of points while keeping the distance between the points as it is set in dX and dY.

Caution: To avoid the deformation of the same area (oversampling), use step size at least two times the contact radius $a = \sqrt{h * R}$, *a* - contact radius, *h* - indentation depth, *R* - tip radius.

If the surface of the sample is not flat, increase "Z at XY move (µm)" to make sure that the probe does not hit the sample and, thus, start indentation from the contact with the sample. Also, use automatic find surface to bring the probe closer to the sample when the surface shape of the sample is not known or it is clearly not flat as the piezo is limited to 100 µm extension distance.
The matrix scan will be initiated from the current location of the probe, thus, you can use a separate Move step to bring the probe to another location. The scan is performed by making the positive steps in Y-axis until the line is complete, then the single positive step in the X-axis, and then the negative steps in Y-axis. Therefore, in the camera view, you need to position the probe at the top left corner of the region that needs to be mapped.
Once the parameters have been set, the scan is initiated by pressing the 'Start scan' button. Before continuing to a new point, including the first indentation of a matrix scan, the probe moves up to a safe transportation height and subsequently moves to the indentation location. After reaching this location, the find-surface procedure is initiated. Using the find-surface again for each point in a grid scan allows the Pavone to scan samples that have a higher degree of surface topography. For very flat samples this 'Auto find surface' feature, can also be switched off so that the probe remains a few microns above the surface when moving between coordinates in a grid scan (see **Figure 33**-a).
The data of the scan is saved in a generated 'matrix_scan_X' folder in the previously specified experimental folder. In this folder, a text file is created for each individual indentation, as well as one file containing the effective Young's moduli per coordinate in a single grid "E-eff vs XY position.txt" file. During data analysis with DataViewer, when using "Save" function, an additional text file is created called "Results.txt" which saves all results in columns that can be used for further statistical analysis.

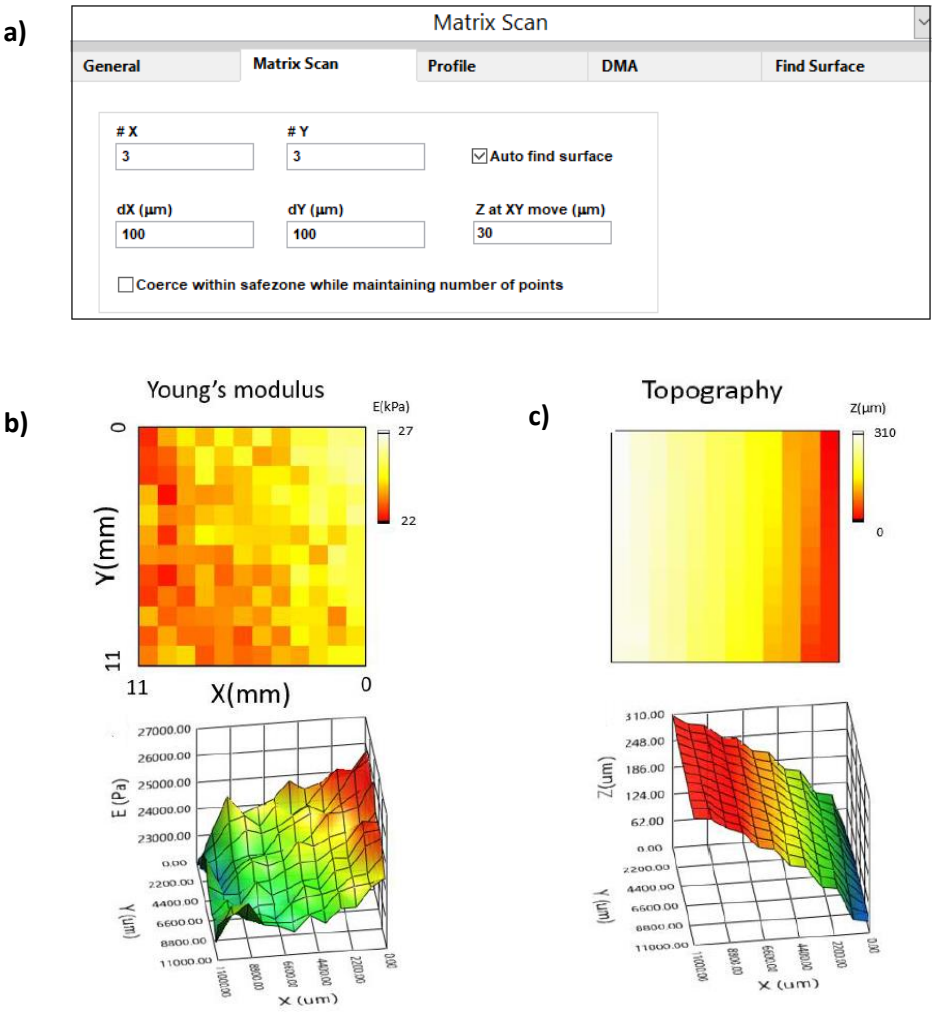


Figure 33: Matrix scan settings and example of matrix scan result on a swelling hydrogel.

3.4.8 Run Coordinate List (Config exp.) and Coordinates (main window)

In order to measure multiple individual objects or regions of interest in a semi-automatic manner, one can record the coordinates of these objects in the "Coordinates" menu (see **Figure 34**). Simply press "Add" and then select a location on the camera image and the XYZO coordinates will be filled in the list. Continue with all the objects/locations of interest. When finished, press "Save" and the Coordlist.json file will be created. To run indentation measurements on selected coordinates, go to "Config exp." and add "Run Coordinate List".

> **Caution: when running indentations on the list of coordinates, make sure that the "Find probe" position is correct and that the Z stage position is not too close to the sample.**

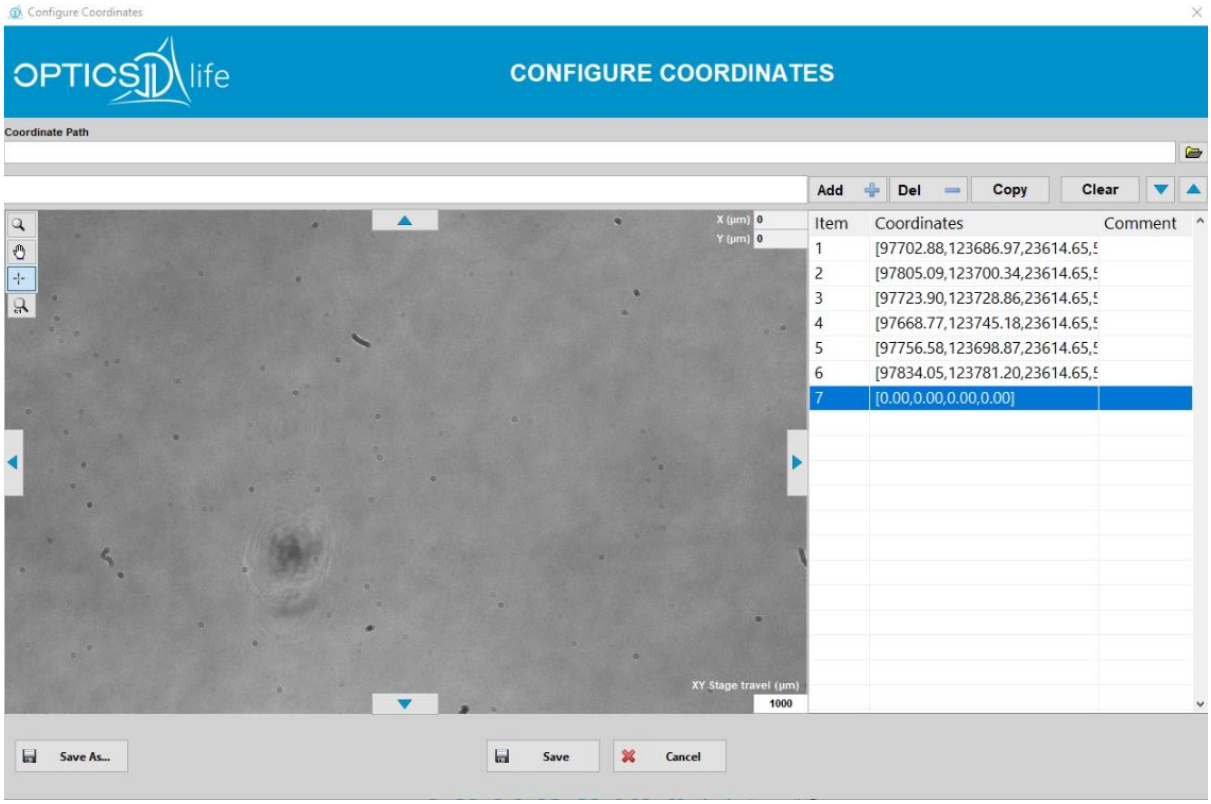


Figure 34: Setting up the coordinate list.

Coordlist.json file is loaded with four coordinates: X position of stages, Y position of stages, Z position of stages, and Objective position (see **Figure 35**). At first, all the stages are moved to the selected coordinate followed by an Automatic find surface (if it is ticked) and then a single indentation is performed. Coordinates can be also manually modified by directly opening the .json file with Notepad. Data will be saved in a separate folder “Coordinates”.

