Tuning Fork Tunes
exploring new scanning probe techniques

Wouter H.J. Rensen
TUNING FORK TUNES
exploring new scanning probe applications
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TUNING FORK TUNES
EXPLORING NEW SCANNING PROBE TECHNIQUES

PROEFSCHRIFT

ter verkrijging van
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door

Wouter Harry Jacinth Rensen
geboren op 15 mei 1973
te Zevenaar
Dit proefschrift is goedgekeurd door de promotor:

prof.dr. Niek F. van Hulst
Set up your microscope and tell me what you see

Sting
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This thesis presents the opportunities, limits and new applications of tuning forks as force sensors in Scanning Probe Microscopy (SPM). This Chapter gives an introduction to SPM and quartz tuning forks as force sensors in SPM. Two schemes of operation can be distinguished, one where forces normal to the sample are detected and one where shear forces parallel to the sample are detected. An overview will be given of the various models presented in literature that describe the involved shear forces. Finally, a short overview of the experimental setups used in this thesis is given.
1 Introduction

1.1 Aim of this thesis

The advent of Scanning Tunneling Microscopy (STM) by Binnig and Rohrer\(^1\) in 1982, awarded with the Nobel prize in 1986, preluded a wide range of types of Scanning Probe Microscopy (SPM). A small probe is used to measure local properties of a sample. Scanning the probe with respect to the sample, or vice versa, results in a property map of the sample surface. Probes have been developed to detect thermal, electrical, magnetic, mechanical and optical properties on a nanometer scale.\(^2,3\) A requirement for all types of SPM is that the probe is in close proximity to the sample. The need to control the probe-to-sample distance implies the need for a sensor that is sensitive for this distance.

Two types of SPM play an important role in this thesis, atomic force microscopy and near-field scanning optical microscopy. In Atomic Force Microscopy (AFM), introduced by Binnig et al.,\(^4\) the distance between a sharp probe and the sample is controlled by detecting interaction forces between probe and sample, such as Van der Waals forces, when the probe approaches the sample.\(^2,5,6\) A feedback loop adjusts the distance between probe and sample in such a way that the probe-to-sample interaction force is constant. Assuming that the probe-to-sample distance is constant for a constant interaction force, the adjustments in sample position with respect to the probe can be used to construct a topographic map of the sample surface. Additionally, mechanical properties like compliance, friction and adhesion can be measured with this type of microscopy.\(^7-9\)

In AFM, micromachined cantilevers are used as sensor.\(^10\) Interaction forces between the probe, that is mounted at the end of the cantilever and the sample bend the cantilever and a laser beam reflecting of the back of the cantilever is deflected. The reflected laser beam is directed onto a position sensitive photodetector, the position of the beam on the detector is a measure for the interaction force.\(^11\) Next to this contact mode of operation, is the noncontact mode,\(^12,13\) where the cantilever is mechanically oscillated at its resonance frequency. Force interaction now causes an amplitude damping of the cantilever or a phase shift of the oscillation of the cantilever with respect to the driving oscillation.

The optical force detection in AFM suffers from a number of disadvantages. Alignment of the laser beam onto the cantilever and position sensitive detector is elaborate. The laser diode is sensitive to mode hopping and interference with reflected light resulting in artifacts in the measurements.\(^14\) The presence of a laser diode, lens and detector prevent the design of a cheap and compact microscope head. Finally, the oscillation amplitude of cantilevers used for noncontact operation is large, in the order of tens to hundreds of nm.
In Near-field Scanning Optical Microscopy (NSOM), introduced by Pohl et al.,\textsuperscript{15} a sub-wavelength sized aperture is scanned over the sample at a distance small compared to the aperture size. Through this aperture, usually at the apex of an aluminum coated, tapered optical fiber,\textsuperscript{16} light can be emitted to or collected from the sample surface under the aperture. As a result, a micrograph of optical properties, like transmission or fluorescence\textsuperscript{17} of the sample is produced with a resolution only limited by the size of the aperture.

The distance between probe and sample is controlled by laterally oscillating the fiber probe at its resonance frequency. When the probe approaches the sample, shear forces interact between probe and sample, damping the oscillation amplitude of the fiber.\textsuperscript{18,19} At the introduction of shear force detection for NSOM, the oscillation of the fiber was detected using optical methods, either by reflecting an external optical beam off the metal coated fiber,\textsuperscript{18} or by detecting the position of the aperture by focusing the light emitted from the aperture on a position sensitive detection system.\textsuperscript{19} To reduce the risk that optical shear force detection disturbs the near-field optical experiment, for example by causing a background, nonoptical shear force detection is desirable.

Karrai et al. introduced tuning forks as shear force detectors.\textsuperscript{20} A tuning fork with a tapered optical fiber probe mounted to one prong, is oscillated at resonance. Upon approach to the sample, shear force interaction between probe and sample reduces the oscillation amplitude of the tuning fork. The lack of optics, small oscillation amplitudes and a high sensitivity have made tuning forks ideal force detectors in NSOM under ambient conditions. The large damping of tuning forks upon immersion in water has prevented the use of tuning-fork-based shear force detection for biological NSOM applications, where the sample needs to be in a liquid environment.

After the first implementation for NSOM applications, tuning forks were introduced to other types of SPM, including Atomic Force Microscopy and Magnetic Force Microscopy,\textsuperscript{21} demonstrating the potential of tuning forks as force sensors.

This thesis explores both new applications and limits of tuning forks as force sensors in SPM. The performance of tuning-fork-based AFM is compared to cantilever-based AFM in terms of sensitivity and speed. Additionally, tuning forks are applied for the first time for shear force detection in a liquid environment to enable biological applications of NSOM. An example of such an application is the localization of single fluorescent molecules attached to DNA.
1.2 Tuning forks

Originally developed for frequency control, the largest application of quartz tuning forks is time control in clocks and watches. Thanks to mass production, they are widely available and cheap. Tuning forks are available in a frequency range from 10 kHz to 600 kHz. For the work described in this thesis, 100 kHz tuning forks are used.

Figure 1-1. Quartz tuning fork with a resonance frequency of 100 kHz. At resonance, the two prongs to the left move in opposite directions, like scissors and induce a piezoelectric current, that is picked up by the gold electrodes on the tuning fork.

Figure 1-1 shows a 100 kHz quartz tuning fork. Quartz is a piezoelectric material: stress in the material results in an electric charge displacement. The charge displacement is picked up by the electrodes on the tuning fork. The layout of the electrodes is such that at resonance, when the prongs move in opposite directions, the charge pick-up is optimized.

Figure 1-2. Cross section of tuning fork prongs, while the prongs move away from each other. The inner sides of the prongs are stretched and the outer sides are compressed. The dashed line indicates zero stress. The electrodes A and B are designed such that the piezo-electric charge displacement, indicated by the arrows inside the prongs, is always directed from one electrode to the other.

Figure 1-2 shows a cross section of the prongs of a tuning fork depicted in Figure 1-1. The direction of the charge displacement is indicated. The layout of the electrodes is designed to maximize the pick-up of charge displacement when the prongs move in opposite directions. If the prongs would move in the same direction, the electrode layout would cause the charge displacement picked up in one prong to be compensated by the charge pick-up in the other prong. At resonance, the charge displacement oscillates with the prongs, resulting in an alternating current.
The current picked up by the electrodes when the tuning fork is oscillating at resonance, is a measure for the deflection of the tuning fork prongs. When a probe mounted on one prong has a force interaction with a sample, the tuning fork is damped and the resonance frequency of the tuning fork shifts. This can directly be observed by changes in the piezoelectric current from the tuning fork.

Tuning forks can be driven using two methods. First, electrical excitation, the method that is normally used when tuning forks are used for frequency control and second, the method more common in SPM, where tuning forks are mechanically driven by an external dither piezo.

1.2.1 Electric drive for tuning forks

The inverse piezoelectric effect can be used to drive a tuning fork. A voltage applied across the tuning fork electrodes will induce a deformation of the tuning fork. If the applied voltage is an AC voltage with a frequency equal to the resonance frequency of the tuning fork, the deformation of the tuning fork has a maximum amplitude. The current through the tuning fork, or the admittance can be used to monitor the tuning fork motion.

Figure 1-3 shows a scheme to drive tuning forks electrically. The tuning fork is driven by an AC voltage. The current through the tuning fork is amplified by a transimpedance amplifier. The amplitude of the resulting tuning fork signal, or the phase with respect to the driving oscillation can be used as a measure for the force experienced by the tuning fork.

The bottom right corner of Figure 1-3 shows an equivalent circuit for the tuning fork: A LCR oscillator with parallel parasitic capacitance. Typical values for the equivalent circuit, representing a 100 kHz tuning fork are: motional resistance $R = 45 \, k\Omega$, motional inductance $L = 2.1 \, kH$, motional capacitance $C = 1.2 \, fF$ and parasitic capacitance $C_{par} = 1.4 \, pF$. Due to the parasitic capacitance, a current runs through the tuning fork at all frequencies and the current caused by the LCR oscillator is superposed on that current. The parasitic capacitance introduces an electronic background signal and reduces the available dynamic signal range.
1.2.2 Mechanical drive for tuning forks

The second method to drive tuning forks, is the use of an external dither piezo as shown in Figure 1-4. The dither piezo excites the tuning fork mechanically at resonance.

The stress induced by the motion of the tuning fork prongs, generates a current thanks to the piezoelectric effect of quartz. The tuning fork current is amplified by a transimpedance amplifier. The amplitude of the resulting tuning fork signal, or the phase with respect to the driving oscillation can be used as a measure for the force acting on the tuning fork.

Because the drive is now mechanical, there is virtually no current generated when the tuning fork is off resonance. Therefore, only current is lost in the parasitic capacitance of the tuning fork, when it is driven at resonance. Although the parasitic capacitance reduces the current available for detection, the dynamic signal range will hardly be affected, as there is no electronic background. Because of the decoupling of the drive and the detection, the mechanical drive is the most used drive method for tuning forks in Scanning Probe Microscopy.

