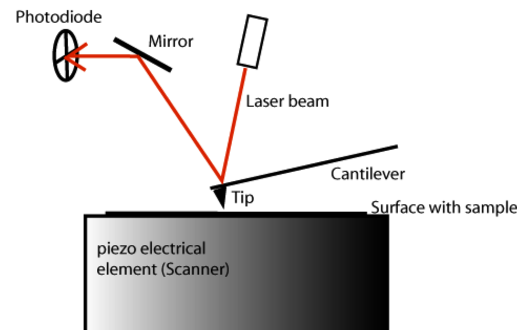
1. Scanning force microscopy (SFM)

Background

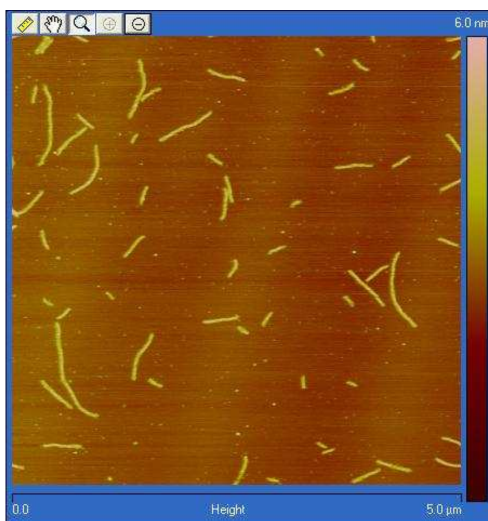
SFM technology's ancestor, STM (Scanning Tunneling Microscopy), was invented in 1981 by Gerd Binnig and Heinrich Rohrer at IBM in Zurich, Switzerland. They went on to win the Nobel Prize for physics with this discovery. Their work has formed the basis for all serious scanning probe microscopy research worldwide ever since.



SFM works like an old fashioned record player.

A scanning force microscope (SFM) basically works like an old fashioned record player, where the movement of the needle sends the recorded impulse through the amplifier and on to the speakers to produce music.

Unlike to the record player a SFM detects the movement of the needle through a laser beam, which is reflected from the top of a cantilever to a photodiode. The bending of the cantilever can be measured by using a four-quadrant photodiode. The strength of the cantilever deflection is allocated/collated to a color scale and the data is used to generate a map of the surface topography.



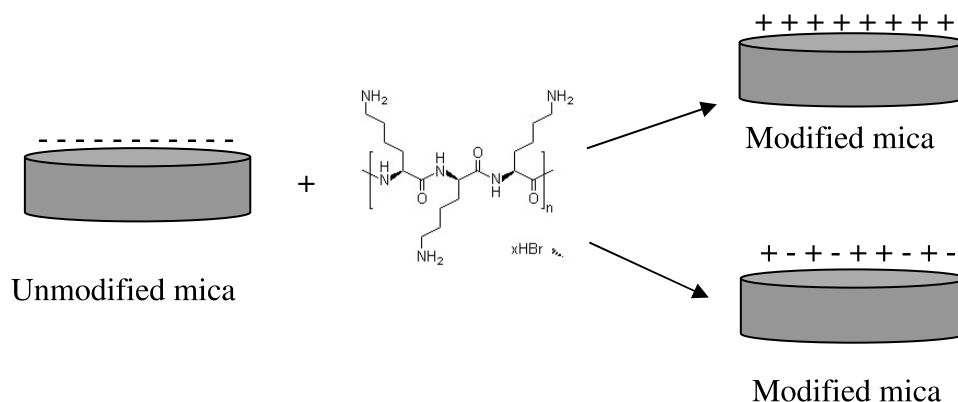
Topographic image of the *in vitro* assembled intermediate filament vimentin.

The surface

Samples that are to be measured must be deposited onto a surface, which is flat on atomic scale. Materials that are used are e.g. glass, gold or mica. We will use mica, which belongs to the group of sheetsilicates (phyllosilicate). These minerals have the property of a highly perfect basal cleavage which is explained by the hexagonal sheet like arrangements of its atoms. Sheetsilicates like mica provide a negatively charged surface.