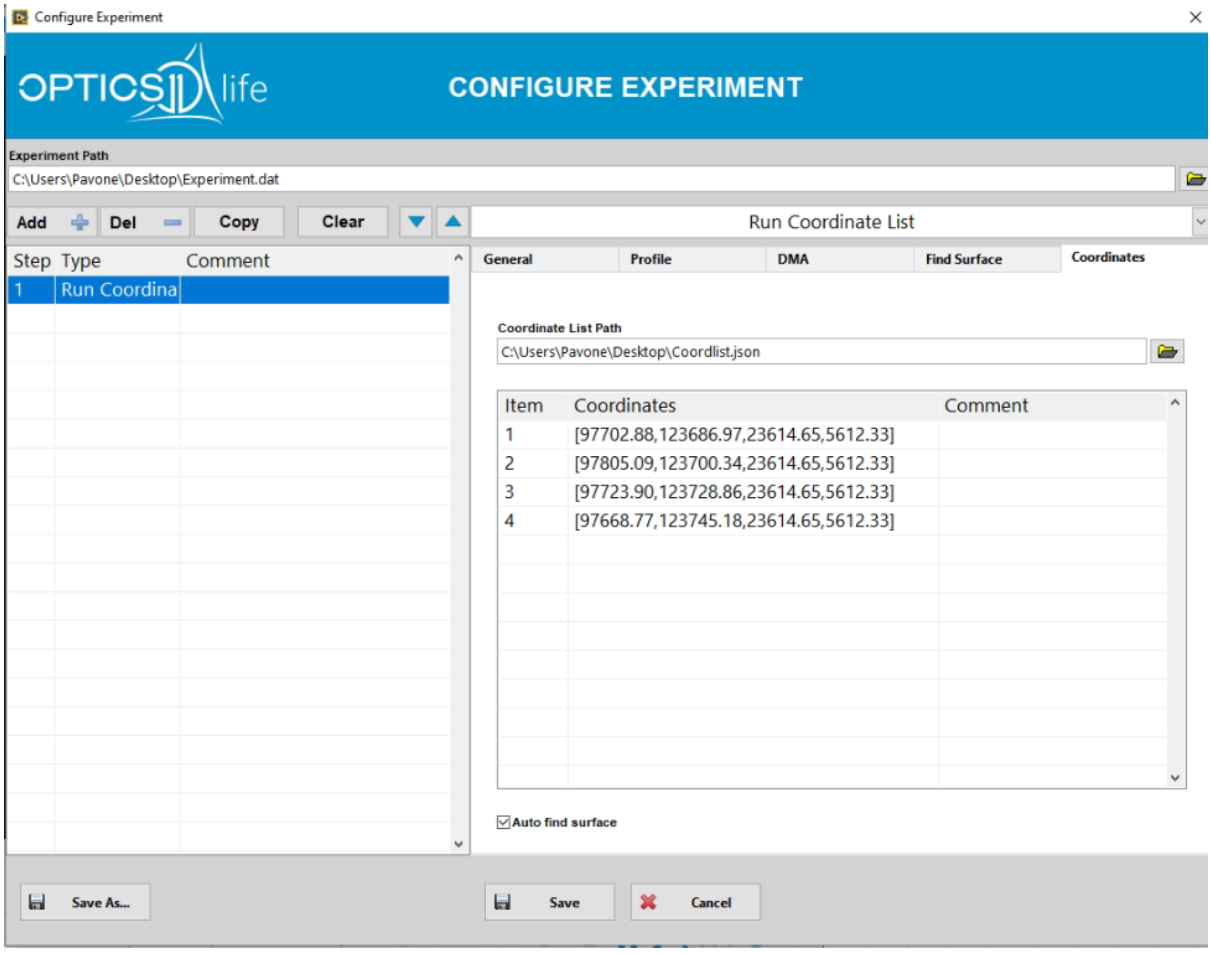


Figure 35: Setting up indentations on a list of selected coordinates.

3.4.9 Environment control

The temperature setpoint (from 15 to 50°C) during the experiment can be set in “Environment control” menu (see **Figure 36**). One should wait 30 min to let the system and the sample plate to warm up to the desired temperature.

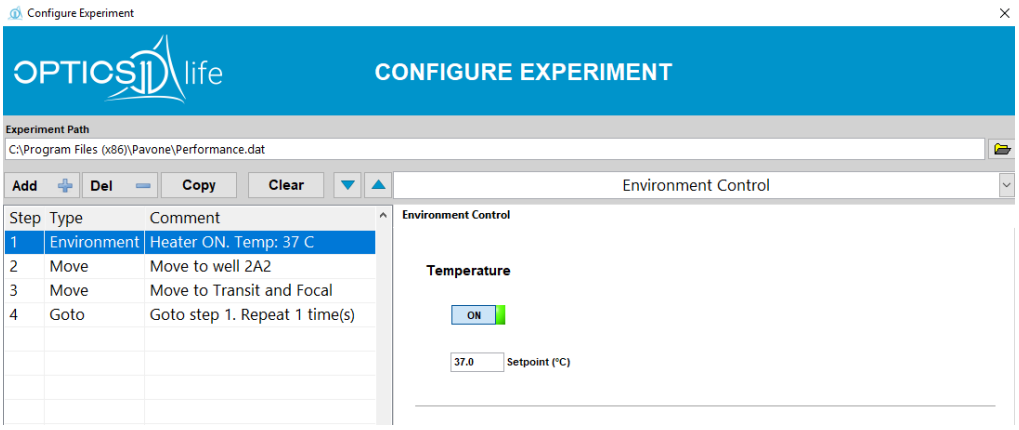


Figure 36: Setting up the temperature set point in the “Environment control” menu.

3.4.10 Configure experiment sequence for microplates

For automatic measurements in wellplates also a sequence can be programmed in the 'Configure experiment' menu. When opening the menu an empty table can be configured to include the steps required in an experiment from six pre-configured actions. To do so, click the 'Add' button, and couple an action type to the step by clicking on it from the drop-down menu on the right (see **Figure 37**).
After selecting the appropriate action type, click on the step in the sequence and browse through the different tabs and parameters on the right side to configure this step. When done, the entire sequence can be saved under a specific name by changing the name in the experiment path and pressing 'Save'.

1. Move to well
2. Move to transit and focal height or Move Absolute or Move Relative
3. Wait
4. Find Surface
5. Matrix scan/Coordinate list/Indentation
6. Go to step 1 and repeat the sequence for "**n**" times ("**n**" represent the number of selected wellplates); more details are described in the following section.

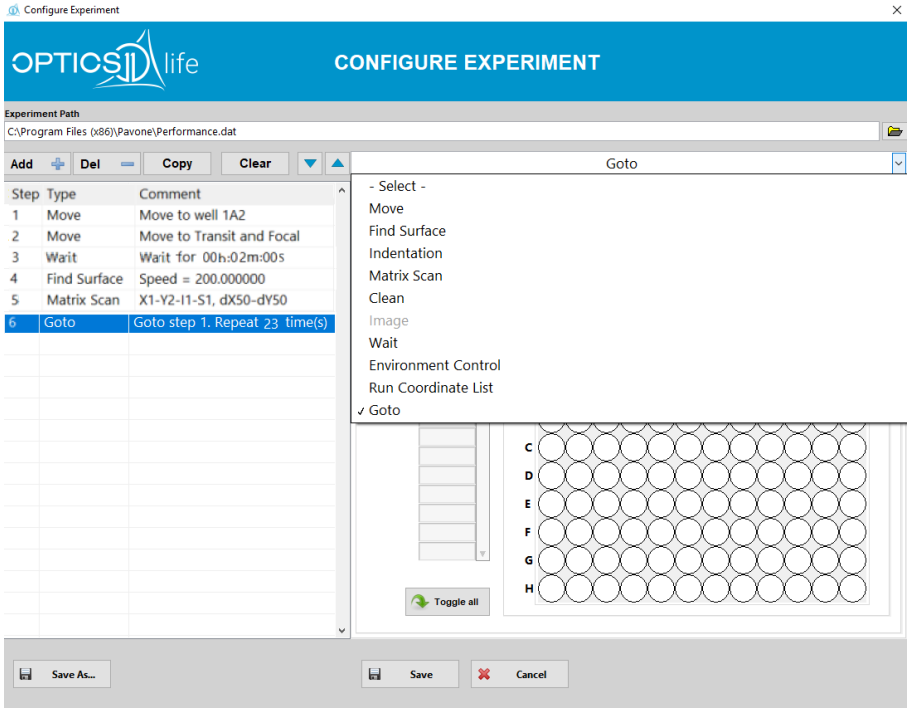


Figure 37: Configure the experiment menu with a drop-down menu, displaying the six action types.

3.4.11 Goto function

The "Goto" function can be used to set up automatic measurements in the whole wellplate (wellplate slot 1 or 2). This function acts as a loop and repeats a sequence of actions that are configured in the previous steps for selected number of wells. To use this function, simply click on "Add" button and select "Goto". In the "Goto" window you can adjust the following options (see **Figure 38**):

- **Go to step** – here you choose from which step the "repeating" process should start. Note that always the selected step should be the step where the "Move to well" action is defined (usually step number "1"), from which, all steps of programmed experiments will be repeated for a new well. The end-point of each single "repeating" sequence is the "Goto" function itself meaning that all steps of the programmed experiment that are defined below the "Goto" function, will be executed during each single "repeating" sequence.
- **Edit step** – this is always set the same as "Go to step". Here the selected step will be edited.
- **Step type** – "Move".
- **Wellplate slot** – here the number of the wellplate slot (number 1 or 2) can be selected.
- **Toggle all** – all wells within the selected wellplate will be selected.