1.3 Tuning-fork-based force detection

Two basic modes of operation can be discriminated for tuning forks as force sensors in Scanning Probe Microscopy. The first method is similar to the method used in Atomic Force Microscopy, where the cantilever oscillates perpendicular to the sample. The second method finds its application in Near-field Scanning Optical Microscopy. The fiber based probes are too long to be oscillated in their length direction that is perpendicular to the sample surface. Instead, the probe is oscillated parallel to the sample surface and shear forces between probe and sample are detected.
1.3.1 Normal force detection

When tuning forks are used for normal force detection, the prongs of the tuning fork are parallel to the sample surface. The tuning fork can be tilted a few degrees, so that the mounted probe can access the sample, without the tuning fork touching the sample. If the probe is protruding a short distance from the tuning fork, the tuning fork can be tilted a bit. Potential probes are AFM cantilever probes or STM probes. Figure 1-5 shows a tuning fork with an AFM cantilever probe mounted for normal force detection.

Normal force detection resembles regular noncontact AFM. A considerable amount of work has been performed to understand probe-to-sample interaction for this type of AFM and therefore, it is well established. Dominating forces in AFM are attractive Van der Waals forces and repulsive interatomic forces.

Normal force detection is performed using a commercial AFM, where tuning forks can replace the regular AFM cantilever. In this setup, the sample is scanned, having the advantage of a simple head, where the AFM cantilever can easily be replaced by a tuning fork probe. A disadvantage is that the sample must have a limited size to fit between scanner and the head holding the probe.

The setup shown in Figure 1-6, uses an electrically driven tuning fork as described in § 1.2.1. However, also a mechanical drive is possible, where the probe is driven by an external piezo. In both cases, the amplitude of the piezoelectric tuning fork signal, or the phase lag between the tuning fork signal and the driving function can be used as a measure for the probe-to-sample interaction force. A feedback loop adjusts the height position of the scanner in such a way that a preset constant amplitude damping or phase shift is maintained. The electronics do not only contain a feedback loop to maintain the probe in close proximity to the sample, the electronics also control the scanning in lateral directions and to collect the desired data. The setup is suitable for comparing conventional AFM and tuning-fork-based AFM, as both types AFM can be performed on the same instrument.
1.3.2 Shear force detection

Shear force detection has been developed mainly for Near-field Scanning Optical Microscopy, where a fiber-based probe is dithered parallel to the sample surface.\textsuperscript{18,19} The first scheme was based on optical detection, where a laser beam is directed onto the oscillating fiber and the shadow of the fiber is projected on a position sensitive photodetector to detect the oscillation of the fiber. When the fiber probe approaches the sample, the oscillation amplitude of the fiber dampens. The damping can be used as a measure for probe-to-sample interaction force.

Regulating the distance between probe and sample, to keep the amplitude damping and the interaction force constant results in a constant distance between probe and sample. The disadvantage of this optical shear force detection is the presence of a laser beam to detect the motion of the fiber. This light could interfere with the light emitted from the aperture of the fiber. Furthermore, elaborate alignment of the optical system is necessary and the free oscillating end of the fiber has to be long to allow the optical beam to cross.

![Figure 1-7. Tuning fork with a fiber probe mounted for shear force detection.](image)

Tuning forks were introduced as non-optical, piezoelectric means for detecting shear force interaction between probe and sample. Although normal force approaches have been reported,\textsuperscript{30} shear force detection is the best suited method for probe-to-sample distance control when long flexible fibers are used as probe, because the motion of the fiber probe is controlled along the tuning fork, where the prongs are oscillating and where the base is stable.

![Figure 1-8. Probe scanning shear force microscope.](image)

A commercial AFM\textsuperscript{12} is modified by removing the laser diode and position sensitive detector. Instead, a holder for tuning forks in shear force configuration is mounted. In the resulting shear force microscope, the probe is scanned over the sample surface. Scanning the probe has two advantages:
• The scanner head contains the complete microscope and can be placed on top of virtually any sample.
• Because only the tuning fork and probe are scanned, the scanned mass is low, enabling high scan speed.

The setup as shown in Figure 1-8, uses a mechanically driven probe as described in § 1.2.2. However, also an electrical drive as described in § 1.2.1 is possible with this setup. As with the normal force configuration, the amplitude of the piezoelectric tuning fork signal, or the phase lag between the tuning fork signal and the driving function can be used to control the probe-to-sample distance. The electronics to control the instrument and to collect the data, is the same as for the normal force configuration.

1.4 Origin of shear force

The physical origin of shear force is still under debate. At the introduction of shear force detection, Van der Waals and capillary forces were suggested as possible interaction mechanisms. Several models have been reported since, based on different types of experiments under different circumstances. The different, sometimes conflicting models lead to the conclusion that there is more than one regime of shear force interaction between probe and sample.

The model that can explain most features observed for shear force microscopy under ambient conditions, assumes a liquid contamination layer that is always present on the sample. Figure 1-9 shows a schematic overview of an air-sample interface under ambient conditions. The molecules on a solid sample surface are depicted by solid black spheres. The sample surface is hydrophilic enough to attract water from the air. Close to the sample surface, the water molecules are arranged in quasi-solid layers of water. Further away from the sample, the water molecules are disordered and behave like a fluid.

In shear force approach experiments, a probe is oscillated parallel to the sample, while being moved towards the sample. When the probe is in air, the air will cause a viscous
damping of the oscillated probe. Long range electrostatic interaction between probe and sample may occur when the probe is within a few hundred nanometer from the sample.\textsuperscript{40-42} When the probe touches the water layer, a meniscus builds up, damping the oscillation of the probe. In the liquid water layer, the probe experiences an increased viscous damping. Closer to the sample, where the water molecules are ordered in layers forming a quasi-solid, viscoelastic force interaction occurs between probe and sample. Finally, the probe is in (intermittent) contact with the sample and short-range repulsive interaction forces damp the motion of the fiber.

This model can explain the following features observed in approach experiments\textsuperscript{33-46} and combined shear force and STM experiments:\textsuperscript{42,46}

- When the probe approaches the sample, the amplitude damps and in the approach curve, a number of damping regimes can be discriminated, such as the build up of a meniscus, the interaction with the ordered water layers close to the sample surface and an intermittent contact with the sample surface.\textsuperscript{33-39}
- The interaction range is larger than the electron tunneling distance.\textsuperscript{42,46}
- The interaction range is usually larger for higher humidity\textsuperscript{15,36,40} and for hydrophilic samples.\textsuperscript{34,37,39,45}

This model can be extended to conditions where the sample is in vacuum or immersed in liquid. In vacuum, the water layer is not present. The interaction is dominated by Van der Waals forces, electrostatic forces and short range repulsive forces. When the sample is immersed in liquid,\textsuperscript{35-37,41} there is no air-liquid interface and no meniscus will build up when the probe approaches the sample. Close to the sample surface, the liquid still forms quasi-solid layers resulting in viscoelastic interaction between probe and sample.

An alternative model for shear force interaction is a so-called ‘knocking’ model.\textsuperscript{41,44} In this model, the probe is in intermittent adiabatic short-range contact with the sample, resulting in a nonlinear bending of the fiber probe. The most important feature of this model is that the distance over which shear force interaction between probe and sample acts, depends on the amplitude of oscillation of the fiber probe and the angle between probe and sample. Other features supporting this ‘knocking’ model are an asymmetric resonance curve when the probe is interacting with the sample and a linear drop of oscillation amplitude upon sample approach.

The features supporting a ‘knocking’ model are only observed for large probe oscillation amplitudes. With the model discussed before, the interaction range is expected to depend on the probe oscillation amplitude only for large amplitudes. For large amplitudes, the probe oscillation is large compared to the water layer of a few nm, and the influence of the water layer diminishes, because the probe will now move
from air through the relative thin water layer and periodically hit the sample. In vacuum, at large oscillation amplitudes, the probe oscillation is large compared to the range of Van der Waals forces and the probe moves through the Van der Waals interaction range and indeed ‘knocks’ the sample.

For low interaction forces it is important to use small probe oscillation amplitudes. Tuning forks allow probe amplitudes of less than 1 nm, where the probe amplitude with optical shear force detection is usually more than an order of magnitude larger.

1.5 Near-field scanning optical microscopy

A new near-field optical microscope is designed, dedicated to biological applications. For this purpose, the near-field optical microscope, based on a conventional inverted microscope (Axiovert 135 TV, Zeiss) is combined with a confocal microscope for fluorescence detection. Special features of this new microscope are the possibilities to change objectives, to use phase contrast and to translate the sample. Figure 1-10 shows a schematic overview of the new instrument.

When the instrument is used as a near-field optical microscope, light from a Ar+-Kr+-laser (Spectra-Physics) is coupled into the fiber probe that will guide the light to the aperture. The sample just under the aperture is then illuminated.

Figure 1-10. Combined confocal and near-field scanning optical microscope, with two channel fluorescence detection.
The microscope objective (Olympus 64x, 1.4 NA) collects the fluorescence light emitted from the illuminated spot. A lens is used to focus the fluorescent signal on the photon counting detectors (APD, EG&G, Perkin-Elmer). A longpass filter blocks residual excitation light and the fluorescence light can be split in two components, for example in two perpendicular polarization components by a polarizing beamsplitter cube, or in two spectral components by a dichroic mirror.

A fluorescent image of the sample can be reconstructed by scanning the sample, while collecting data at every point. To maintain optical alignment of the aperture of the fiber probe and the detector in NSOM, the sample needs to be scanned instead of the probe. Tuning-fork-based shear force detection is used to maintain the aperture of the fiber probe in close proximity to the sample. In this setup, tuning forks can be driven both electrically and mechanically as described in § 1.2.

In the confocal mode, the laser light is reflected by a dichroic mirror into the back of the microscope objective. This excitation light is focused on the sample. The fluorescence is collected by the same objective and now transmitted through the dichroic mirror. The detection path is the same as for the near-field optical microscope.

The scan range of this microscope is 32 μm×32 μm×26 μm in lateral directions (X,Y) and vertical direction (Z) respectively. The exceptionally large scan range in the vertical direction, is useful for samples that show large height variations.

### 1.6 Outline

This thesis explores the limits and new applications of tuning forks. In Chapter 2 a model for the sensitivity and operation of tuning forks is developed and a comparison is made between conventional Atomic Force Microscopy (AFM) and tuning-fork-based AFM.