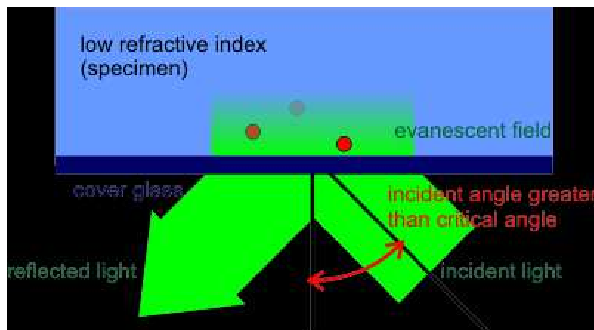
Imaging of macromolecules on a mica surface

The intermediate filament (IF) proteins vimentin and desmin are partial positively charged and can therefore be attached on negatively charged mica. Other molecules like DNA are negatively charged, so a mediator is needed to facilitate the binding of DNA to the mica surface. As mediators metal ions can be used as well as modifications of the surface properties by coating the mica surface with positively charged substances like polylysine. Applying different polylysine concentrations the net-charge on the mica can be modified and thereby the interaction strength between molecule and surface can be altered.



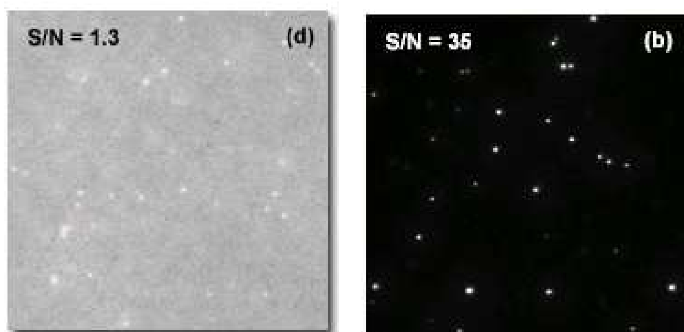
2. Total internal reflection fluorescence microscopy (TIRFM)

Total internal reflection fluorescence microscopy (TIRFM) is a method of imaging molecules at or near the surface of a glass slide. TIRFM functions by introducing laser light at a high angle to the glass surface. Under these conditions the light is totally internally reflected at the glass water interface due to the difference in the refractive index. When light is totally reflected an evanescent wave front occurs which penetrates the medium about 50 to 150 nm with an exponential decay. Therefore only molecules that are close to the surface are exited by the incoming laser light.



The benefit of using TIRF over normal widefield illumination is the enhanced signal-to-noise ratio. This is because with widefield illumination a large region of sample buffer is illuminated leading to a high background signal, whereas with TIRFM only a thin layer is illuminated which reduces the background. This is important for single-molecule detection where the fluorescence of each single molecule should not be overwhelmed by the background signal.

As an example, below two images are shown, the first from normal epifluorescence and the second from TIRFM.

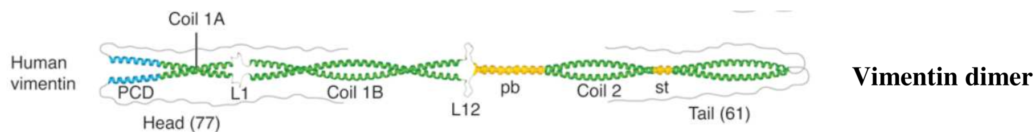


Source:
http://www.microscopyu.com

For further information, both Nikon and Olympus have very informative tutorials online:
http://www.olympusmicro.com/primer/techniques/fluorescence/tirf/tirfintro.html
http://www.microscopyu.com/articles/fluorescence/tirf/tirfintro.html

