Attaching microspheres to cantilevers using the NanoWizard® Life Science stage and AFM head

Microsphere cantilever tips

AFM force spectroscopy can be used to obtain sensitive measurements of the stiffness of soft materials such as living cells and polymer gels. The surface inhomogeneity can be mapped and changes measured *in situ*, as living cells respond to the addition of particular drugs, for instance. The AFM is also a useful tool for measuring adhesion on a nano-scale. The tip can be functionalised with molecules for ligand-receptor experiments, or whole cells can be picked up to measure the adhesion with other cells or solid surfaces.

It is vital to know the tip geometry for indentation and elasticity measurements, and the surface contact area for adhesion measurements. The standard pyramidal tip shape is designed for high-resolution imaging, and a spherical tip shape is often more suitable for force measurements over inhomogeneous surfaces. For single molecule ligand-receptor recognition measurements, the tip end should be as small as possible to reduce the chance of multiple interactions, but sometimes investigators wish to have a statistical average over a small region, to enable comparison with different areas, or to monitor changes due to stimulation.

Microspheres are often used to modify AFM cantilevers to provide consistent and reproducible tip shapes. The smooth spherical surface can be used to reduce the indentation depth and localised pressure when measuring cell elasticity, for example, or to increase the interaction area in a controlled way for adhesion measurements.

Modified cantilevers can either be purchased, like particle probes from sQube (http://www.sqube.de, in range of sub-micrometer to micrometer scale) and NanoWorld, (http://www.nanoworld.com/) or they can be produced in the lab when needed. Nanoworld offers spherical tips in range of 1µm radius, or rounded tips from 50-500nm as well as plateau tips from 1-8 µm. Silica spheres (e.g. http://www.microparticles.de or http://www.polysciences.com) are well suited. It can be difficult to attach very small beads to the cantilever without enveloping them in glue. For applications requiring spherical indenters with a diameter of less than 2 um it may be desirable to purchase probes from the sources mentioned above.

NanoWizard® Life Science setup

The standard procedure for attaching spheres to cantilevers is to use some form of glue to attach them to the tip. Other attachment methods are also possible, such as to sinter silica spheres directly to the cantilever, for example. It is common for a separate micromanipulator to be purchased for putting spheres on cantilever tips, but this is not needed when using the NanoWizard® AFM and Life Science stage on an inverted optical microscope.

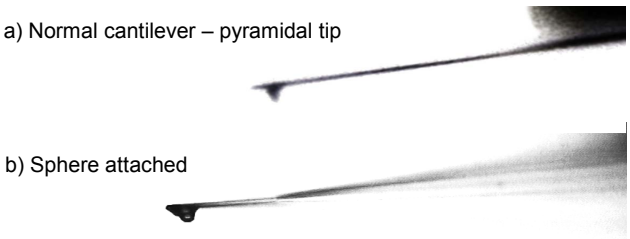


Fig 1. Optical images of a standard cantilever (pyramidal tip) and one with a sphere glued at the end. Note that the one with the sphere was a longer cantilever.

The NanoWizard® Life Science stage is designed for mounting on an optical microscope, such as the Zeiss Axiovert 200. Coarse positioning in the micron to centimetre range is done with the separate positioners for moving the sample and the AFM head. Fine positioning of the cantilever in the range from microns to nanometers can be carried out using the 100 x 100 x 15 micron piezos and the freehand positioning available through the software interface, although not in the case of the CellHesion 200 system. When mounted on an inverted optical microscope, the NanoWizard® AFM and Life Science stage provide all the tools necessary for cantilever modifications such as gluing spheres to the tip, making further equipment

purchases unnecessary. Performing the manipulation with the NanoWizard AFM has the advantage of completely flexible and accurate positioning through the linearized piezo system, and the forces applied to the cantilever can be monitored using the laser deflection.

This report provides a practical guide for people wishing to use their NanoWizard® Life Science stage and AFM head to attach spheres to cantilevers. The AFM cantilever remains within the field of view of the optical microscope, while the stage micromanipulators are used to move the sample to a patch of either glue or spheres. The cantilever is then positioned precisely over the edge of the glue or over a sphere using the x-y piezos and the software interface. The z-position can be brought into range using the stepper motors, and the z-piezo used to touch the glue or spheres with the cantilever tip in a controlled manner. The technique can also be extended to other applications, for modifying the tip surface with molecules or attaching other objects to the cantilever.