Chapter 3 describes the application of tuning fork as shear force sensors in liquid environments. High resolution results and images on soft biological samples are presented.

In Atomic Force Microscopy (AFM), recently a circuit was introduced to enhance the sensitivity of AFM cantilevers. Chapter 4 examines the potential application of a similar circuit for tuning-fork-based shear force detection, to enhance the sensitivity in liquid. The fundamental limits of sensitivity enhancement are investigated.

The localization of single fluorescent molecules is an important application in biology. Chapter 5 shows the advantages of Near-field Scanning Optical Microscopy (NSOM) for localization of single fluorescent molecules.
1.7 References


1 Introduction

31. Discoverer, TM-Microscopes, 1171 Borregas Ave. Sunnyvale, CA 94089, USA. Scan range of the scanner is 6 μm × 6 μm × 2 μm in lateral directions (X,Y) and vertical direction (Z) respectively.
32. Explorer, TM-Microscopes, 1171 Borregas Ave. Sunnyvale, CA 94089, USA. Scan range of the scanner is 100 μm × 100 μm × 10 μm in lateral directions (X,Y) and vertical direction (Z) respectively.
1 Introduction
Tuning forks as force sensors

This chapter explores the operation of tuning forks as force sensors, the way they move and their sensitivity. A finite element model is developed to simulate tuning fork motion. With the finite element model, it becomes clear that the tuning fork prongs are coupled and that a harmonic oscillator model of only one prong fails to explain the influence of the fiber on the tuning fork motion. Taking this limitation into account, the harmonic oscillator model can still be used to estimate interaction forces between probe and sample, based on changes in the amplitude of the piezoelectric tuning fork signal or the phase shift between the tuning fork signal and the drive function. The performance of tuning forks in tuning-fork-based Atomic Force Microscopy (AFM) is compared to the performance cantilever-based sensors in conventional AFM.

2.1 Introduction

Near-field Scanning Optical Microscopy (NSOM) can only work if the probe-to-sample distance is small compared to the aperture of the probe. The resolution is then equal to the aperture size. For a ~70 nm aperture, the maximum distance needs to be in the order of a few nanometers. In addition, the probe, a pulled glass fiber coated with an aluminum layer of 100-150 nm and with an aperture of ~70 nm is extremely fragile. A single uncontrolled contact with the sample renders the probe useless. The most common technique to control the probe-to-sample distance is to measure interaction forces between probe and sample. For this purpose, the probe is dithered lateral to the sample. At close proximity to the sample, shear forces act between probe and sample and the oscillation is damped. Several techniques can be used to detect the damping of the fiber. Tuning-fork-based shear force detection, as explained in § 1.3.2 has become the most widely used method within a few years from the introduction by Karraï et al. in 1995.

Within a few years after introduction for NSOM, tuning forks were introduced as an alternative for cantilever-based force sensors in Atomic Force Microscopy (AFM). Despite different mechanical properties, like stiffness and mass of the sensors, tuning forks as force sensors can be compared to cantilevers in AFM. The fact that tuning forks are cheap, sensitive, easy to use and reliable explains their popularity.

2.2 Finite element tuning fork model

![Figure 2-1. Finite element mesh with nodes having mass \( m_n \) and elements consisting out of springs \( k_{nn} \) and dampers \( \gamma_{nn} \).](image)

Generally, tuning forks in scanning probe applications are modelled as harmonic oscillators just as cantilevers in atomic force microscopy. One tuning fork prong is considered as a simple beam with an effective mass and spring constant that can be calculated from the material properties of quartz. The damping is a fitting parameter for the Q-factor of the tuning fork, which is defined as the full width of the resonance peak at half its maximum, when the amplitude versus frequency is plotted. This model is sufficient to explain numerous aspects of tuning fork behavior, but fails to recognize some important features, like the interaction between the two prongs and prong deflection versus drive amplitude. For a correct estimation of the interaction forces, knowing the probe amplitude is essential.
2.2.1 Finite element method

For a more accurate study of tuning forks, both prongs should be taken into account. Analytical solution of the motion of a 3D oscillator with two prongs is complicated. A practical alternative is finite element analysis (FEA), a suitable method to analyze mechanical properties of complex structures like tuning forks.

For finite element analysis, a complex structure is converted into a mesh as shown in Figure 2-1, where the nodes have a mass, and every element between two nodes is a combination of a spring and a damper. The spring and damping constants of the connecting elements are chosen such that together they exhibit the same behavior as the whole structure. Figure 2-2 shows a 100 kHz tuning fork to the left. The finite element mesh used to simulate the properties of the tuning fork is shown to the right. In the center, the mesh is superimposed on the tuning fork.

The spring constants of the elements and the mass of the nodes are deducted from the dimensions and material properties of 100 kHz quartz tuning forks as listed in Table 2-1. The damping of a tuning fork in ambient conditions, is dominated by viscous damping caused by the environment of the tuning fork. The damping constants in the model are chosen to match the observed damping of the tuning fork.

Table 2-1. Dimensions and material properties of 100 kHz quartz tuning forks

<table>
<thead>
<tr>
<th>Dimension</th>
<th>Value</th>
<th>Dimension</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length B</td>
<td>4.42 mm</td>
<td>Prong width T</td>
<td>0.406mm</td>
</tr>
<tr>
<td>Width H</td>
<td>0.889 mm</td>
<td>Density ρ</td>
<td>2.65·10^3 kgm^{-3}</td>
</tr>
<tr>
<td>Thickness W</td>
<td>0.127 mm</td>
<td>Young's modulus E</td>
<td>7.87·10^{10} Nm^{-2}</td>
</tr>
<tr>
<td>Prong length L</td>
<td>1.575mm</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2 Tuning forks as force sensors

A simple linear equation of motion is the result for each element and node. Together these equations of motion can be written in matrix form:

\[
\begin{bmatrix}
  m_{00} & 0 & 0 & \cdots & \xi_0 \\
  0 & m_{11} & 0 & \cdots & \xi_1 \\
  0 & 0 & m_{22} & \cdots & \xi_2 \\
  \vdots & \vdots & \vdots & \ddots & \vdots \\
  \xi_0 & \xi_1 & \xi_2 & \cdots & \xi_n
\end{bmatrix}
+ 
\begin{bmatrix}
  0 & \xi_0 \\
  0 & \xi_1 \\
  \vdots & \vdots \\
  \gamma_{n0} & \xi_0 \\
  \gamma_{n1} & \xi_1 \\
  \gamma_{n2} & \xi_2 \\
  \vdots & \vdots \\
  \gamma_{nm} & \xi_m
\end{bmatrix}
+ 
\begin{bmatrix}
  k_{00} & k_{01} & k_{02} & \cdots & k_{0n} \\
  k_{10} & k_{11} & k_{12} & \cdots & k_{1n} \\
  k_{20} & k_{21} & k_{22} & \cdots & k_{2n} \\
  \vdots & \vdots & \vdots & \ddots & \vdots \\
  k_{mn} & k_{mn} & k_{mn} & \cdots & k_{mn}
\end{bmatrix}
+ 
\begin{bmatrix}
  \xi_0 \\
  \xi_1 \\
  \vdots \\
  \xi_0 \\
  \xi_1 \\
  \xi_2 \\
  \vdots \\
  \xi_m
\end{bmatrix}
= 
\begin{bmatrix}
  F_0 \\
  F_1 \\
  \vdots \\
  F_0 \\
  F_1 \\
  F_2 \\
  \vdots \\
  F_m
\end{bmatrix}
\] (2.1)

Where \( \xi_n(x,y,z) \) is the generalized coordinate of node \( n \), \( \xi_n \) and \( \xi_n \) are the first and second order time derivatives of \( \xi_n \) respectively. And where \( m_{nn} \) is the effective mass of node \( n \), \( \gamma_{nn} \) and \( k_{nn} \) are the damping and spring constant between nodes \( n \) and \( m \) respectively and \( F_n \) is the driving force. Equation (2.1) can be rewritten as:

\[
\dot{M} \ddot{\xi} + \Gamma \dot{\xi} + K \ddot{\xi} = \ddot{\hat{F}} \] (2.2)

Where \( M \) is the matrix representing the mass of the tuning fork model, \( \Gamma \) is the matrix with all damping elements and \( K \) is the matrix with all spring constants, \( \ddot{\xi} \) is the vector with the generalized coordinates of all nodes and \( \ddot{\hat{F}} \) is the vector with all driving forces. The boundary conditions still have to be set. These boundary conditions ensure the model to be connected properly to the outside world. Individual nodes can eventually be exposed to set displacements. Together these forces constrain the model according to the connection of the tuning fork to the driving piezo element and the driving displacement of this element.

2.2.2 Solving the motion of a tuning fork using a finite element model

Several routes can be taken to resolve the motion of a tuning fork using a finite element model. It is possible to extract the oscillation modes of a tuning fork, by eliminating all driving terms in the boundary conditions and by setting \( \ddot{\hat{F}} \) to zero. Assuming only harmonic time dependence for \( \ddot{\xi} : \dot{\xi}_n(t) \sim \dot{\xi}_n e^{\omega t} \), equation (2.2) reduces to an eigenvalue problem:

\[
\dot{\omega}^2 \dddot{\dot{\xi}} + K \dddot{\dot{\xi}} = -\omega^2 M \dddot{\dot{\xi}} \] (2.3)
If the eigenvalue problem is well-posed, meaning that a finite non-zero number of solutions exist, each solution has a specific eigenvector \( \Xi_p \), where \( p \) is the number of the solution. This eigenvector corresponds to a specific position of all generalized coordinates or a specific mode shape of the tuning fork and there is a specific frequency associated with this solution; the eigenvalue or resonance frequency \( \omega_p \). The solutions to the eigenvalue problem form a complete and orthogonal set. As a consequence, any motion of the tuning fork can be described as a superposition of different modes. Figure 2-3 shows four different modes with different resonance frequencies as a solution to the eigenvalue problem in equation (2-3).

### 2.2.3 Drive efficiency

Different modes respond different to specific ways of driving the tuning fork. With some combinations of mode and drive function, energy is effectively transferred from drive to tuning fork motion, while other combinations hardly result in a motion of the tuning fork.