To select the wells individually, you can simply first choose the slot of the wellplate, then wellplate type (e.g. 6well, 12well, 24well and, etc.) and then click on the wells that the programmed experiment needs to be performed at. By selecting each well, the color of that specific well will turn black (see **Figure 38**). Note that you can use more than one "Goto" function to program your experiments with different configurations for different wells or wellplates. The important point is to select an appropriate start-point for each "Goto" function by referring the "Go to step/ Edit step" to the correct step. Choosing a wrong start-point ("Go to step/ Edit step") for each "Goto" function may lead to an unwanted sequence of experiments.
In case of any question, get in contact with support@optics11life.com to get more information and training.

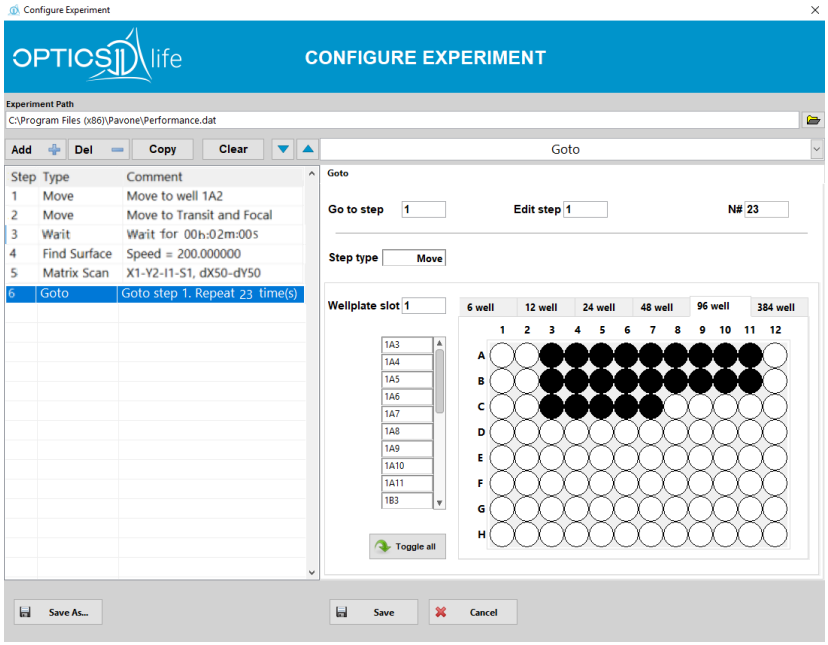


Figure 38: "Goto" function window and selected wells in black.

Caution: make sure to select the right wellplate slot and wellplate type. Only single wellplate can be programmed for a single 'Go to function'. Make sure that no wells are selected in other wellplate tabs.

3.5 Camera settings and Microscopy

To have a good image of your sample or probe, camera settings need to be adjusted by the user in the “Camera” option in the main window (see **Figure 39**). One can select parameters to be adjusted automatically by ticking ‘Automatic’ or use a one-time automatic adjustment from the main software window “Auto Setup Camera”. The maximum ‘Frame rate’ of the camera is 47 fps and it is dependent on the exposure time e.g. exposure time of 100 ms (0.1 s) limits the frame rate to 10 fps (1/0.1=10). To achieve higher frame rates, set shorter exposure time. From the main window, one can select to turn on 3 different LED rings: Brightfield, Phase one and Phase two. “LED intensity” sliding bar in the main window allows to adjust the intensity of LED rings.

For saving the images, “File format” of the “Camera” window (see **Figure 39**) allows setting image saving options when using “Save image“ button from the main window (‘Front panel’) or automatic saving of images or videos “During experiments”. Images taken during the experiment will be saved in the same location as the text files of indentation. Moreover, the objective type (4x, 10x, 20x and 40x) can be selected in the Objective menu. If another objective is used, um/pixel value can be calibrated using XY stage movement in the camera view.

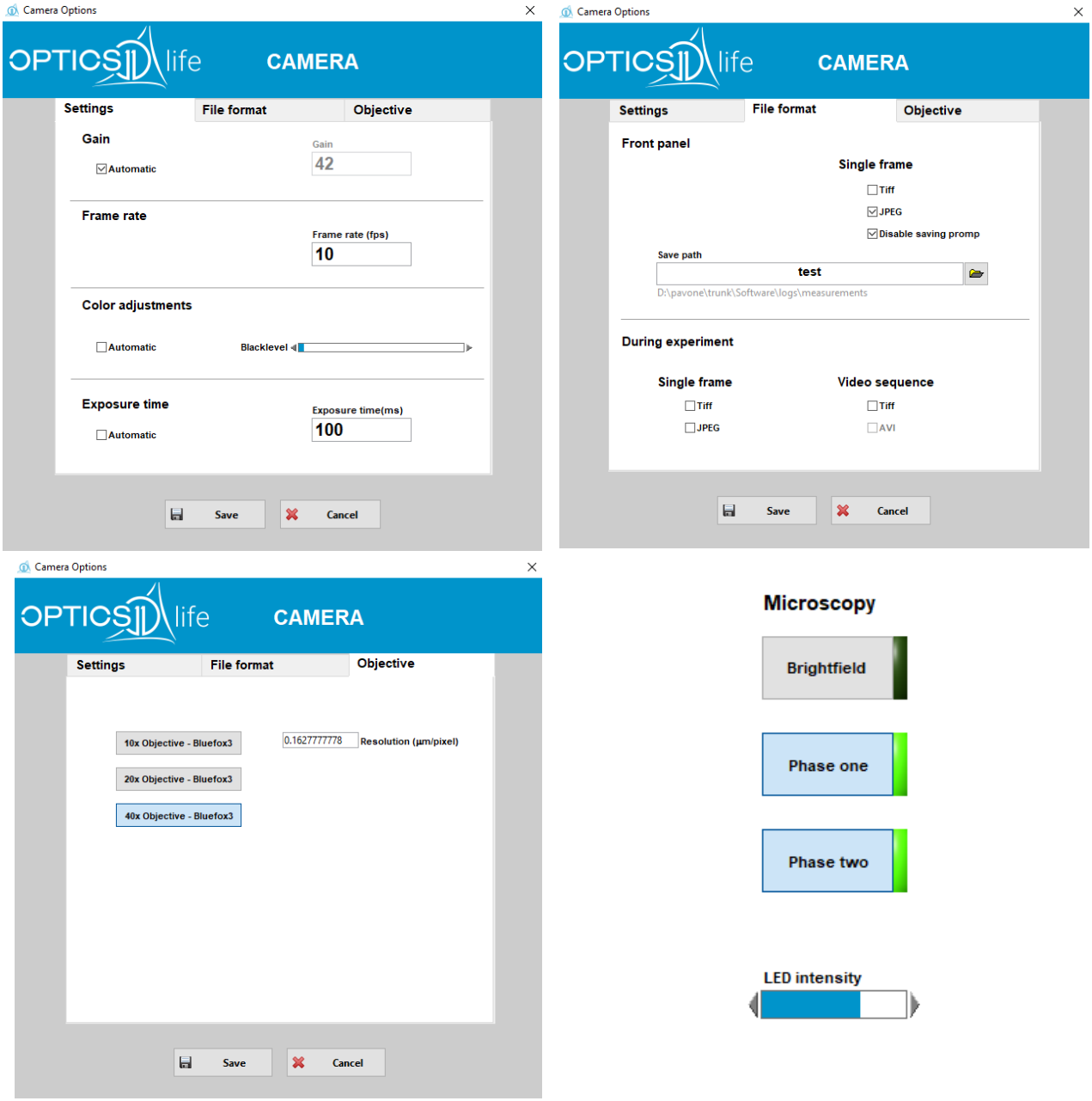


Figure 39: Camera settings and image saving options.