Materials

In this case, a two-part epoxy was used to attach silica spheres to normal cantilevers. The viscosity and surface tension of the glue are important, particularly for soft cantilevers which can get "sucked" into the glue layer. Some experimentation may be required to find the ideal glue and conditions. The working time is also important to allow easy handling. An Ultraviolet-curing glue may also be suitable and will extend the working time for aligning spheres and cantilevers. The glue used here was a two-part solvent-free marine sealant (SP Systems Handipack). This had low viscosity, a working time of around 15-20 minutes, and a hardening time of 1-2 hours.

The amount and distribution of the glue can have a strong effect on the handling. Small, relatively thin and flat regions of glue may be better, since with higher, more rounded droplets, there may be more chance of the glue pulling in the cantilever due to surface tension. For low-viscous glue, thin spreading using a sharp tip can leave small trails or droplets of glue at the edge of the patch which will make it easier to pick up the correct amount of glue.

The cantilevers used here were OMCL (Olympus), with a spring constant of 0.06 N/m. Tipless cantilevers are useful for cell attachment or when very small spheres are used, but if the sphere is larger than the pyramid tip. With a tipped lever you will pick up more glue and so increase the risk of enveloping the bead. The Arrow TL-1 (NanoWorld) cantilevers are a good example of tipples cantilevers with a very low spring constant (0.03 N/m).

The spheres were a poly-disperse dry sample of silica spheres, with a diameter range between 3 and 10 microns. A plain glass slide was used as a substrate for the glue and spheres, and was cleaned with ethanol before use. Some spheres can be transferred dry onto the glass slide, or dispersed in ethanol. The glass slide will then usually have a mixture of clusters and individual spheres on the surface, from which individual spheres can be selected optically. A small amount of the glue was transferred to the glass slide directly after mixing, using a pipette tip. The glue was spread thinly close to the patch of spheres. Thin glue "trails" left small droplets that could easily be used. Larger patches of glue tended to flow onto the cantilever and result in too much glue being transferred. The NanoWizard® AFM and Life Science stage were mounted on a Zeiss Axiovert 200 inverted optical microscope.

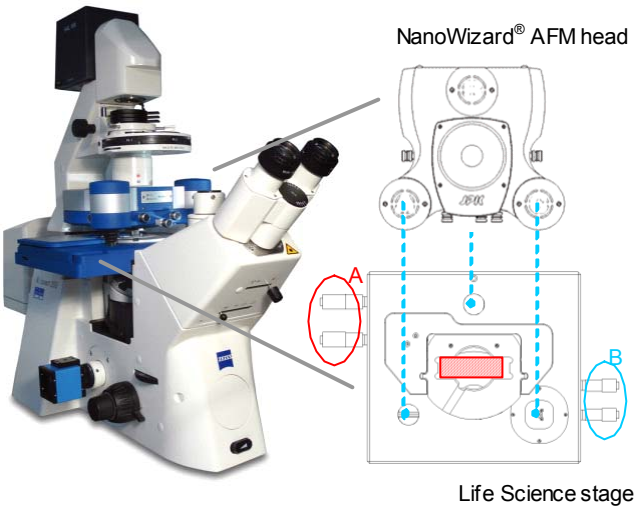


Fig 2. NanoWizard® Life Science setup. The stage and AFM head are sketched separately. The micro-positioners to move the sample (represented by a red glass slide) are ringed in red, and the ones for the AFM head (three blue dots for the AFM feet positions) are ringed in blue

Coarse positioning

The NanoWizard® Life Science stage and AFM head are shown mounted on the optical microscope and also in outline form in Figure 2. In this case, the glass slide sample is represented by the red rectangle and its position is controlled by the micromanipulators on the left hand side ringed in red and marked A. The three feet of the AFM head sit on the points marked by the three blue dots, and the AFM head (and therefore cantilever) position is controlled by the micromanipulators on the right hand side, ringed in blue and marked B.

The cantilever and AFM system is set up as normal, with the laser focused on the end of the cantilever, and the cantilever positioned within the field of view of the optical microscope. The positioning screws B can then be used for coarse positioning of the cantilever tip within the field of view, and the positioning screws A can be used to move the sample between the areas of glue or spheres.