**MECHANICALLY DRIVEN TUNING FORKS.** In SPM, tuning forks are mostly driven by mechanically dithering the base of the tuning fork, as shown in Figure 2-4. With this method of exciting the tuning fork, mostly the center of gravity is excited. The resulting motion of the tuning fork depends on the overlap of the specific tuning fork mode and the drive function:

\[
\ddot{\Xi}(t) = \sum_p \langle \Xi_p | D(t) \rangle
\]  

(2-4)

For example, dithering the tuning fork’s base drives modes a and d in Figure 2-3 more efficiently than modes b and c. Because of this, mode a has been reported in literature\(^\text{11}\) to be the actual mode of tuning forks in experiments. The discrepancy
between the calculated and measured resonance frequency proves this assumption to be incorrect. At the resonance frequency of mode c in Figure 2-3, the tuning fork will oscillate in this asymmetric mode.

The amplitude however, will be smaller than expected when only one prong of the tuning fork is considered in a harmonic oscillator model. The drive efficiency of a mechanically excited tuning fork can be defined as the amplitude of a tuning fork prong divided by the amplitude of a single beam with the same dimensions and the same driving amplitude. In the case of a perfect symmetric tuning fork and an excitation of only the center of gravity, the amplitude would even be infinitely small, resulting in a zero drive efficiency. Tuning forks can be excited mechanically with a nonzero efficiency because the attached fiber renders the tuning fork prongs asymmetric. In addition, the drive is not completely limited to the center of gravity, because tuning forks are dithered from one side resulting in small asymmetries in the drive.

ELECTRICALLY DRIVEN TUNING FORKS. The drive efficiency as defined for mechanically excited tuning forks cannot be applied for electrically excited tuning forks. Yet, also for electrically driven tuning forks, some modes are more efficiently excited than others. Here, the drive efficiency can be defined as the tuning fork prong amplitude divided by the driving voltage. The electrode layout is an important factor in the drive efficiency. Tuning forks have an electrode layout that is designed for a maximum drive efficiency of mode c in Figure 2-3. This is also the operational mode of the tuning fork.

2.2.4 Tuning fork signal strength

As discussed in § 1.2, tuning forks are made of quartz, a piezoelectric material. Stress in such a material results in a charge displacement. An oscillating tuning fork results in an oscillating charge displacement, or an alternating current. This current is picked up by the electrodes on the tuning fork. The stress in a specific point in a tuning fork is a function of the mode shape. To ensure the maximum signal strength, the electrodes have to be optimized for the desired mode of the tuning fork. Figure 2-5 shows the electrode lay-out as present on the used tuning forks. This lay-out is optimized for mode c in Figure 2-3, where the prongs move in opposite directions. In
other modes, for example modes a and d in Figure 2-3, the prongs move parallel and the current picked up in one prong will be compensated by the current picked up in the other prong and a net signal will only be obtained if the prongs are not completely symmetric.

The total signal is the piezoelectric current picked up by the electrodes. This is a function of stress in the material and the electrode layout. The stress in the material depends on the deformation of the tuning fork prongs, which depends on the overlap between drive function and the tuning fork modes. Together, the total signal S can be written as:

\[ S = \sum_p \langle E|\Sigma_p|D \rangle \]  \hspace{1cm} (2-5)

Where E is the electrode lay-out, \(X_p\) is the tuning fork mode with number \(p\) and \(D\) is the drive. When the tuning fork amplitude signal is recorded as a function of drive frequency, usually, several resonance peaks can be observed. Each peak corresponds to a specific tuning fork mode that is excited at its own resonance frequency. Despite its low drive efficiency, the peak corresponding to mode c in Figure 2-3 has the largest amplitude, emphasizing the importance of the electrode lay-out.

2.2.5 Influence of the fiber on tuning fork modes

Mode c in Figure 2-3 is the mode that is normally used when operating tuning fork based shear force microscopy. As discussed in the previous paragraph, this mode is normally not efficiently driven with mechanical excitation of the base of the tuning fork. However, the asymmetry induced by the fiber allows a relatively efficient drive of this tuning fork mode.

The driving efficiency can be illustrated by calculating the tip amplitude of a fiber attached to a tuning fork as a function of drive frequency. The tuning fork model described in equation (2-2) is solved for increasing fiber cross sections. For every cross section, the boundary conditions include a drive with an amplitude of 1 nm and the model is solved for 50 drive frequencies. Again, only harmonic time dependence is assumed. For simplicity, the model uses square fiber cross sections. A round cross section reduces the contact area between fiber and tuning fork to zero and would impose infinite stress in the material. For a round fiber also the glue has to be accounted for in the model, which would unnecessarily complicate the model.

Figure 2-6 shows the effect of increasing fiber sizes. For a tuning fork without fiber, the tip amplitude at a resonance frequency of 97.4 kHz is 1.3 nm. At this small amplitude, other modes, although not at resonance contribute to the amplitude. Still, the tuning
Figure 2-6. Tuning fork amplitude for tuning forks with increasing fiber cross sections. The drive amplitude of 1 nm is indicated by the lower horizontal line. The tip amplitude obtained by a simple beam with the same Q-factor of 580 is indicated by the upper horizontal line. For model simplicity calculations are made using fibers with a square cross section. From lower frequency to higher frequency, tuning forks are simulated with fiber width of 0, 10, 20, 40, 60 and 130 μm. For the first three tuning forks, the drive contributes significantly to the tip amplitude. To illustrate the resonance of the mode and to eliminate contributions of other modes, the amplitude of the prong separation is indicated by the dashed curve.

fork mode c depicted in Figure 2-3 is at resonance. To illustrate this, the amplitude of the prong separation, a typical feature of this specific mode, is plotted with a dotted line in Figure 2-6.

The tip amplitude is only 1.56 times the drive amplitude, where a simple beam would have an amplitude of the Q-factor times the amplitude. In these simulations the Q-factor was 580, making the drive efficiency 1.56/580 = 0.0027. As mentioned before, this can be explained by the small overlap between the drive and the tuning fork mode. Already for a thin fiber of 10 μm, the tip amplitude increases to 2.0 nm at a resonance frequency of 98.2 kHz. Also here the prong separation amplitude is plotted. The fiber adds mass to one of the prongs and increases the prong’s stiffness. As a result, the asymmetry of the tuning fork increases and drive efficiency is enhanced. Added mass will lower the resonance frequency of the tuning fork, while increased stiffness will increase the tuning fork resonance frequency. The simulation shows that the increased stiffness has the strongest effect.
The calculations are repeated for fiber widths of 20 μm, 40 μm, 60 μm and 130 μm. As for tuning forks without fiber and with a fiber of 10 μm, the prong separation amplitude is plotted for a fiber size of 20 μm to separate the motion of the tuning fork mode at resonance from other motions of the tuning fork. For larger fiber diameters, the tip amplitude in the tuning fork mode at resonance is large enough so that the drive amplitude can be neglected. The resulting tip amplitude can be found in Table 2-2. For the simulated fiber sizes, the resulting drive efficiency is calculated. The drive efficiency is defined here as the ratio between the tip amplitude of the tuning fork in the desired mode and the tip amplitude that would be observed if the oscillator was a simple beam, where the tip amplitude is Q-factor times the drive amplitude.

Table 2-2 shows that for a tuning fork that has fiber with a width of 130 μm attached to it, is 330 times more efficiently driven than a tuning fork without a fiber.

<table>
<thead>
<tr>
<th>fiber size [μm]</th>
<th>res. freq. [kHz]</th>
<th>tip amplitude [nm]</th>
<th>drive efficiency&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>97.41</td>
<td>1.56</td>
<td>0.0027</td>
</tr>
<tr>
<td>10</td>
<td>98.18</td>
<td>2.05</td>
<td>0.0035</td>
</tr>
<tr>
<td>20</td>
<td>98.77</td>
<td>3.94</td>
<td>0.0051</td>
</tr>
<tr>
<td>40</td>
<td>99.51</td>
<td>20.7</td>
<td>0.034</td>
</tr>
<tr>
<td>60</td>
<td>100.69</td>
<td>100</td>
<td>0.17</td>
</tr>
<tr>
<td>130</td>
<td>102.34</td>
<td>191</td>
<td>0.33</td>
</tr>
</tbody>
</table>

<sup>a</sup> drive efficiency is calculated as the tip amplitude of the tuning fork divided by the tip amplitude as obtained for a simple beam oscillator.

### 2.2.6 Observed tip amplitude

Normally fibers with a diameter of 125 μm are glued to tuning forks. With fibers of this size and the contribution of the glue to the asymmetry of the tuning fork, the drive can be expected relatively efficient. A setup as shown in Figure 2-7 was constructed to verify the actual tuning fork tip amplitude. A cleaved fiber is glued to a tuning fork that is mounted on a calibrated scanner. The light coming from the fiber is focused on a position sensitive silicon photo-detector using a microscope objective (Nikon 20x, 0.40 NA). A motion of the cleaved fiber tip in a plane parallel to the plane of the photo-detector results in a motion of the light spot on the detector. The scanner can be used to calibrate the motion of the tuning fork tip.
The Q-factor of the mounted tuning fork in this experiment was 154 at a resonance frequency of 101.98 kHz. The tuning fork was mechanically excited with a piezo element, with a piezoelectric constant of 0.3 nm/V. At frequencies lower than the resonance frequency of this element which is in the MHz-range. The driving amplitude can be calculated with this constant and the tuning fork tip amplitude can be observed with the setup in Figure 2-7.

Figure 2-8 shows the tuning fork tip amplitude as a function of drive amplitude. It should be noted that the relation between tip and drive amplitude is linear over more than three decades. Only for tip amplitudes in the order of 0.2 nm, noise causes an offset on the observed tip amplitude. In Figure 2-8, the tip amplitude is 110 times the drive amplitude. The drive efficiency is then 0.71. This is a factor of two larger than the calculated drive efficiencies. In the simulation, the effect of glue used for attaching the fiber to the tuning fork is not taken into account. Apparently, the glue will increase the asymmetry more, explaining the larger drive efficiency.