3.6 Data processing and analysis

For every single indentation and matrix scan, all the load-displacement data is stored in a tab-separated text file (see **Figure 40**). By using this text file, Time, Load, Indentation, Cantilever, Piezo and Auxillary data can be imported into any program for post-processing. Also, the DataViewer program can be used to quickly load all data and make small adjustments in the fitting where necessary.
For each matrix scan, a tab-separated text file is saved with a matrix of the Effective Young's Modulus per coordinate. Additionally, per indentation, a separate text file is saved that contains all information for this indentation. When reanalyzing the data in DataViewer, "Save" option will create a new folder "_corrected" with the new "Results.txt" and "E-eff vs XY position.txt" files. The header of the file contains

all essential information about the measurement. Please read the DataViewer manual regarding data analysis.

```
Date    10/02/2021  Time    07:23:16    Status  OK
0.2kPa_Twente
Scan (#)    1   X (#)   1   Y (#)   1   Indentation (#) 1

X-position (um) 55863.4
Y-position (um) 71501.7
Z-position (um) 20782.4
Z surface (um)  20802.4
Piezo position (nm) (Measured)  -13.3

k (N/m) 0.028
Tip radius (um) 22.000
Calibration factor  2.428
SMDuration (s) 8.3

Device: Pavone
Software version:  V1.5.2

Control mode: Indentation
Measurement: DMA

Profile:
Time 6.000 Freq 0.100 Periods 5.000 Amp 50.000 Relaxation 2.000
Time 58.000 Freq 1.000 Periods 5.000 Amp 50.000 Relaxation 2.000
Time 65.000 Freq 5.000 Periods 5.000 Amp 50.000 Relaxation 2.000
Time 68.000 Freq 10.000 Periods 10.000 Amp 50.000 Relaxation 2.000
Time 71.000 Freq 20.000 Periods 20.000 Amp 50.000 Relaxation 2.000
Time 74.000 Freq 50.000 Periods 50.000 Amp 50.000 Relaxation 2.000
Depth (nm) 5000.0
Loading / unloading time (s)    1.0
Initial relaxation (s) 5.0
DMA absolute start times (s)    14.283,75.001,82.343,85.343,88.343,91.343
DMA absolute end times (s)  72.999,80.342,83.342,86.342,89.342,92.338


Model: Hertz
P[max] (uN) 0.177
D[max] (nm) 0.000
D[final] (nm)   0.000
D[max-final] (nm)   0.000
Slope (N/m) 0.000
E[eff] (Pa) 2961.425
E[v=0.500] (Pa) 2221.068
Frame rate (FPS)    30.000
Comment:

Time (s)    Load (uN)   Indentation (nm)    Cantilever (nm) Piezo (nm)  Auxiliary
```

Figure 40: Typical indentation file showing the header lines and the start of the five data columns.

4 ADDITIONAL SYSTEM CALIBRATION AND SETTINGS

4.1 Wellplate calibration - adjusting the safety areas

Each wellplate has safety areas, which are determined by the dimensions of the well plate (see **Figure 41**) and calibrated by the user. This guide will show how to add well plates and how to adjust safety areas.

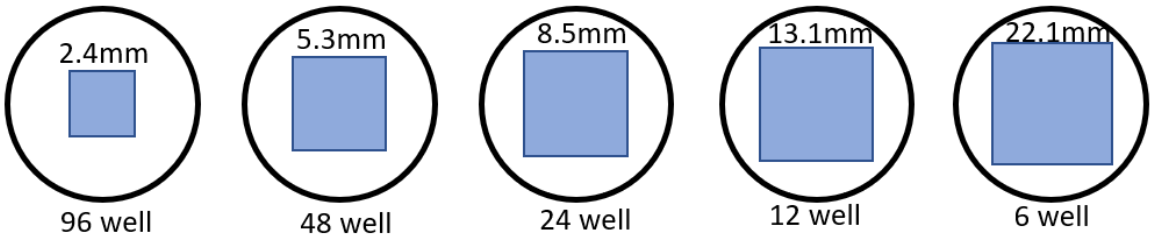


Figure 41: Example of the safe areas for different wellplates.

Follow these steps:

1. Mount two same wellplates or custom plates on Pavone, open the “Wellplates” menu from the main window and select the “calibrate” option (see **Figure 42**-a). Make sure that the probe is aligned with the objective (“Find Probe” function) and then press “continue”.
2. Press the “Add” button and from the right menu on the right side select the “Wellplate” if you want to calibrate a wellplate or “Custom” if you want to calibrate a custom plate (e.g. glass slide or a petri dish holder) (see **Figure 42**-b). In following the steps for calibrating a wellplate are described. The same steps should be performed for calibrating the custom plates.
3. In the “General” menu, choose a “Name” and select the type of the “Plate” (see **Figure 42**-c).
4. The calibration process should be done for four wells i.e. top-left corner and bottom-right corner wells of wellplate in slot 1 and slot 2. These four wells can be recognized by red squares (showing the safe area of the well) (see **Figure 42**-d).
5. Select the first well from the “Plate” menu which is well A1 from the wellplate slot 1 (“Well 1A1”).
6. Now calibrate the “Top left Position” and “Bottom right Position” of this specific well by moving the stage in “X” and “Y” direction and the probe in “Z” direction to see that the probe can enter safely the well. The step size can be set in “Travel (µm)”. You can move XY stages by clicking on the blue arrows on the bottom side of the current window (see **Figure 42**-d). First, move the stage to a place where the probe can safely enter the well in the “Top left position”. Then by choosing small steps sizes (“Travel (µm)”), slowly move the Z-stage down with the probe and enter the well. If the probe can safely and freely (without collision with the well edges) enter the well, you can press the “Save Position”. By this, the current “X” and “Y” coordinates of the stage will be saved as a safe “Top left position”. In the lower menu, repeat the same procedure for the “Bottom right Position”. In case of any doubt, you can press the “Move to point” option and double-check the “Top left position” and “Bottom right position” before proceeding to the calibration of the next well. Note that the movement of the stage and probe during the calibration process can immediately be terminated by pressing the “Stop” button (see **Figure 42**-d).
7. Proceed and select the next well from the “Plate” menu.
8. Repeat step 6, and calibrate the “Top left position” and “Bottom right position” for the selected well.
9. Complete the calibration process for all four wells and press “Save” (see **Figure 42**-d).
10. The calibration process is completed meaning that the calibrated wellplate can be selected from the left-side menu in the “Wellplates” window for future experiments.

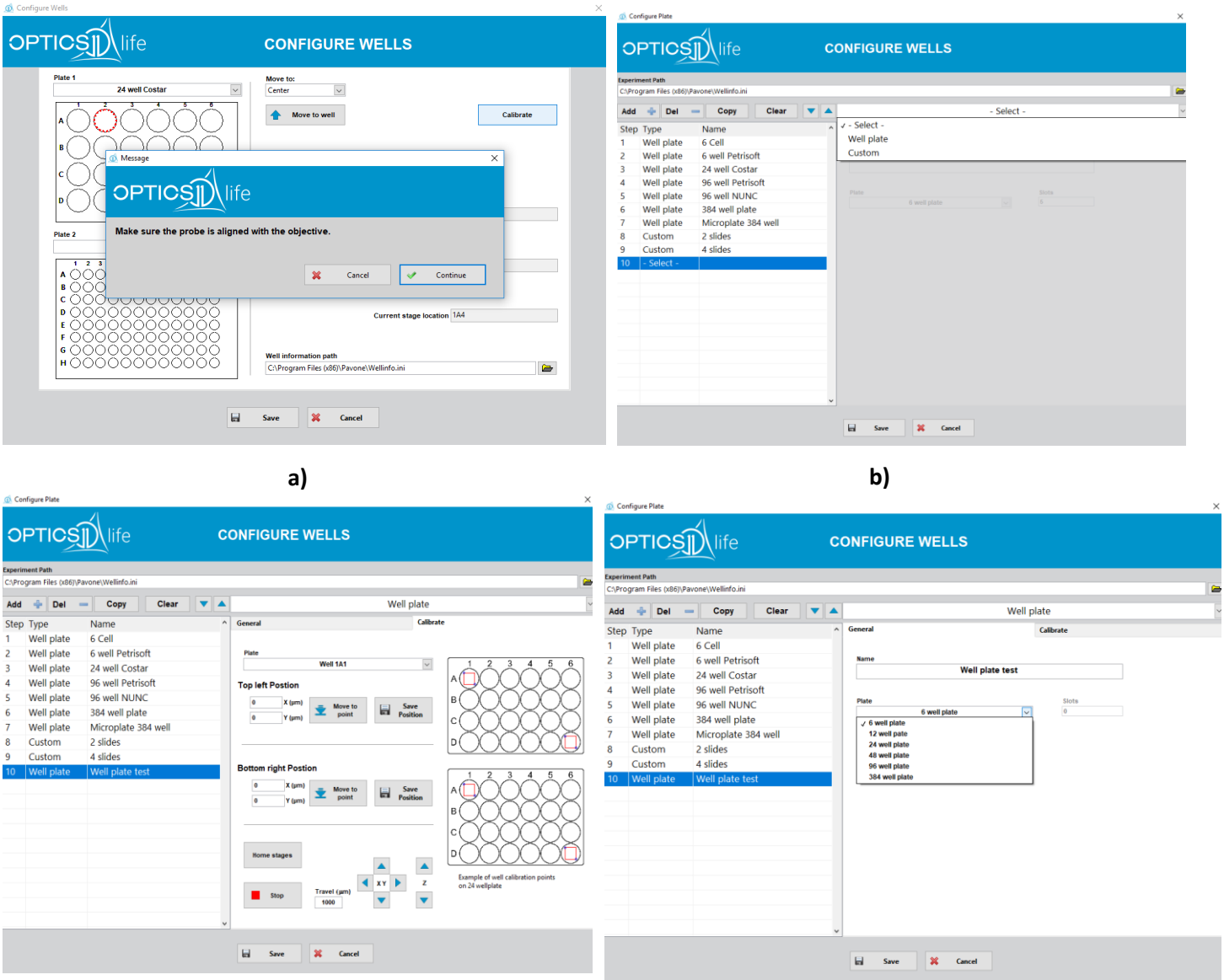


Figure 42: Different stages of wellplate calibration process.