Figure 3 shows optical images of the spheres (a) and cantilever (b) using a 40x lens. The green-blue colour is caused by the AFM laser safety filter. In b) the pyramidal tip shape can be seen at the very end of the long OMCL cantilever as a characteristic X-shape.

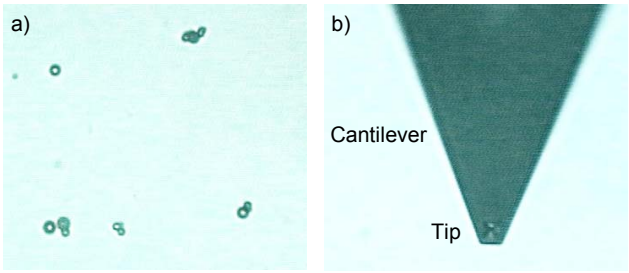


Fig. 3 Optical images of the silica spheres a) and cantilever tip b) with the 40x lens. The pyramidal tip shape can also be seen at the end of the cantilever in b).

The first stage of the process is to apply glue to the end of the cantilever tip. Use the software to land the cantilever onto an area of the glass slide without glue or spheres. When the cantilever has found the surface, clicking the retract button once moves the cantilever using the z-piezo only. If the sample was in the middle of the 15 micron piezo range, then the tip will move 7.5 microns from the glass. This is sufficient for small sample movements. Clicking the retract button again moves the cantilever with the stepper motors by the distance set in the motor panel, (e.g. 50-100 microns) to move the sample larger distances. It may be easiest to use the screws marked B in Figure 2 to move the AFM head small distances, within the field of view of the optical microscope. The cantilever should be positioned near the selected patch of glue.

Fine positioning – glue

The SPM software can be used in force spectroscopy mode to control the fine position of the cantilever using the x, y and z piezos. The cantilever can be moved over the surface by setting spectroscopy points in the Image Viewer window. New points can be set individually, or the mouse can be used to click and drag a selected point in the Image Viewer to move the cantilever freehand over the surface.

In force spectroscopy mode the cantilever does not need to start from the approached position. The cantilever can be retracted a safe distance, positioned over the glue or sphere and then the spectroscopy z-piezo movement used to "fish" for the surface, without needing to engage on it as for imaging. The cantilever position can be monitored using the CCD camera optical image, which is displayed within the SPM software. The z-stepper motors can be used to move the cantilever up and down if required during the alignment process.

a)

b)

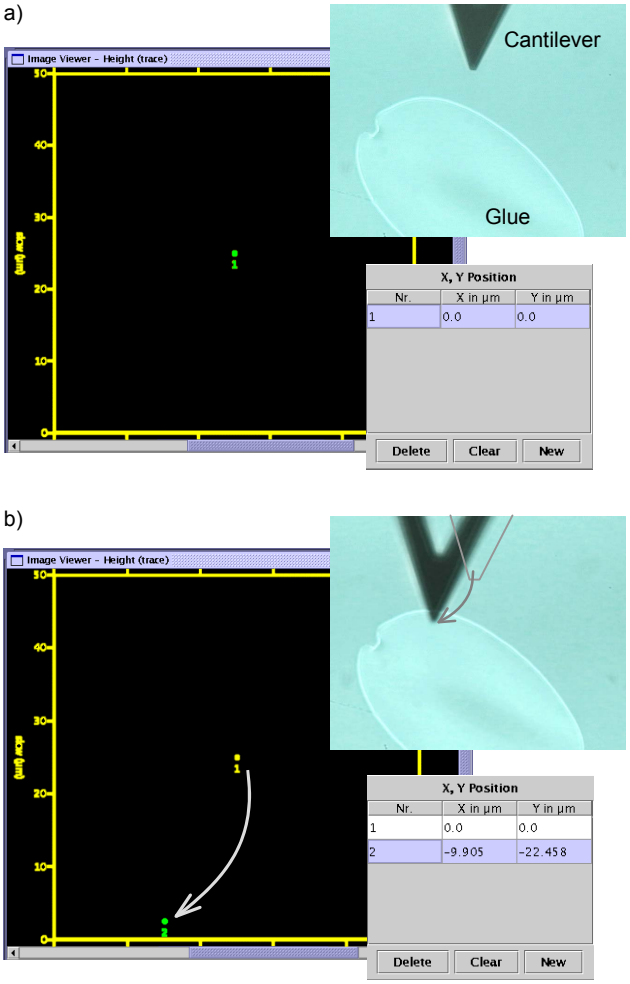


Fig. 4 Simultaneous images of the software and optical microscope view (20 x lenses). In a) the cantilever is at the centre of the scan area (position 1), which is to one side of the glue patch. In b) the cantilever has been moved using the software control, by setting a new spectroscopy point 2 over the glue patch.