For 32.768 kHz tuning forks, the tip amplitudes have been recorded in a similar way. From the reported tip amplitude, a drive efficiency of 0.031 can be calculated. This is about a factor of 20 lower than 100 kHz tuning forks with the same fiber attached. To explain this, the size of the 32.768 kHz tuning forks should be considered. These tuning forks are much heavier and larger than 100 kHz tuning forks. The same fiber will make a 100 kHz tuning fork relatively more asymmetric than a
32 kHz tuning fork and the drive will be more efficient for a 100 kHz tuning fork accordingly. A correct estimate of the tip amplitude is especially important when estimating interaction forces, where the tip amplitude is an important parameter. Overestimating the tip amplitude leads to an overestimation of the interaction force.

The electrical driving efficiency for the same tuning fork can be determined with the same setup, in case the tuning fork is electrically driven.

Figure 2-9 shows the tip amplitude for an electrically driven tuning fork. Also here the relation between tip amplitude and drive voltage is linear over three decades, until it levels off at low driving voltages because of noise in the amplitude detection. As with mechanical drive, it is important to know the tip amplitude to estimate the interaction forces. The amplitudes observed for both mechanical and electrical drive are similar and both types of driving tuning forks can be used in Scanning Probe Microscopy.

2.3 Harmonic oscillator tuning fork model

Although finite element analysis is necessary to completely understand the motion of the tuning fork, a harmonic oscillator model is still useful. The modes of the tuning fork are well separated in frequency. If a specific mode of a tuning fork is efficiently excited at resonance, the tuning fork motion will be confined to that specific mode and a harmonic oscillator model can be used to represent the tuning fork in that mode. Care should be taken that the properties of the harmonic oscillator are chosen so that it matches the results of the finite element analysis.
2.3.1 Harmonic oscillator model

A simple harmonic oscillator as shown in Figure 2-10 with a mass $m$, spring constant $k$ and damping $\gamma$ is driven through the spring with a displacement $d$, resulting in a displacement of the oscillator with $x$. This is a model for a tuning fork, where also an effective mass is displaced through a cantilever spring, i.e. the prong of the tuning fork. The most important source of damping for a tuning fork in ambient conditions is friction between the tuning fork and the environment of the tuning fork.

When choosing the harmonic oscillator parameters, the mode shape of the tuning fork is important. As discussed in the previous paragraph, the tuning fork is oscillating in the mode shown in Figure 2-11. In this mode, the prongs are moving in opposite directions, where the bases of the prongs are fixed. To make the model consistent with the finite element method, only one prong can be considered as a cantilever beam deflecting in one direction. Inspired by Rayleigh\textsuperscript{22} and Karrai et al.,\textsuperscript{9,11} the equation of motion for a harmonic oscillator representing a 100 kHz tuning fork can be written down.

$$m\ddot{x} + \gamma \dot{x} + k(x - d) = 0$$  \hspace{1cm} (2-6)

Where the effective mass $m$ and the effective spring constant $k$ of the tuning fork prong have to be calculated. The damping can be used as a fitting parameter to match the observed $Q$-factor of a tuning fork with a fiber mounted. For one prong, fixed at the base of the tuning fork, the effective mass equals:

$$m = 0.2427 \rho L W T$$  \hspace{1cm} (2-7)

Where the values of the mass density $\rho$ and the dimensions of the prong $L$, $W$ and $T$ can be found in Table 2-1. The effective spring constant of one prong is:

$$k = \frac{E W T^3}{4L^3}$$  \hspace{1cm} (2-8)
Where \( E \) is the Young’s modulus of quartz. Assuming only harmonic time dependence, \( i.e. \, x(t) = x_0 e^{i\omega t} \), where \( \omega \) is the radial frequency, equation (2-6) can be solved. The relation between the prong deflection and the effective drive is:

\[
\frac{x}{d} = \frac{k}{k + i\omega\gamma - m\omega^2}
\]  

(2-9)

At resonance, the amplitude of the oscillator is at maximum:

\[
\omega_{\text{res}} = 2\pi f_{\text{res}} = \frac{\sqrt{k}}{\sqrt{m}}
\]  

(2-10)

For large \( Q \)-factors \( (Q \gg 1) \), the ratio between the prong amplitude and the effective drive amplitude at resonance equals the \( Q \)-factor:

\[
Q = \frac{\Delta \omega_{\text{FWHM}}}{\omega_{\text{res}}} = \frac{\sqrt{km}}{\gamma}
\]  

(2-11)

Where \( \Delta \omega_{\text{FWHM}} \) is the full width of the resonance peak at half the maximum amplitude.

### 2.4 Probe-to-sample interaction forces

A particular strength of the harmonic oscillator model, is the possibility to calculate forces necessary to disturb the oscillator in scanning probe microscopy.\(^9\)\(^\text{-}\)\(^\text{20}\) In such calculations, the interaction forces are usually simulated by adding a small extra damper or spring to the harmonic oscillator.

Figure 2-12 shows a tuning fork with both types of interaction. A small extra spring \( \Delta k \) is a model for conservative probe-to-sample interaction and a small extra damper \( \Delta \gamma \) is a model for dissipative probe-to-sample interaction. The combination of these two interaction types can be a model for the actual probe-to-sample interaction. The effects of the two interaction types will be investigated separately.

Both the amplitude and the phase response spectrum for various values of \( \Delta k \) and \( \Delta \gamma \) have been simulated. Figure 2-13 shows the effect of an extra damping term and of an extra spring constant in the equation of motion (2-6).
2.4.1 Dissipative probe-to-sample interaction

With the extra damping $\Delta \gamma$ in Figure 2-13, representing the case of dissipative probe-to-sample interaction, the total damping in the system changes to $\gamma + \Delta \gamma$. The most noticeable effect on the resonance curve is a drop of the Q-factor, or a drop of the amplitude at resonance. Also the resonance frequency shifts to a lower frequency, however this effect is neglectable for oscillators with $Q \gg 1$ and $\Delta \gamma \approx \gamma$. The phase of oscillator with respect to the drive remains at $-90^\circ$ or $\pi$. For dissipative interaction, the amplitude of a harmonic oscillator is the proper parameter for representing the probe-to-sample interaction.

The interaction force in this case can be calculated by calculating the force on the extra damping element:

$$F_{\text{dis}} = \Delta \gamma \omega (x - \Delta x)$$

(2-12)

Where $\Delta \gamma$ is the small damper representing probe-to-sample interaction. $F_{\text{dis}}$ is the dissipative force acting on this damper and $i\omega(x-\Delta x)$ is the velocity of the oscillator. Normally, an amplitude damping $\Delta x$ is chosen as a setpoint for probe-to-sample interaction. From equation (2-12), it is clear that the force is $90^\circ$ out of phase with the displacement of the harmonic oscillator. Only the amplitude of the interaction force is of interest, so from now, only the absolute value of the interaction force will be calculated. The damping constant $\Delta \gamma$ is unknown, but follows implicitly from the difference in amplitude at resonance:

$$\frac{x - \Delta x}{x} = \frac{\gamma}{\gamma - \Delta \gamma}$$

(2-13)

Solving for $\Delta \gamma$ and combining with equation (2-12), the amplitude of the interaction force becomes:

$$F_{\text{dis}} = \gamma \omega \Delta x = \frac{k x \Delta x}{Q x}$$

(2-14)
The latter expression is more useful, since the spring constant can be calculated, the Q-factor and prong amplitude can be observed directly and the relative change in amplitude is normally used as setpoint for probe-to-sample interaction. This in agreement with results published for tuning forks and conventional atomic force microscopy.9,11

2.4.2 Conservative probe-to-sample interaction

With the extra spring Δk in Figure 2-13, representing the case of conservative probe-to-sample interaction, the total stiffness of the system changes to k + Δk. There are two effects on the resonance curve, the Q-factor will rise and -most noticeably- the resonance frequency will shift to a higher frequency. As tuning forks are normally driven at a constant frequency, the phase with respect to the drive will, being at a maximum slope, have the strongest response to a change in resonance frequency and is the proper signal as a measure for conservative probe-to-sample interaction. Analogous to dissipative probe-to-sample interaction, conservative interaction will be represented by a small spring resulting in a chosen phase shift. Once the spring constant of the interaction is known, the amplitude of the force on this spring can be calculated with:

\[ F_{cons} = x \Delta k \]  

(2-15)

Adding a small spring Δk, with Δk ≪ k to the harmonic oscillator, results in resonance frequency of:

\[ \omega_{res} + \Delta \omega = \sqrt{\frac{k + \Delta k}{m}} \Rightarrow \Delta \omega = \frac{\omega_{res}}{2k} \Delta k \]  

(2-16)

For a harmonic oscillator, the derivative of the phase with respect to the frequency equals:

\[ \frac{\partial \phi}{\partial \omega} \bigg|_{\omega = \omega_{res}} = \frac{1}{Q} + \frac{2Q}{mQ + \omega} = -\frac{2Q}{\omega} \]  

(2-17)

Where \( \phi \) is the phase in radians and assuming that Q ≫ 1 and \( \frac{\gamma}{m} \ll \omega \). The latter approximation can be verified intuitively. The phase shifts almost from 0 to \( -\pi \) in a frequency band of Q/\( \omega \). For small changes in phase, equation (2-17) can be written as:

\[ \Delta \omega = \frac{\omega_{res}}{2Q} \Delta \phi \]  

(2-18)
Combining equation (2-15), (2-16) and (2-18) gives:

\[
F_{\text{cons}} = \frac{k x}{Q} \Delta \phi
\]  

(2-19)

As mentioned in § 2.4.1, the prong amplitude \(x\) and the Q-factor can in principle be observed directly. The spring constant \(k\) can be calculated and the phase shift \(\Delta \phi\) is the setpoint for probe-to-sample interaction.