4.2 Camera calibration

The camera must be aligned properly to the stage movement to ensure the precision of move-to-point function. This guide will enable one to set up the camera to ensure smooth operation.
The camera is affixed with three set screws that must be loosened to adjust the rotation of the camera (see **Figure 43**). The camera can be roughly positioned using two white bars that should be touching, then it will be fine-tuned by monitoring the motion of the stages.

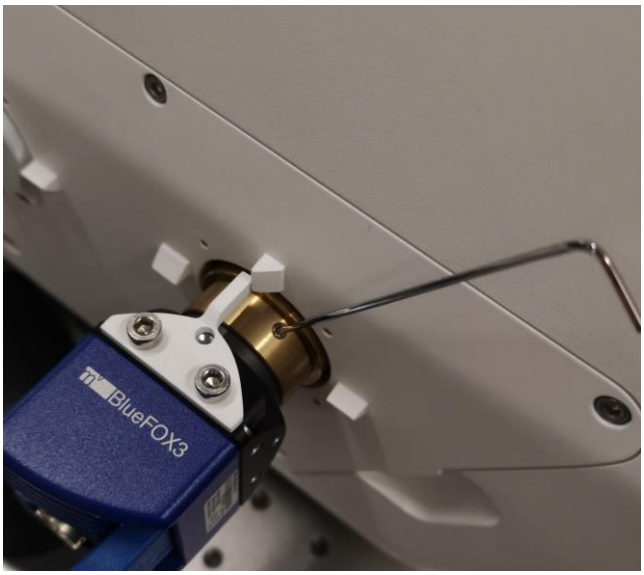


Figure 43: Camera alignment.

Follow this procedure (Figure 44):

1. Adjust the camera to have the mount aligned as shown in the picture. Tighten up screws but not strong, so that you can easily readjust them.
2. Place any type of well-plate and focus on a small object such as dust. You do not need to mount the probe.
3. Press with the cursor on the small object and note the coordinate Y of the green cursor.
4. Move the stage in Y direction across the field of view so that you can still see the small object.
5. Press with the cursor on the small object and note the coordinate Y of the green cursor. Compare it with the one noted before.
6. Rotate the camera accordingly so that the Y coordinate of the object, measured at the two sides of the field of view, matches.
7. To check that you succeeded, open “find probe” position window, draw a small circle anywhere on the screen. Then, select a small object and press “move to point”. Next, open “find probe” window and check whether the small object is in the circle.

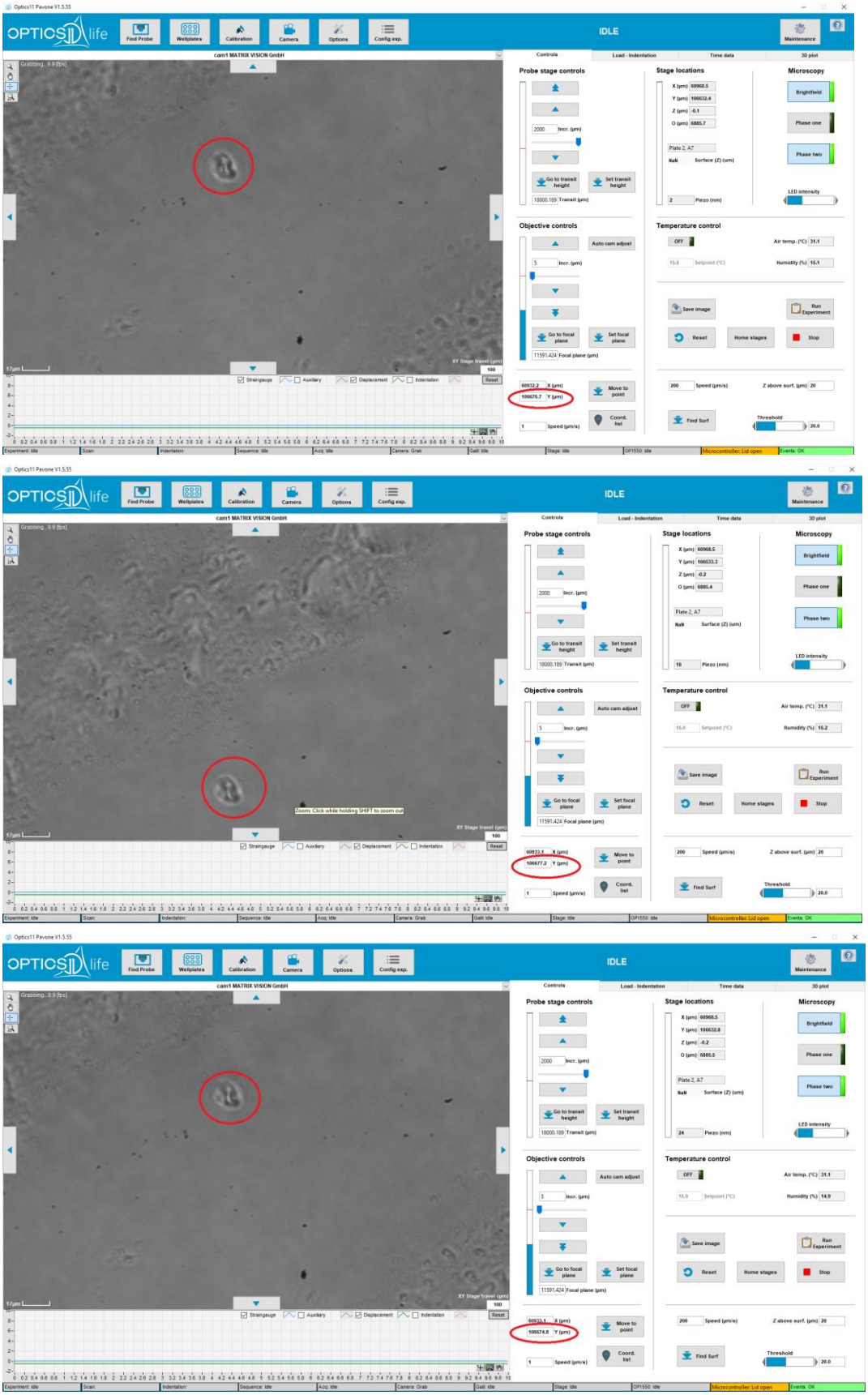


Figure 44: Camera alignment (top to bottom): choose a point with a green cursor, Y coordinate 106676.7. Move up the Y stage and press on the same object, Y coordinate 106677.2. Rotate the camera clockwise. Move stage down again, Y coordinate 106674.6. Continue until the Y coordinate is aligned.

4.3 Maintenance settings

Maintenance settings are not intended to be changed by the user unless they are specially trained to do it by one of the application specialists of Optics11 Life (see **Figure 45**). The default maintenance settings are saved in the desktop folder 'settings'.

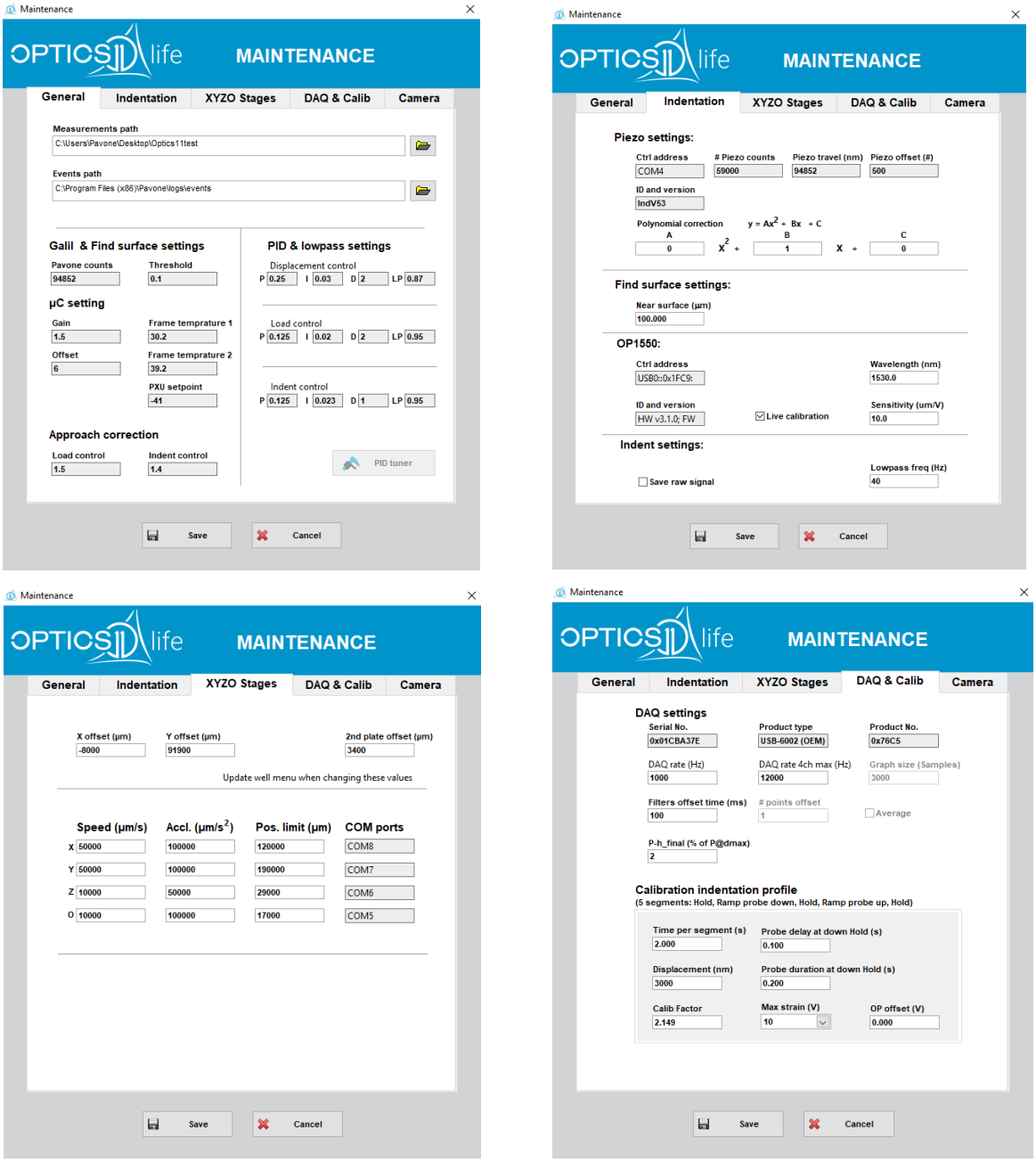


Figure 45: Example of maintenance settings.