Figure 4 shows screen clips from the AFM software window, along with the corresponding optical images (20x objective lenses). In the optical images, a small amount of glue at the edge of the glue patch can be seen, along with the cantilever. The software screen clips show the Image Viewer window, and the part of the spectroscopy control panel where the physical coordinates of the selected spectroscopy points are displayed.

In Figure 4 a), the cantilever is over the glass, at the centre of the scan area (0,0) marked Position 1 in the spectroscopy control panel, and displayed in green in the Image Viewer screenshot. In Figure 4 b), the cantilever has been moved over the edge of the glue patch by setting Position 2 in the Image Viewer window. Choosing a part of the glue where the edge is perpendicular to the cantilever direction, as shown here, can limit the contact between the tip and glue. The cantilever can be moved so the tip only overlaps the glue area by a few microns, as in Figure 4 b).

The cantilever moves real-time as the points are selected or moved in the software, and the position can be checked in the optical image. Once suitable points have been set, the cantilever tip can be moved back and forth between them by moving through the spectroscopy points list displayed in the control panel. Spectroscopy can be performed from a retracted position, and an initial approach is not required. This is particularly useful here, where the cantilever needs limited contact with the glue.

Relative force spectroscopy mode moves the cantilever towards the surface using the z-piezo until a particular setpoint deflection value is reached. This can be used to feel when the cantilever reaches the glue surface. The amount of force, the indentation depth, and an optional waiting time at the surface can all be set to optimise the transfer of glue to the tip region of the cantilever. It is recommended to set "Single scans" in the options menu, so that the movements are performed once only when *Run* is clicked, to give full control over the tip interaction.

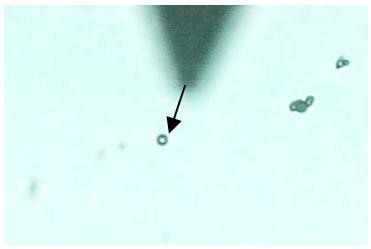
For soft cantilevers and low-viscosity glues, the tip may not come free at the end of the force spectroscopy movement. The stepper motors can be used to move the cantilever away from the glue, say by stepping up in 5 micron steps until the glue capillary between the tip and surface breaks. If too much glue flows onto the cantilever tip, some can be deposited back onto the glass by performing another spectroscopy curve over a bare glass region next to the glue patch. This can be repeated on clean areas until no droplet of glue is left on the glass.

Fine positioning – spheres

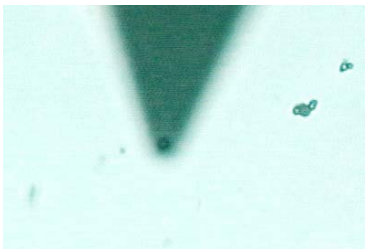
Once the tip is coated with glue, a sphere can be picked up with the cantilever tip. The tip should be retracted a safe

distance and then the sample positioning screws (marked A in Figure 1) can be used to move some spheres under the tip. It is best to find isolated spheres with as much space around them as possible, so that extra spheres are not picked up elsewhere along the cantilever arm.

a)



b)



c)

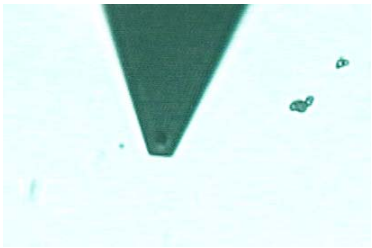


Fig. 5 Optical microscope images (40 x lenses) of the cantilever and a patch of spheres. In a), the cantilever is at the centre of the scan area, over the glass. In b) the cantilever has been moved over the chosen sphere using the software control. In c) the cantilever is in the approached position, touching the sphere.

Figure 5 shows the cantilever next to a suitable region of spheres, imaged using the 40x lens. The same positioning procedure can be performed as for the glue. The cantilever is held at the retracted position on the z-piezo and the spectroscopy mode position control is then used to place the cantilever tip over the chosen sphere. Force spectroscopy in relative mode is then used to pick up the sphere – again force and waiting time can be varied to optimise the attachment. The positioning is shown in Figure 5 for the cantilever over the glass in a) and over the chosen sphere in b). The spectroscopy mode can now be used to "fish" for the sphere. In the image in c), the cantilever is shown in the approached position, touching the sphere.