### 2.5 Interaction forces with tuning-fork-based AFM

Now that interaction forces can be calculated, the performance of tuning forks will be compared with noncontact atomic force microscopy (nc-AFM). In the experiment performed to compare the two techniques, the cantilever based AFM is operated with a standard Si-cantilever probe reflecting the light from a laser diode. The reflected light is detected by a position sensitive photo diode. The signal from the photo diode is amplified by a trans-impedance amplifier and then demodulated and further processed by the electronics of the instrument. The amplitude of the cantilever deflection was used for tip-sample distance control. For the comparison, the tuning fork is used in a normal force configuration. An AFM cantilever is mounted on the tuning fork in such a way that it is oscillated normal to the sample surface. As the tuning fork is an appealing nonoptical force sensor, several tuning-fork-based atomic force microscopes have been developed.

#### 2.5.1 Instrumental aspects

For the tuning-fork-based setup, the same type of Si-cantilever is glued on top of a tuning fork, as shown in Figure 2-14. The free length of the cantilever is made short enough to make it rigid at the resonance frequency of the tuning fork. A trans-impedance amplifier amplified the piezo-electric signal from the tuning fork and a lock-in amplifier extracted the amplitude or phase. Ruiter et al.\(^{13,14}\) have found that during approach towards the sample with a tuning-fork-based system, mostly a shift in resonance frequency occurs, corresponding to an increase in spring constant. Given the frequency shift and the relatively high Q-factor of the tuning fork, the choice for this experiment is to use the phase signal for the tuning-fork-based setup.

Except for the probes and the detection electronics, the same instrument\(^{23}\) was used for the comparison. Several parameters and operating conditions for the two different techniques are given in Table 2-3.
Table 2-3. Selected parameters on cantilever and tuning fork based AFM.

<table>
<thead>
<tr>
<th>sensor parameters</th>
<th>cantilever</th>
<th>tuning fork</th>
<th>operating conditions</th>
<th>cantilever</th>
<th>tuning fork</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k$</td>
<td>48.0N/m</td>
<td>45.0 kN/m</td>
<td>$x_{\text{res}}$</td>
<td>5.4 nm</td>
<td>0.1 nm</td>
</tr>
<tr>
<td>$Q$</td>
<td>360</td>
<td>2600</td>
<td>$\Delta x$</td>
<td>2.7 nm</td>
<td></td>
</tr>
<tr>
<td>$\omega_{\text{res}}$</td>
<td>143.0kHz</td>
<td>97.7 kHz</td>
<td>$\Delta \phi$</td>
<td>1.0°</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$F_{\text{interaction}}$</td>
<td>360pN</td>
<td>30pN</td>
</tr>
</tbody>
</table>

2.5.2 Si(111) sample

As a test object a Si(111) sample was used, provided by the National Institute of Standards and Technology (NIST).\textsuperscript{31} It is prepared in a way that stable monatomic steps with a step height of 0.314 nm are present in ambient conditions.\textsuperscript{32} In air there will be an oxide layer, but this layer has a constant thickness and does not affect the step heights in the sample.

2.5.3 Results and discussion

Figure 2-15 shows the topography measured with tuning-fork-based AFM, and Figure 2-16 shows the topography of the same sample, however measured with cantilever based AFM. No image processing is done, except for subtracting a parabola at each scan line (second order line leveling) to compensate for sample tilt, angular scanner movement and drift due to temperature fluctuations during scanning. The observed step height is 0.33 nm for both techniques with an RMS noise over the area of 0.09 nm for the cantilever based AFM and 0.14 nm for the tuning-fork-based AFM.

Figure 2-15. Mono-atomic steps on Si(111) measured with a tuning fork based set-up. 1 μm × 1 μm scan area, 500 × 500 pixels, line frequency 0.25 Hz. The measured step height is 0.33 nm with a RMS noise over the area of 0.14 nm. A double step height is measured at step crossings. Small particles are clearly visible suggesting small interaction forces. The calculated interaction force is 30 pN.
In the comparison, two different signals have been used for tip-sample distance control. The amplitude of the cantilever for the cantilever based AFM and the phase of the tuning fork in the tuning-fork-based AFM. To make the comparison still fair, the operation conditions as given in Table 2-3, were chosen so that the RMS noise over the area was approximately the same for both measurements.

In both images, the monatomic steps are clearly visible. The step height is twice the step height of one single step at places where two steps coincide, an indication that these are indeed monatomic steps. Also the measured height of the steps is in agreement with the expected value of 0.314 nm for both techniques. The interaction forces calculated with the harmonic oscillator model indicate a lower interaction force of 30 pN for the tuning-fork-based setup compared to the interaction force of 360 pN obtained with the cantilever based AFM. The results with the tuning-fork-based AFM are close to the thermal limit of 5.3 pN at room temperature.\(^9\) The cantilever based AFM is still far from its thermal limit of 1.3 pN. For a more detailed discussion of thermal noise, see Chapter 4. More small particles are visible in the measurement with the tuning-fork-based AFM. A possible explanation is that the cantilever based AFM swept the dust away. This would confirm that the forces applied to the sample by the tuning-fork-based setup are indeed lower.

The measurements of monatomic steps on a Si(111) sample demonstrate the sensitivity of the tuning fork as a height detector. In the experiments performed, the sensitivity is one order of magnitude better than noncontact AFM cantilevers. Choosing smaller amplitude damping as the feedback set point will lower the interaction forces of the cantilever based AFM ultimately down to the thermal limit. Cleveland et al. have shown that the thermal limit can indeed be the ultimate limit for AFM.\(^{33}\) Increasing the Q-factor of the tuning fork can lower the interaction forces and the thermal limit for force detection of the tuning-fork-based AFM. With quartz tuning
forks, Q-factors in the order of $10^4$-$10^5$ can be obtained, which would result in a thermally limited interaction force of 1-10 pN. In the present setup that uses a constant driving frequency, a higher Q-factor will prevent fast scanning, but implementing a self-oscillating circuit and using the resonance frequency as the tip-sample distance signal, will circumvent this problem.

2.6 Conclusions

Tuning forks are sensitive and reliable force detectors in Scanning Probe Microscopy (SPM). Finite element analysis has shown that a harmonic oscillator model of only one prong is too simplistic. With a model of only a single prong of a tuning fork, the influence of the fiber size on the drive efficiency of the tuning fork cannot be explained. Also the presence of multiple resonance peak in the response spectrum of a tuning fork cannot be explained. However, using the proper drive amplitude, the harmonic oscillator allows estimation of the interaction force between tip and sample.

Using this model for cantilever-based and tuning-fork-based Atomic Force Microscopy (AFM), it is possible to compare the two techniques. With tuning-fork-based AFM, interaction forces of 30 pN have been observed, which are close to the thermal detection limit of tuning forks.

Because tuning forks do not need any optics for height detection, they are used in near-field optical microscopy. However, the lack of optics also makes probe replacement and even multiple scan heads on one sample possible. The high sensitivity and the lack of optics can make a tuning-fork-based setup the preferred choice in industrial applications, where simple operation, quick probe replacement and throughput are important.
2.7 References

23. The instrument used as a basis for these experiments was a Discoverer manufactured by TM-Microscopes, 1171 Borregas Avenue, Sunnyvale, CA 94089, USA
31. NIST, 100 Bureau Drive, Gaithersburg, MD 20899, USA
2 Tuning forks as force sensors
Liquid immersion of tuning forks

This Chapter presents a study of the dynamic behavior of tuning forks and the feasibility of tuning-fork-based shear force microscopy in liquid. At increasing immersion depths, the resonance characteristics of 100 kHz tuning forks are obtained. A shift in resonance frequency and a partial recovery of the tip vibration amplitude have been observed upon immersion in liquid. The conservation of the vibration mode upon immersion is confirmed by both direct stroboscopic observation and by detection of the tip vibration amplitude of the tuning fork. Thanks to the partial recovery of the Q-factor upon complete immersion in liquid, it is possible to obtain high-resolution images on annealed gold and to image soft samples in liquid without apparent sample damage. Living cells still impose more stringent demands on the environment to be imaged reliably for longer periods of time. Conventional light microscopy shows that living cells detach from the sample surface before they can be imaged. Based on the results in this Chapter, it can be expected that imaging living cells should be possible with the described methods.

3.1 Introduction

The application of Near-field Scanning Optical Microscopy (NSOM) in biology requires the immersion of the sample and thus the probe in aqueous solution. Optical shear force and normal-force detection schemes have already shown potential for imaging in liquids.\textsuperscript{1-6} A nonoptical method, based on bulky piezo elements, has also been demonstrated.\textsuperscript{7,8} A method to control the probe-to-sample distance based on ion-conductance has been reported,\textsuperscript{9,10} where the resolution is still limited due to the large aperture sizes and the large probe-to-sample distance. Tuning-fork-based shear force detection is preferable, as tuning forks are small, sensitive, reliable and cheap force detectors as discussed in Chapter 2. Until now, the huge damping of the tuning fork movement, occurring upon immersion in liquid and a lack of understanding of the involved vibrational modes have prevented the use of tuning-fork-based shear force detection in liquids.

In principle, liquid immersion of the tuning fork can be avoided by immersing only the fiber tip.\textsuperscript{11,12} Such a method however requires some means of controlling the tip immersion depth into a liquid layer with micrometer stability. If the immersion depth of the fiber varies, the tip-sample interaction cannot be distinguished from interaction between fiber and liquid. Although controlling the liquid level is feasible, this method introduces complications. A concentration gradient may emerge: water evaporates at the liquid surface and is replaced by water from a reservoir. For thin layers of water, diffusion is limited and a significant concentration gradient arises. This complicates the operation of the microscope and the exchange of fluids, making the immersion of the whole tuning fork preferable. An extra challenge raised by complete immersion of the tuning fork is an eventual short-circuit of the tuning fork electrodes by a saline solution.

In this Chapter, first the dynamic behavior of the tuning fork in water is studied. Second, the consequences for the tuning fork electrodes upon immersion in physiological buffer solution (PBS) are discussed.

3.2 Tuning fork immersion

Full immersion of the tuning fork might be preferable for shear force microscopy in liquid. However, immersion renders tuning forks useless for shear force detection, if the viscous damping of immersed tuning forks is too large.
To gain insight into the effect of damping, the dynamic response of 100 kHz tuning forks\textsuperscript{18} at increasing immersion depths in water is examined. Figure 3-1 shows a tuning fork before and after immersion. At full immersion, the prongs and a large part of the tuning fork is immersed. The part of the base of the tuning fork remaining above the fluid level does not significantly contribute to the motion of the tuning fork.