5. OPTIMIZING INSTRUMENT STABILITY AND SAMPLE MOUNTING

Optimizing the experiment conditions and sample mounting can greatly improve the quality of the indentation. During an indentation, environmental stability, sample stability and probe suitability define the quality of the experimental dataset. In case the indentation results show irregularities or high noise, it is worth considering the factors as described below.

5.1 Environment stability

The Pavone is engineered to operate as independently from the environment as possible. Nonetheless, it is still possible for the environmental conditions to influence and compromise measurement results.

Temperature

In order to minimize the measurement error due to temperature drift, sudden room or medium temperature changes should be avoided when measuring. Verify that the Pavone is mounted away from any air-conditioning, heating, or ventilation vent. The built-in temperature control system can be used to measure samples at physiological temperatures. Be aware that the temperature system can also introduce drift in the measurement if the sample or medium temperature has not yet reached an equilibrium state. **As a result, you will see that the signal is not on the demodulation circle and requires recalibration.**

Vibrations

To minimize the measurement error due to mechanical disturbances, such as ground vibrations from other labs, other equipment, or appliances, ensure that the Pavone is mounted on a stable bench. Preferably this bench is not shared with other vibrating machines such as vortexes, pumps, or large fans. Optionally the Pavone can be bolted to the top of a third-party stabilization platform, such as an air table. Make sure that the air table is inflated when using Pavone.
Mechanical instability can also originate from the sample container or sample substrate contact with the Pavone sample stage. In order to get the best measurement possible, the complete 'measurement loop', i.e. everything between the probe and the sample stage base plate, should be free from instability and be as stiff as possible. This can be achieved by ensuring good and stable contact between the sample carrier/wellplate and the Pavone sample stage, and by using stiff substrates and containers. Less stiff sample carriers, such as trans-well membranes, may be used, but it is advised to check the stability of the sample carrier or the insert when mounting it into the Pavone.

5.2 Probe stability

Solution temperature

When immersing the probe in a liquid of a different temperature, such as refrigerated liquids or liquids obtained from the incubator, wait upon immersion before starting calibration. The probe and medium must reach equilibrium in temperature. This can be observed by the interferometer signal: if the voltage is changing when the probe is immersed, i.e. the signal is drifting, it means that the probe and medium

are still adjusting to its new environment. When using temperature control of Pavone, wait until the set temperature is reached. Also, when submerged the probe into the medium, wait at least 5 minutes before calibration to make sure that the probe “gets wet”.

> **Caution: Probe calibration should be performed at the same temperature as the experiment.**

Air bubble

As the probe and cantilever are made of glass, an air bubble may get trapped between the probe and the cantilever: this will result in a failed probe calibration or excessive noise in the measurements. Please proceed with the removal of the air bubble as described in the steps below.



Figure 46: Side-view of an Optics11 probe while immersed in a transparent medium. As indicated by the red arrow, an air bubble is present at the base of the cantilever (left) and, Removing the air bubble by drying the probe (right).

To remove an air bubble, lift the probe out of the medium and carefully take it out of the probe mount. Now draw the liquid out of the cantilever-probe gap by holding a tissue to the side of the probe. Once the bubble pops, the surface tension is released, and when immersed again, the air bubble will not form as the previously dry surface got wet after bursting the bubble. Note that you should avoid directly touching the cantilever: the cantilever is very fragile and can break easily when touched. Holding the tissue at a safe distance will also allow you to dry the probe without touching the cantilever (see **Figure 46**).

Suitable probe cantilever stiffness

To perform successful indentation experiments, it is necessary to have a probe cantilever with a suitable stiffness compared to that of the sample. When the probe cantilever is too soft, the cantilever bends a lot but does not indent enough on the sample resulting in very low indentation depths. When the cantilever is too stiff, the cantilever does not bend enough and indent too much into the sample, resulting in very large indentation-depths. Read Section 2.2 for more information on how to select optimal probe or use an excel document called “Probe selection calculator”.

Suitable probe tip size

Choosing an appropriate tip size is related to the structure of the sample and the kind of experiment one wishes to perform. When measuring the global elasticity of a sample, a larger tip would be more suitable, since the applied and recorded force of each indentation would effectively average over a larger area. For measuring small spatial features, a smaller tip could be more suitable. Tip size should be carefully considered when working with porous materials: to determine the overall structural properties of a

porous material, select a tip size several times larger than the largest pore. For examining local features within one pore, select a tip size that is several times smaller than the pore diameter.
There are a few other considerations regarding the tip radius:

1. The maximum indentation depth shouldn't be more than 16% of the tip radius. This is the Hertz model assumption of the parabolic indenter used to calculate the contact area during indentation: $a = \sqrt{h * R}$, a - contact radius, h - indentation depth, R - tip radius.
2. The maximum indentation depth shouldn't be more than 5-10% of the sample thickness to do not sense the substrate underneath the sample. Furthermore, the substrate should be much stiffer than the sample itself.
3. The sample is also assumed to be an infinite half-space, thus, tip size should be chosen so that this assumption is true.
4. When performing indentation mapping, the step size should be at least two times the contact radius *a*, to avoid oversampling.

Regarding the tip size, you should balance between the spatial resolution of the mapping which depends on the heterogeneity of the sample and the scale of the measurement in terms of depth. If you only want to sense surface properties, a smaller indentation depth is desirable. If you want to sense more bulk/averaged properties, you should consider measurements at larger depths with a larger radius. However, when using larger spheres, step size needs to be higher and, thus, you lose spatial resolution during indentation mapping (see **Figure 47**).

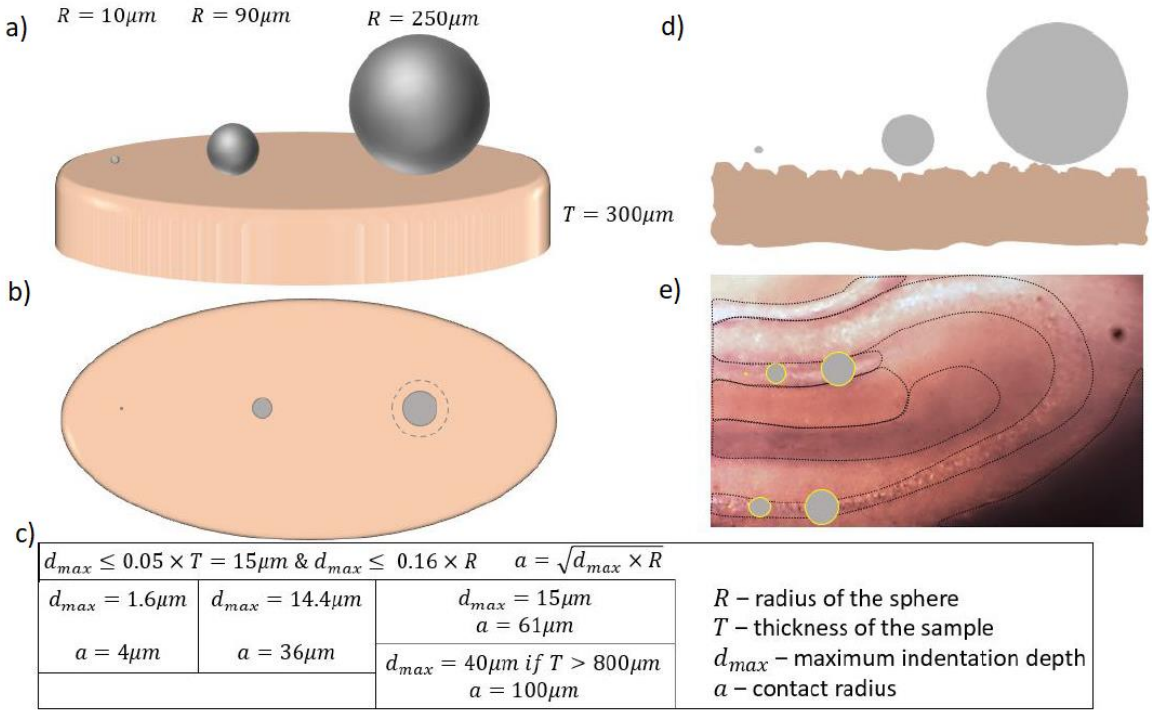


Figure 47: Example of maximum indentation depth $\mathbf{d_{max}}$ and B) contact radius a for three tip radius R in relation to the A) sample thickness T, D) surface roughness and E) regional heterogeneity (hippocampus).