Application Examples

There are many applications for microspheres attached to cantilevers, and only a few examples are given here. Most applications are in force measurement, either indentation for elasticity or rheology measurements, or as adhesion to measure specific or nonspecific binding. The cantilever can be used to mechanically stimulate a particular response in a living cell.

Sphere-modified cantilevers can be used to measure the viscoelastic properties of whole cells and of particular parts of cells [1,2]. The known surface area and indentation shape can be used with the standard Hertz model to calculate a value for the Young's modulus of the cell. Microspheres can also be used with the AFM to study frequency-dependent rheological properties of the cell [1]. More complex mathematical models can also be developed to model the indentation of thin sections of the cell, such as the advancing lamellipodium. Here the AFM cantilever feels the substrate below the cell as a contribution to the apparent stiffness, and this can be used to also give a measure of whether the particular part of the cell is well-adhered to the surface [2].

Indentation of living mechanosensitive cells can stimulate specific responses. Osteoblasts are cells that form bone, and are part of the complex system that the body uses to adapt the skeleton to the demands on it. Increased strain on the skeleton generally results in the body increasing the bone mass, but there are many questions remaining as to how this is sensed or controlled on a cellular level. A known spherical tip shape allows the calculation of the actual strain exerted on the cell by a given indentation force [3]. Osteoblasts have been mechanically stimulated with microsphere AFM tips and the stimulation was monitored using fluorescence to visualise the increase in calcium ion concentration [4]. The calcium response could also be transmitted by the cells to their neighbours, who had not been directly stimulated. Microspheres have also been used to measure specific binding on osteoclast cell

surfaces, using the controlled surface contact area to improve the statistics for the binding events [5].

Conclusions

The NanoWizard® Life Science stage and AFM head can be used to easily align and manipulate the tip and sample to glue spheres to cantilever tips. This does not require any separate equipment or manipulators. The other advantages of working with this setup are the high-resolution optical microscope image, and the control and monitoring of the forces on the cantilever during the process. The sample and head manipulators give a working range of centimetres to quickly move between the glue and the spheres. The software interface makes precise and dynamic fine positioning of the cantilever straightforward using the piezo system. The use of spectroscopy mode from the retracted position allows a completely controlled approach of the cantilever to the glue or spheres.

Literature

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[5] Lehenkari P.P. and Horton M.A. "Single integrin molecule adhesion forces in intact cells measured by atomic force microscopy" Biochem. Biophys. Res. Comm. 259(3): 645-650 (1999).

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[7] J.W.G. Tyrell, P. Attard, “Atomic Force Microscope Images of Nanobubbles on a Hydrophobic Surface and Corresponding Force-Separation Data” Langmuir 18/1: 160-167 (2002).

[8] H.-J. Butt, B. Capella, M. Kappl “Force measurements with the atomic force microscope: technique, interpretation and applications” Surface Science Reports 59: 1-152 (2005).

List of tested glues

I. **SP Systems Handipack** (www.gurit.com), is an all-purpose two-part solvent-free marine sealant. This has low viscosity, a working time in thin film of around 15-60 minutes, and a hardening time of 6-8 hours. The setting time depends on the temperature. The bead-cantilever unit is ready to use after heating up to about 70-80°C for 1 hour. The fresh, non hardenend adhesive is for use in air only, and cannot harden in liquid.

UHU-Endfest 300 (www.uhu.de) is a 2-component adhesive on an epoxy resin base with a working time of up to 2h. Higher ambient temperatures will reduce the setting time as for SP Systems Handipack. The non-hardenend adhesive is for use in air only, and cannot harden in liquid. In liquid the glue drop forms a coarse-grained cluster, and the bead can not be attached. It is important to mix the hardener and resin very accurately in the correct proportions, otherwise the adhesive is not stable enough and in some cases the attached bead will be lost.
An alternatively is **UHU-Plus schnellfest**, though the working time is much reduced (to about 5 minutes). The UHU-Plus schnellfest is easier to mix homogeneously than Endfest 300, and therefore the final bonding strength is much higher.