The tuning fork is mechanically excited by an external dither piezo with a constant driving amplitude in the order of 10 pm. The driving frequency is swept from 78 kHz to 98 kHz. A transimpedance amplifier amplifies the piezoelectric tuning fork signal and the amplitude of the signal is recorded for each driving frequency.

Figure 3-2 shows the dynamic response of one tuning fork for increasing immersion depths. The curve up front is obtained with the tuning fork in air, as shown in Figure 3-1(a), while the curve at the back is obtained with the prongs completely immersed in water, as shown in Figure 3-1(b). The resonance frequency in air is close...
to 96.7 kHz with a high Q-factor of about 1500. The next four curves correspond to an increasing immersion of only the fiber tip, which results in a slight shift in resonance frequency and a drop of Q-factor. As soon as the prongs touch the water surface, a meniscus builds up, resulting in a sudden decrease in frequency and Q-factor for the sixth resonance curve. The frequency shift and the drop in Q-factor continue for further immersion of the tuning fork. With the prongs more than halfway immersed, the decrease in resonance frequency continues, however the Q-factor starts to recover again to a value around 60. After complete immersion of the prongs, the resonance frequency stabilizes at 85.4 kHz and a Q-factor of 62 and the fluid level is not critical anymore. The recovery of the Q-factor and the shift in resonance frequency upon complete immersion raises the question whether the tuning fork in water is moving in the same vibration mode as in air.

As discussed in Chapter 2, tuning forks exhibit many vibrational modes. The resulting tuning fork signal is a function of the drive function and frequency, the vibrational modes and the electrode layout on the tuning fork. The recovered peak that has a shifted resonance frequency, could be explained by a different vibrational mode having a larger overlap between drive, mode and electrodes in water compared to the normal vibrational mode observed in air.

3.3 Tuning fork motion in liquid

It is important to know the specific vibrational mode of the tuning fork, because not every mode will be equally suitable for shear force microscopy. The model developed in Chapter 2 is only applicable to the mode where the tuning fork prongs move like scissors. For other modes, even if these modes are suitable for shear force microscopy, the models needs to be adapted to give an accurate estimation of forces.

3.3.1 Direct observation

The most straightforward way to investigate the vibrational mode of an immersed tuning fork is direct observation. The tuning fork is mounted on a SPM head that is placed on top of an inverted optical microscope to observe the underside of the two prongs. The oscillation frequency of the
tuning fork of ~100 kHz is too high follow the motion by eye. A light emitting diode, flashing at a few Hz off the driving frequency, is used as a strobe light to illuminate the tuning fork prongs. Their movement of 1-2 µm can easily be seen if the tuning fork is driven at a high amplitude of 10-20 nm.

At different driving frequencies, various modes are visible. In air, at 96.7 kHz, the two prongs move towards and away from each other, as expected. The motion after immersion of the tuning fork is the same, however shifted to a resonance frequency of 85.4 kHz. This leads to the conclusion that the tuning fork is oscillating in the same mode, both in air and in water at these two frequencies. The shift in resonance frequency can be explained by the fact that there is a water layer around the tuning fork prongs that is moving along with the tuning fork, increasing the effective mass of the tuning fork prongs.

### 3.3.2 Observation of tip oscillation amplitude

Second, the relation between the tip amplitude, or prong deflection of the tuning fork and the driving amplitude of dither piezo is investigated. As discussed in § 2.2, every vibrational mode of tuning forks is associated with a specific drive efficiency. Different modes will have different drive efficiencies, related to differences in overlap between drive, mode and electrodes. If tuning forks are moving in the same vibrational mode in air and water, the drive efficiency remains the same. The prong amplitude for a constant driving amplitude, scales only with the inverse of the viscous damping, or the Q-factor.

Similar to the method described in § 2.2, a cleaved fiber, instead of a regular tapered fiber, is attached to the tuning fork for detection of the tip oscillation amplitude, resulting in a Q-factor of 189. A red HeNe-laser is coupled into the fiber and the scanner head is placed on top of the inverted optical microscope, with a position sensitive photodetector mounted to one of its output ports. With this

![Figure 3-4. Setup for detecting tuning fork tip amplitude.]()}
detector, movements of the tuning fork prong from less than a nanometer to several microns can be detected.\textsuperscript{20} The detector was calibrated using the scanner in the SPM head.

Figure 3-5 shows the results for the tuning fork movement in air and water. The movement of the tuning fork is linear over a wide range of amplitudes. For small driving amplitudes, an offset of the oscillation amplitude is visible, resulting from noise in the amplitude detection. The graph shows that in water, the amplitude drops with the same factor as the Q-factor does, as expected for a tuning fork in the same vibration mode. This result and the direct observation confirm that the tuning fork is moving in the same mode, at around 97 kHz in air and at 85 kHz in water.

### 3.4 Shear force microscopy in liquid

With the acquired knowledge that vibrational modes in water are similar to modes in air, where the forks can be successfully operated, tuning forks can be expected to work reliably in a liquid environment. A tuning fork that is completely immersed in water has a typical Q-factor around 60 and is driven such that the tip amplitude is in the order of 1 nm. The phase of the piezoelectric tuning fork signal with respect to the drive is used as measure for the tip-sample distance.\textsuperscript{20} With both prongs of the tuning fork fully immersed in water, the system is independent of the fluid level compared to other setups that are extremely sensitive to the immersion depth of the probe.\textsuperscript{1,4,7}

#### 3.4.1 Annealed gold

To test the achievable resolution on a hard sample, an annealed gold surface\textsuperscript{13} is measured with a tuning-fork-based shear force microscope.\textsuperscript{19} Annealed gold exhibits domains of crystalline gold. The surface of these domains are atomically flat. The topography of such an surface is obtained with a tuning-fork-based shear force microscope.

A drop of water, large enough to immerse the prongs completely, is deposited on the sample. Figure 3-6 shows the topography of annealed gold, obtained with shear force microscopy at a line frequency of 0.5 Hz. The observed surface roughness of these domains, if larger than the expected atomic scale, is caused by noise in the detection and vibration of the setup and is an indicator for the performance of the shear force microscope.
In Figure 3-6, different gold domains are clearly visible. The RMS vertical noise in this image is 1.5 nm over the area of a domain. This is the noise figure that can be expected for the used scanner and feedback settings. The ultimate performance of tuning forks for high resolution imaging could be even better. This result shows for the first time that with a tuning-fork-based setup high-resolution images can be obtained in water.

### 3.4.2 Cytospin sample

To test whether this setup allows low interaction forces and can handle soft biological samples, a cytospin sample\(^4\) of bone marrow cells is prepared. The cytospin preparation, where cells are spun on a cover glass and then dried, has several advantages:

- Cells can be imaged in air and liquid.
- Cells show a clear topography.
- The sample is stable at various temperatures.
- The sample is preserved for a long time.

These properties make a cytospin an ideal test sample for shear force microscopy in air and liquid. The topography of such a cytospin sample is first obtained using shear force detection with the sample in air. As the cells on this sample are dried, the membrane follows the solid remaining contents of the cell.
A large area scan of the sample as shown in Figure 3-7(a), shows several cells, having various morphologies. A large fraction of the cells, have approximately the same height (≈ 150-250 nm). The cell contents are visible for this group. More details of one cell from this group can be seen in a zoom-in. Figure 3-7(b), shows a cell, where the membrane follows the topography of the contents of the cell. The nucleus of the cell is clearly visible, as are several organelles. The average height of the nucleus is 270 nm and the average height of the organelles is 160 nm above the glass. Immersing the sample in water may cause extreme damage to the cells, due to osmosis. Figure 3-7(c) shows an example of a cell where the cell contents are spread over a large area. Most cells fortunately showed a similar topography after immersing in water and drying, indicating that these cells are not damaged by osmosis.

Dry cytopspins are rugged, hard samples, where the interaction force should only be low enough to prevent damage to the probe while scanning. The interaction forces are estimated to be in the order of 400 pN.
To test shear force imaging on a soft sample in liquid, the topography of the same sample can now be obtained after placing a large enough drop of water onto the glass slide to immerse both prongs completely. A new scan is made on the same sample, however on a different area. The overview given in Figure 3-7(a) indicates that a large fraction of the cells that are displayed, shows a similar average height, despite different lateral sizes. Because a large fraction of cells show a similar topography, the measured cells in air and water can still be compared keeping in mind that small differences can be caused by normal differences between cells.

![Shear Force Microscopy in Liquid](image)

Figure 3-8. Topography on cytospin samples in water. All images show unfiltered data, except for subtraction of a straight line at each scan line. Line frequency is 0.5 Hz.
(a) A 100μm by 100 μm scan showing several cells.
(b) A 50 μm by 50 μm zoom in on one cell. In water, the average height of the cell is 380 nm above the glass.
(c, d) A further zoom in on the cell and the edge of the cell, indicating that high resolution can still be obtained in liquid.

The large area scan in Figure 3-8(a) shows the topography of a number of cells immersed in water. As before immersing the cells in water, a large fraction of the cells shows a similar height. These cells showed the cell contents before immersion in
3 Liquid immersion of tuning forks

water. Figure 3-8(b) and (c) show a zoom-in on a single cell. When immersed in liquid, in most cells, the organelles cannot be seen as clearly as in Figure 3-7(b). Zooming in further, in Figure 3-8(d), shows that details on the side of the cell are still visible. The interaction forces are estimated to be in the order of 4 nN.

The average height of an immersed cell is 380 nm above the glass. This is 37% more than similar cells before immersing in water. This can be explained by absorption of water in the cell, which will make the sample soft compared to a dry cytospin and which will raise the cell membrane provided that the absorption is not strong enough to break the membrane. Or this can be explained by a damaged cell membrane, where water can flow in and out freely. Either way, shear force microscopy is performed on a floating cell membrane. The topography of the cell membrane can be repeatedly imaged without apparent damage to the cell. After drying, the topography becomes similar again to Figure 3-7(a). Clearly, the interaction forces between tip and sample are small enough to prevent damage to the cell membrane. This is an encouraging step towards tuning-fork-based shear force microscopy on living cells.