Suitable indentation profile

Please request the chapter “Mechanical testing of soft tissues by indentation” (Nelda Antonovaite, "Exploring the mechanical microenvironment of the brain by dynamic indentation”) which gives the background for selecting indentation profile.

5.3 Sample stability

Additional to the environment, the sample itself can also introduce a measurement error.

Sample temperature and integrity

When attempting to measure samples that come fresh out of the incubator please allow some time for readjustment to the Pavone temperature. This applies even more so to larger temperature gradients: if medium or samples come out of a refrigerator, let the sample temperature stabilize to room temperature before initiating the measurement.

The sample itself can also have unstable mechanical properties. It might be swelling if just hydrated or if there is a change in the osmolarity of the medium or if the sample is degrading. When measuring a highly heterogeneous sample, such as decellularized ECM, porous scaffolds or 3D-printed structures, extra care should be taken in selecting the appropriate cantilever stiffness, tip size and indentation settings. For example, when working with samples that have a porous surface, selecting a tip diameter that is either much smaller or larger than the average pore size avoids having the tip trapped in the sample.

Sample immobilization

Next to a stable sample, it is important to immobilize the sample to eliminate lateral and height drift during and between indentations. Indenting drifting or floating samples results in unreliable indentation measurements such as high noise or irregular curves. Especially when performing automated matrix scans make sure the sample is immobilized correctly. For more information about how to mount the sample, please read the separate document called “Sample preparation protocols”.

Adhesive samples

Some hydrogels and tissues are very sticky which will result in a large distance needed for “Z above surf” in order to perform the measurements out of contact. Also, there might be some snap-on behavior in the loading curve which makes the calculation of the contact-point less precise. To decrease the effect of adhesion, you can use a stiffer probe as the attractive forces will have a smaller effect on the cantilever. However, using stiffer probe results in less precision for the soft samples at low indentation depths. Furthermore, adhesion can be minimized by cleaning the probe from the dirt, coating the sample with 5% BSA or coating the tip with 1% pluronic solution, where both of them make it hydrophobic.
Furthermore, the Hertz model assumes that the sample is not adhesive, thus, if adhesion is large, please use the JKR model available in the DataViewer as it can estimate the contact area better. For more information about adhesion and coating, please read the separate document called “Sample preparation protocols”.
Finally, ‘live calibration’ function in the maintenance should be turned off when measuring adhesive samples (see Section 6.2).

6. THE OP1550 INTERFEROMETER

The interferometer operates as a stand-alone device and can be switched on or off at any time. The OP1550 interferometer contains a tunable laser source, modulation options, a high-speed photodiode, and data acquisition electronics (see **Figure 48**).
The OP1550 interferometer is configured using the five buttons on the front panel that allows you to walk through the menus and select and alter menu items. In order to select a field or button on the OP1550 screen, move the cursor over the button or field and press the center button (enter). In case a field is selected, the field will turn blue and the up and down buttons can be used to alter the value the cursor highlights.
The interface is built up out of two menus. The first menu (Measurement, Wavelength scan, Demodulation, and Settings) can be accessed using the buttons on the top of the screen. The second menu (Laser, ADC, Output, Modulation, and Maintenance) can be accessed by scrolling from left to right while keeping the cursor on the right-hand side of the screen. The settings described below might differ depending on the model of the interferometer while working principles are the same in all of them.

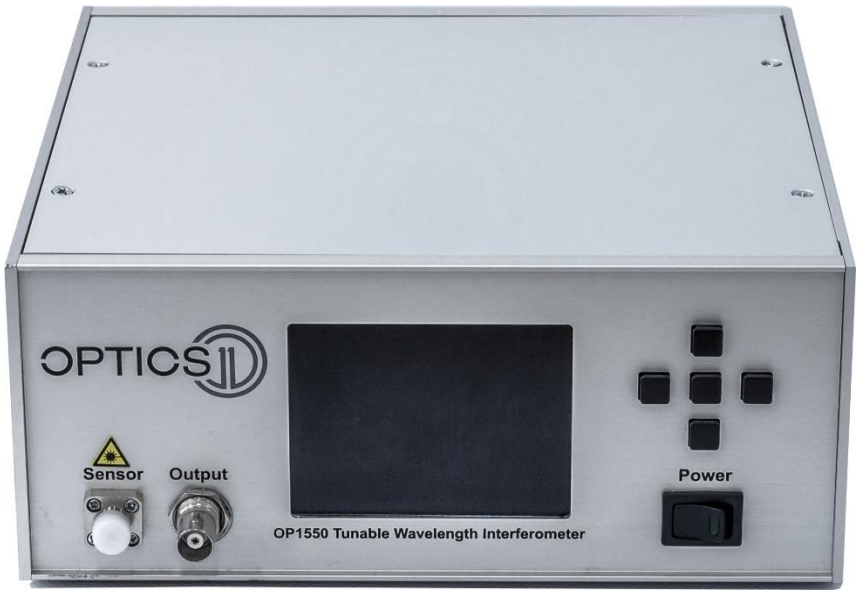


Figure 48: The OP1550 interferometer.

6.1 Using the OP1550 interferometer

The measurement, wavelength, demodulation and settings menu

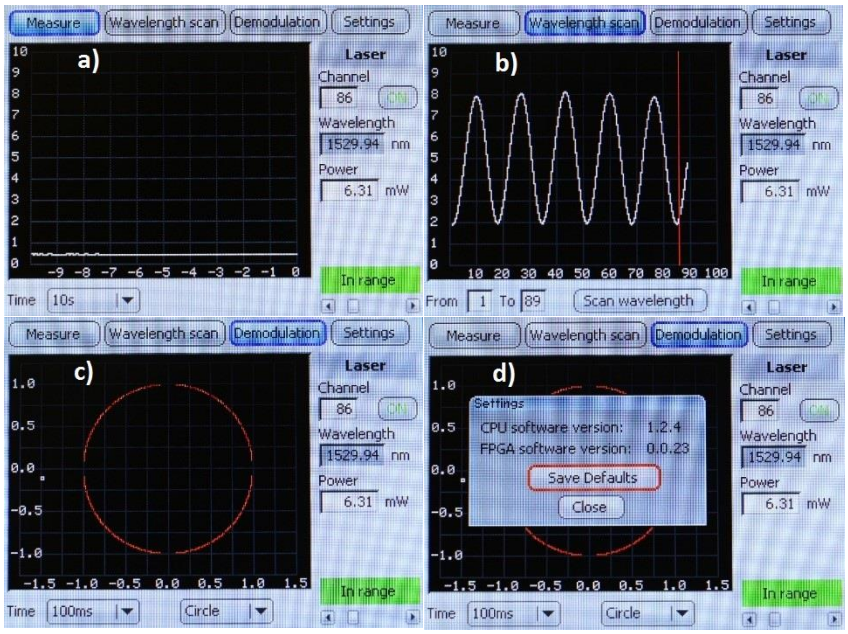


Figure 49: Measurement (a), Wavelength scan (b), Demodulation (c) and Settings menu (d) of the OP1550 interferometer.

The **'Measure'** tab displays the actual intensity that reaches the photodiode, which converts this intensity into a voltage. During indentation, when the cantilever bends, you can observe interference fringes which are the results of fiber/medium and cantilever/medium reflections (see **Figure 49**-a).

The **'Wavelength scan'** menu shows the resulting interference fringes after a wavelength scan is performed during which the wavelength of the laser is swept within ~50 nm range. This step is required to assess the gap between the cantilever and the fiber tip. During this step, the gain and offset (see ADC menu) of the interferometer are set so that the visibility of the interference signal, i.e. peak to peak signal of fringes, is optimal. Wavelength scan can be performed by pressing the 'Scan wavelength' button at the bottom of the screen in the 'Wavelength scan' menu or through the software in the 'Initialize' window. A good wavelength scan results in a sinusoidal curve as shown in **Figure 49**-b.

The **'Demodulation'** tab displays the linearized signal of interference fringes presented on a unit circle which is achieved through high-frequency wavelength modulation. It can be very useful for observing the cantilever bending, while manually approaching the surface of a sample. When the tip is moving out of contact, the white signal is going to stay in the same position. As soon as the tip touches the sample and starts loading the sample, the white dot will start moving clockwise around the circle. When the probe is retracted during unloading, the signal will move counter-clockwise (see **Figure 50**).

The **'Settings'** menu allows setting all current parameters as default values. When restarting the OP1550 interferometer the last saved values will be loaded.