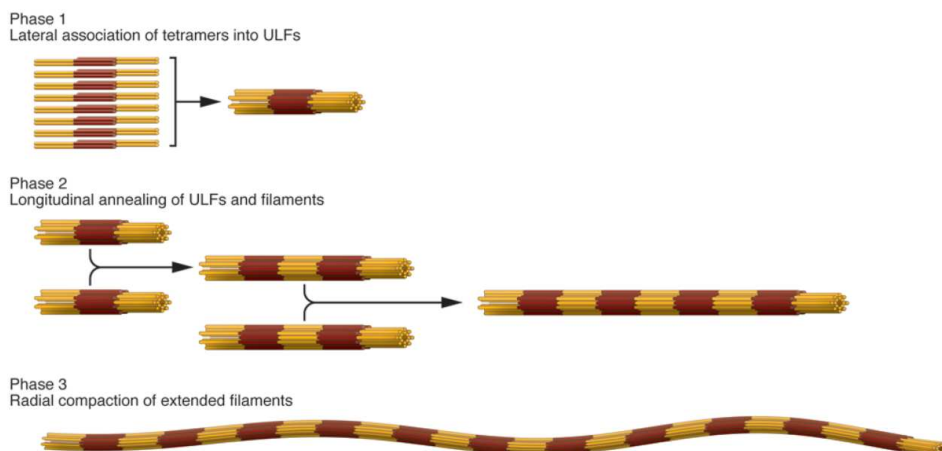
3. Intermediate Filaments

Intermediate filament (IF) proteins form together with microtubules and actin filaments the cytoskeleton of metazoan cells. IFs are known as cellular stress absorbers and are involved in the cell's plasticity. In contrast to microtubules and actin filaments, which consist of globular subunits, the building blocks of IFs are fibrous proteins. All IF proteins exhibit a characteristic tripartite structure which includes an α-helical "rod" domain flanked by non-α-helical "head" and "tail" domains. The elementary building block of all IFs is a dimer.



Vimentin dimer

In preparation for assembly, IF proteins reconstituted from 8 M urea into low-salt buffer organize into relatively uniform tetrameric complexes of approximately half-staggered, antiparallel coiled-coil dimers. IF assembly can be initiated by salt addition or a pH shift and follows three distinct steps: 1. Tetrameric subunits associate laterally and form "unit-length filaments" (ULFs); 2. ULFs and existing filaments assemble longitudinally; 3. Filaments compact radially to a uniform diameter of ~ 11 nm.



The IF vimentin is primary found in mesenchymal, endothelial and hematopoietic cells. Desmin is a major constituent of the extra-sarcomeric cytoskeleton in myoblasts. During myogenesis vimentin is downregulated and replaced by desmin. Due to their high sequence homology both proteins belong to the IF sequence homology class 3.

In the course of this practical we will perform co-assembly studies with vimentin and desmin.

Read more: Herrmann H, Strelkov SV, Burkhard P, Aebi U. Intermediate filaments: primary determinants of cell architecture and plasticity. *J Clin Invest*. 2009 Jul;119(7):1772-83

4. Tasks during the lab course:

In this part of the practical we will co-assemble the intermediate filaments vimentin and desmin and check if they form co-polymers. For this purpose we will apply SFM and TIRF microscopy. The assembly will be initiated by adding a salt containing sodium phosphate buffer. In order to distinguish both filament species with the TIRFM both the vimentin and the desmin filaments will be assembled in the presence of either Alexa 488 or 647 labeled subunits.

1. To check whether the fluorescent IFs form smooth elongated filaments that are comparable with unlabeled ones we will first visualize labeled and unlabeled species separately by SFM.

In order to measure if vimentin and desmin form co-polymers we will mix both species at different assembly states. First we will mix vimentin and desmin at the tetrameric state, start the assembly and observe the elongated filaments. Secondly, we will mix pre-elongated filaments of both species and let them further assemble. Due to the fact that desmin has a bigger diameter than vimentin both filament species should be distinguishable by the topographic SFM image. For the evaluation we will measure the full width at half maximum (FWHM) of some selected filaments.

2. In the first part of the practical we have solved the co-polymerisation question by topographic SFM images. In this part of the practical we will visualize the co-assembled filaments with TIRFM. For this purpose we will use the fluorescently labeled IFs.