II. **Araldite 2000 Plus** cited e.g. from M. Krieg [6]. (http://www.bm-chemie.de, Bodo Möller Chemie GmbH; Senefelderstraße 176-178; D-63069 Offenbach/Main). The bead can be attached in air and can then be stored in air for a week. According to M. Krieg (personal communication) it is also possible to use Araldite 2000 Plus in high humidity or indeed under water.

III. **Dymax OP** e.g. OP29 (www.dymax.com) can also be used in air. The adhesive is optically clear, and is characterized by low shrinking and an excellent solvent resistance. The adhesive cures upon exposure to moderate visible light. The process can be sped up by illuminating the cantilever with the fluorescence lamp of the microscope. If the cantilever

is removed from the chip holder before sufficient illumination then the bead may fall off.

IV. **Flex+bond** (Weicon GmbH, 48157 Münster, Postfach 8460, www.weicon.de) Flexbond is a 1-component glue, based on polyoxypropylene (POP), and resistant to temperature, UV and salt solution. Flexbond is recommended, if the functionalized bead has to be attached as well as stored in liquid. The paste-like composition of the adhesive minimises surface contamination of the bead.

V. **Some other adhesives**: Please note, that the list is not complete. Perhaps other adhesive products from other companies can also be used, e.g. UV-curable glue from **Norland** (http://www.norlandprod.com/). Tyrell and Attard [7] used a high melting point wax (**Shell Epikote 1004**) to attach a 15 µm silica sphere onto a cantilever. However, the adhesive has to be heated up to 100 °C to be liquid. Butt [8] reviewed different ways to attach a microsphere to a cantilever. To avoid any glue based contamination Butt [8] attached a silica bead to tipless cantilever by first dipping in a glycerol droplet and picked up the particle from the glass slide. The bead-cantilever unit was heated in an oven to the softening point of the glass bead of 700-800°C. All of glycerol evaporated without any residue.

Quick Checklist

1. Prepare a glass slide with glue and spheres

- Clean the slide with Ethanol and remove any dust by air flow
- **Sphere in liquid**: dilute the stock solution in ultrapure water and ultrasonicate for 5-10min. Put a droplet of the sphere solution on the glass slide and let the water evaporate
- **Dry spheres**: dip a clean pair of pointed tweezers or a micro pipette tip into a silica sample and tap it on the glass slide
- Dip a pipette tip into the glue and thinly spread close to the patch of spheres
- Mount the slide to the sample holder on the microscope

2. Put some glue on the cantilever

- Mount the cantilever and align the laser
- Orientate on the sample, find the spot with the spheres using the sample positioning screws (Fig.2).
- Approach to the slide on a clean region between spheres and glue
- Retract using the piezo only (7.5 microns clearance) (Optional – retract a further 5-10 microns using the stepper motor. In case of CellHesion module or CellHesion 200 the target height can be 10µm and the retract height about 100 µm.)
- Position the cantilever tip over the glue
- Select the “single scan” in the Options menu to dip the cantilever into the glue only once
- Run a force spectroscopy measurement (force mode) to dip the cantilever into the glue using a relatively low setpoint (0.5-1V) and a contact time = 0sec.
- If the cantilever does not break free of glue, use the stepper motors with 5 µm steps to move upwards.
- To remove any excessive glue, perform one or more additional force distance curves on a clean spot of the slide

3. Attach sphere to the cantilever

- Move the sample in order to position the cantilever over the spot with the spheres and decouple the sample.
- Fine positioning of the cantilever directly over the sphere can be done by moving the head (Figure 2, micro positioners B) or with the NanoWizard option: Click and drag with the mouse on the Image Viewer area to move the tip freehand. The selected point can be repositioned by clicking and moving after it has been set.
- Perform a force distance curve using a bit higher setpoint (1-1.5V), pulling length 5µm and a contact time of about 3-5sec.
- For a better optical control of the position of the cantilever above the spheres it can be useful to activate the “Cell capture” mode (where available) and move the cantilever to the sample.

4. Demounting the cantilever and storage

- If low viscous optical glue was used, proceed with light induced curing before removing the cantilever from the system. All other adhesives are viscous enough to allow a carefully removal of the cantilever immediately after the bead attachment.
- Let the glue harden according to the instructions.
- For most of the adhesives the setting time can be reduced by applying heat.
- The bead-attached cantilevers should be stored inside a clean incubator or box.