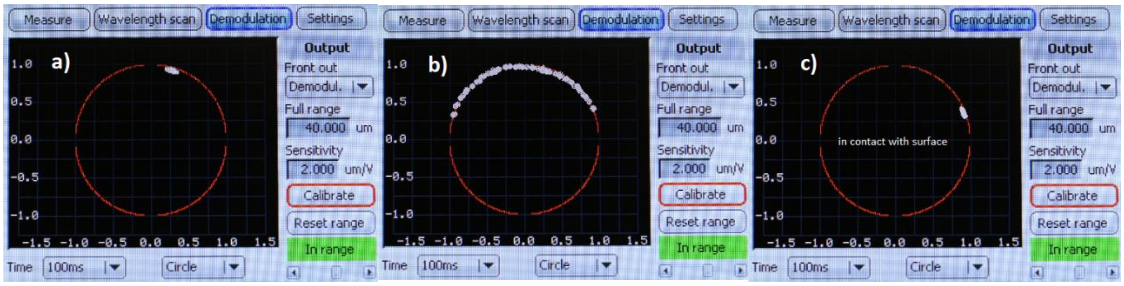


Figure 50: Datapoint changing its position after the tip is getting in contact with a surface: probe out of contact (a), noise caused by probe moving during automated find surface procedure or by manually moving the Z-stage (b), probe in contact with surface resulting in datapoint position change (c).

The Laser, ADC, Output, Modulation, and Maintenance menu

The right side of the screen is reserved for the **Laser**, **ADC**, **Output**, **Modulation**, and **Maintenance** menu. To scroll through the menus, move the cursor to the section right of the graph area, and press the left or right arrow (see **Figure 51**).

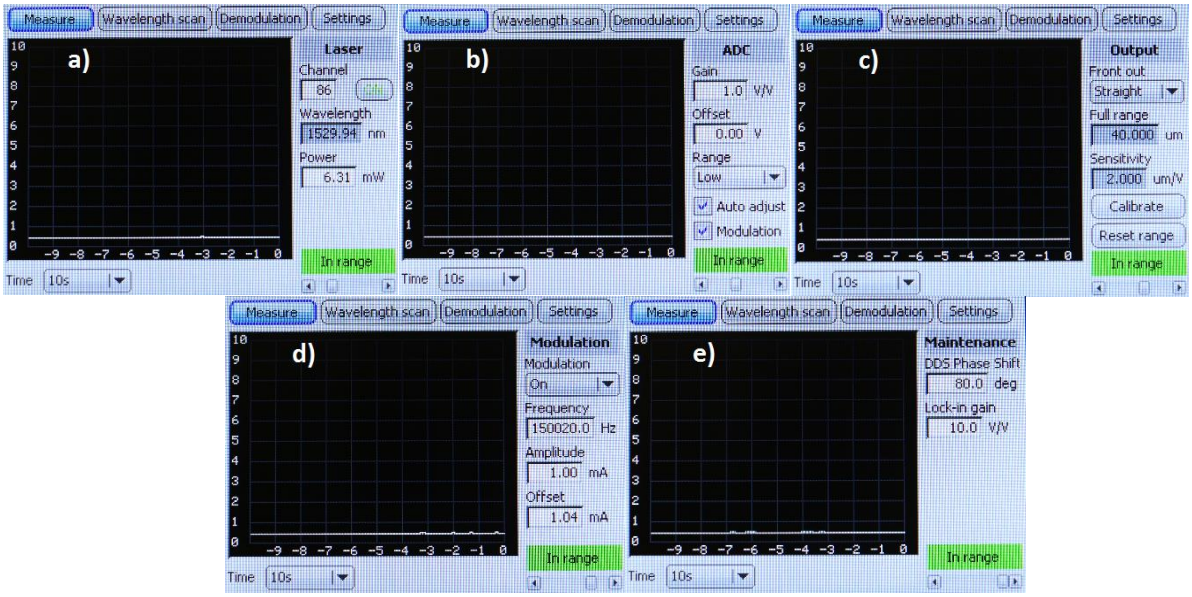


Figure 51: Laser (a), ADC (b), Output (c), Modulation (d) and Maintenance menu (e) of the OP1550 interferometer.

The '**Laser**' menu displays the laser controls. Always operate channel 86 of the laser: this corresponds to 1530 nm. The minimum laser power is 6.31mW and the maximum one is 25mW.

> **Caution: Please consider increasing laser power to a maximum one when measuring in the medium as it will increase your signal-to-noise ratio (see the video: https://youtu.be/coKxhkDsS6Q). However, for the wavelength scan in the air, please decrease the laser power as there might be too much signal which would result in a saturated signal (flat line after wavelength scan).**

The **'ADC'** menu displays the photodiode settings. By using the 'Scan wavelength' feature in the 'Wavelength scan' menu, the Offset and Gain values for the conversion of the raw photodiode voltage to fit the 0-10V range are automatically performed.
In case the automatic procedure fails, the ADC menu provides the opportunity to manually adjust the offset and gain settings. Also, the automatic adjustment of the offset and gain settings can be switched off by unticking the 'Auto adj.' box. If this is done, the 'Scan wavelength' function in the **'Modulation'** menu will tune the laser without setting offset and gain values, which can be convenient for manual setting offset and gain values. Make sure the 'Modulation' box is ticked before performing an experiment.

In the **'Output'** menu you can adjust the signal output at the 'Front out' either as a 'Straight', which displays the modulated signal, or as a demodulated by switching to 'Demodul.' The range and sensitivity parameters can be changed in the maintenance menu in the software, but it is recommended to keep them at the default settings.

The '**Modulation'** menu contains the controls to induce a small modulation on top of the laser output signal by quickly and continuously modulating the laser wavelength. This modulation is required for the Pavone to function properly and should always be turned on when performing indentations. The modulation is active when the mode is set to 'On'; the menu will now show the controls to change the frequency, amplitude and offset of the modulation signal. In normal operation, these will not have to be changed.

In the **'Maintenance'** menu the modulation phase shift and the lock-in amplification can be adjusted to certain parameters. The default settings are 80.0 degree for the 'DDS Phase shift' and 10.0 V for the 'Lock-in gain' and should not be changed.

6.2 Live calibration

You can turn on the "live calibration" function in the "Maintenance" in the software. This enables the real-time correction of scaling factors of the demodulation circle so that the signal stays on the demodulation signal and load-indention curves do not look wavy at large cantilever bending. This function is required when the wavelength scan is not horizontal but on an angle as shown in **Figure 52**.

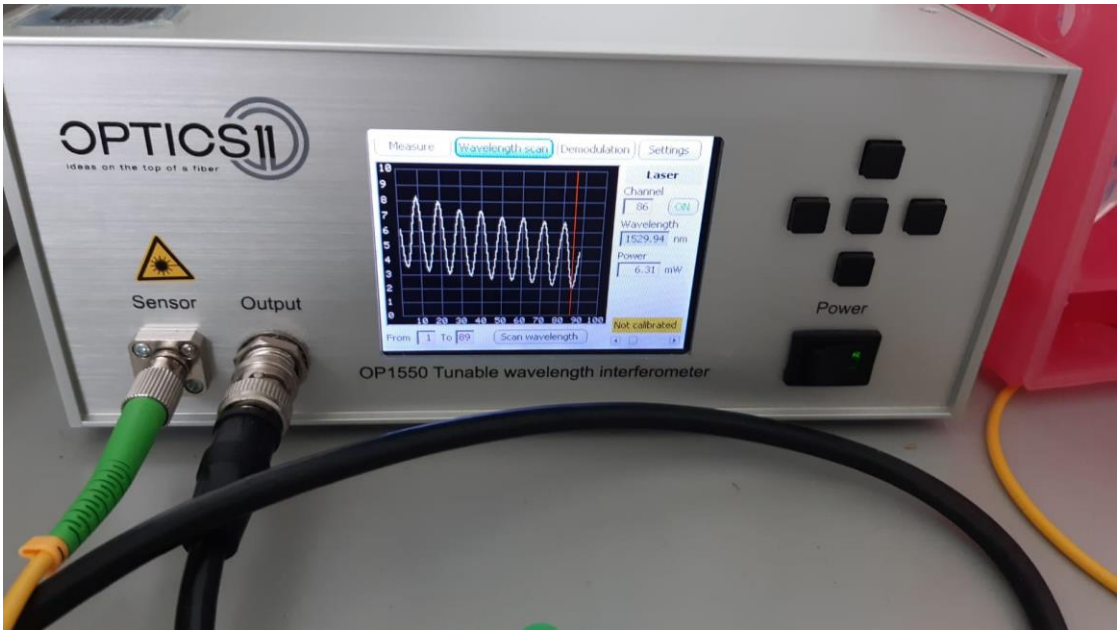


Figure 52: Tilted wavelength scan.

"Live calibration" should be enabled before starting the calibration procedure. Furthermore, calibration should be done to no more than 3um piezo displacement (you can change that in Options).
Do not use "live calibration" if the samples are sticky as it will cause a reset of scaling factors to the over-bended position. In general, better to use a stiffer probe for very sticky samples or contact us for ideas on how to decrease the stickiness.

Please see the videos on how demodulation signal looks with and without "live calibration":
https://youtu.be/1VL6cXOqMsE - live calibration on
https://youtu.be/IPfTWs02yQ8 - live calibration off

For troubleshooting, support or questions, while working with the Pavone, please contact Optics11 Life at:

Tel.: +31 20 5987917
E-mail: support@optics11life.com

Office hours are between 9h and 18h, CET.