3.5 Living cells

So far, only cytospin prepared samples have been measured in a drop of water. Most biological relevant samples need immersion in a physiological buffer solution (PBS). In such a solution, the pH is controlled and ions are present to mimic a natural situation for the sample of investigation. The ions also provide osmotic pressure, to balance the osmotic pressure that can be found inside intact cells.

For living cells, cell medium and serum is added to the PBS, to provide nutrition to the cell. The cells are normally also kept under a 5% CO₂ atmosphere. The medium, serum and CO₂ are necessary to keep cells alive for extended periods of time. During the relative short time to perform microscopy on living cells, it is essential to immerse them in PBS, where the temperature of the buffer is controlled at 37°C. For other biological systems, other temperatures can be necessary. Both the PBS and the temperature requirements impose extra demands on the setup and the sample preparation.
3.5.1 Tuning fork coating

When a tuning fork is immersed in PBS, the electrodes are short-circuited by the saline solution, even at the extremely low voltages of a few microvolts that is on the electrodes during operation of tuning forks. For operation in PBS, it is necessary to coat the tuning fork electrodes.

Electrocoating\textsuperscript{15} is a method where two electrodes are immersed in an emulsion of organic resins in de-ionized water.\textsuperscript{16} When a sufficiently high voltage is applied across the electrodes, water is electrolyzed at the electrodes. The surroundings of the cathode become alkaline and the surroundings of the anode become acidic. At high pH, the emulsion of organic resins is unstable and the resins precipitate on the cathode. The newly formed coating insulates the covered parts of the electrode, favoring the coating process of uncoated areas of the electrode. The result of this method is an even and smooth coating layer, even in gaps and recessed areas. This is particularly useful when coating tuning forks, where the gap between the prongs is only 0.076 mm. The thickness of the coating is controlled by the voltage and, to a lesser extent, the coating time. After coating, the electrode is cured for 30 minutes in an oven at 170°C.

Tuning forks are coated using both electrodes on the tuning fork as a cathode. The anode is made of stainless steel and has an area much larger than the cathode ensuring that the build-up of the coating is not limited by the size of the anode. Tuning forks are coated for 20 s at a voltage of 20 V. After coating and before curing, the forks are gently rinsed with de-ionized water, to remove residual coating material that might fill the gap between the tuning fork prongs. After curing, the resistance between the tuning fork electrodes, when the tuning fork is immersed in a saturated saline solution, is beyond the measuring capability of a multimeter and must be in the order of several tens of MΩ. Coated tuning forks have been tested successfully as force sensors for shear force microscopy on dry samples. With coating, the operation of tuning forks immersed in saline solutions will be possible.

3.5.2 Liquid cell

A sample with living cells needs to be preferably in a closed liquid environment. For relevant biological studies, the sample needs to be handled and placed in the microscope routinely. A common way to prepare samples, is to grow them on a 170 μm thick coverglass. During the transfer, the cells are not immersed and will dry out. Therefore, the transfer of the sample from the Petri dish to the microscope needs to be fast.
A liquid cell is designed where the coverglass is placed in the base of the liquid cell. The cover glass may vary in size from round glasses with 15 mm diameter to square glasses with 25 mm sides. A lid is placed on top, sealing the glass with a rubber ring. The lid is held in place by spring plungers. Now the liquid cell can be filled with PBS, or a cell medium until the fluid surface is on the beveled edge on the topside of the lid, which is an indicator for the fluid level. As long as the fluid is on the beveled edge, the fluid level is such that the tuning fork is immersed far enough to make the resonance curve of the tuning fork relatively independent of the fluid level, as discussed in § 3.2. On the other hand, the fluid level is not too high, where the uncoated dither piezo would be short-circuited. The assembly can be placed on the inverted microscope. The hole in the base of the liquid cell allows access for microscope objectives. The top side of the liquid cell is open allowing transmission illumination and access for tuning forks with mounted fibers for tuning-fork-based shear force microscopy.

3.5.3 Sample preparation

With the liquid cell, living cells can be studied using shear force microscopy. Results on cytospines encouraged measurements on living cells. In principle, a wide range of cells is available for study. For the experiments Chinese Hamster Lung Cells are used, because of their biological interest and proven suitability for scanning probe techniques.

These cell lines are normal, adherent, epithelial cells, growing in monolayers. They are cultured in culture flasks and for experiments on glasses. For subculturing the medium is removed, cells are washed twice with PBS (Phosphate Buffered Saline pH 7.35) and then incubated for approximately 5 minutes at 37°C with trypsin-EDTA solution (Life Technologies 35400) to a final concentration of 0.5 g trypsin and 0.2 g EDTA per liter. Fresh medium is added, cells are aspirated and dispensed in new flasks or counted and put on glasses.
3.5.4 Shear force microscopy on a cluster of CHL cells

A sample with living CHL cells was imaged with a tuning-fork-based shear force microscope,\(^\text{19}\) using only a small amount of PBS. Figure 3-11 shows the topography of a cluster of at least six cells. Five cells are almost completely visible. When the tuning fork was stable in the solution, the cluster could repeatedly be imaged. As the amount of PBS was low, evaporation caused the salt concentration outside the cells to rise. Due to osmosis, the cells lose water, causing them to shrink and finally to die. Several scans could be made to follow this process. Obviously, the biological relevance of this process is low, however the shear force imaging of intact cells, that are significantly softer than cytospins, proves the ability of tuning forks to detect the surface of these cells.

Repeated scans of the cluster show that the cells do lose their volume. The lateral size of cells is constant making the height an indicator for the volume of the cells. Figure 3-12 shows the average height of cells in the cluster versus the time between the measurement and the sample preparation. It should be noted that there is an hour between the sample preparation and the first stable measurement. In that time, most of the liquid has gone and the absence of most of the liquid made the shear force detection stable again. However, the cells are slowly drying out. For biological relevance, the cells need to be in a constant liquid environment.

Figure 3-11. Topography of a cluster of 6 CHL cells. Scan speed was 0.8 lines/s. The cells were immersed in a small amount of PBS.

Figure 3-12. Height of cells in a cluster as a function of time between the measurement and preparation of the sample.
3.5.5 Shear force microscopy on living CHL cells

CHL cells are stored and cultured at 37°C. For imaging, the cells should also be at this temperature, however at present, the liquid cell and sample cannot be heated during imaging. The liquid cell can be heated up before the experiment. This will slow down the cooling of the sample. After the cells are placed inside the liquid cell and on top of the inverted microscope, the temperature will still drop to room temperature and eventually will cause the cells to retract their cell extensions and to detach from the surface as shown in Figure 3-13. The available time window for shear force measurements is still limited. Figure 3-13 shows that within half an hour, the cells are in a state unsuitable for shear force measurements.

Figure 3-13. Phase contrast microscope images of living cells in the liquid cell. Image (a) is recorded at t = 0, image (b) at t = 1 min, image (c) at t = 21 min and image (d) at t = 45 min. The retraction of the cell extensions is clearly visible. The round cells show little change, however, these are cells that already have a low attachment to the cover glass.
Living cells should be measured within less than half an hour, when using the open liquid cell. Unfortunately, during this time, the tuning-fork-based shear force system is not stable, due to temperature differences between the tuning fork and the liquid, and the drop in temperature of the liquid cell to room temperature. With continuous adjustments to the settings of the shear force feedback loop, shear force feedback system should be able to maintain a constant probe-to-sample distance for short time despite the change in temperature.

Figure 3-14 shows the topography of a living CHL cell, just after sample preparation. The horizontal lines are occasions where the feedback loop started to retract the probe away from the sample. Adjusting the feedback settings could prevent this retraction. Halfway during the acquisition of the image, the changes in temperature were such that the retraction of the probe could not be prevented anymore.

For imaging living cells, the requirements on the environment of the cell are such that the cell stays alive for a much longer time, allowing the tuning-fork-based shear force system to stabilize before obtaining the image. However, the results shown in Figure 3-14 also indicate that it is in principle possible to obtain the topography of living cells, using tuning-fork-based shear force microscopy, with the tuning fork fully immersed.

3.6 Conclusions

In conclusion, 100 kHz tuning forks have proved their capability as tip sample distance detectors in a liquid environment. Immersion experiments showed that a full immersion of both prongs of the tuning fork resulted in a partial recovery of the Q-factor. The vibrational mode of the tuning fork is conserved upon full immersion in water. The Q-factor is determined by the dominant viscous damping in water and has a typical value around 60. It is possible to obtain topography with good resolution and relative low interaction forces with both prongs of the tuning fork immersed in water. Reproducible topographic images have been obtained on annealed gold, cystospins and a cluster of cells. Obtaining the topography of living cells is in principle possible with tuning-fork-based shear force detection. However, for reliable and prolonged measurements on living cells a closed liquid cell with temperature control needs to be developed. Such a closed liquid cell not only allows a controlled temperature, but also prevents the evaporation of liquids.
3.7 References

13. Annealed gold sample kindly provided by D. Nikova, Biophysical techniques group, University of Twente, Enschede
14. Cytospin sample kindly provided by C.G. Figsor, Dept. of Tumor Immunology, University Medical Center, Nijmegen
16. Used coating material: Tuning fork coat, TM-Microscopes
18. Model number 1640-00 from ThermoMicroscopes.
19. Explorer from ThermoMicroscopes
This Chapter describes an electronic circuit that is used to actively influence the effective Q-factor of tuning forks used for shear force detection in order to improve the sensitivity. First, the scheme of such a circuit is introduced. The implemented circuit is modelled and analyzed in terms of signal-to-noise ratio and sensitivity. The model and simulations are valid for any force sensor that can be described by a harmonic oscillator. This leads to conclusions on where the application of such a circuit could be useful and where not.
4.1 Introduction

Tuning forks are sensitive force detectors, despite their high spring constant - in the order of 45 kN/m - because their Q-factor is very high, ranging from several hundreds to over one thousand in air. As described in Chapter 2, the sensitivity of a tuning fork to external force scales with the Q-factor. For application in biology, it is necessary to immerse the sample and thus the tuning fork in a buffer solution. Immersing these tuning forks in liquid results in a dramatic drop of the Q-factor, as described in Chapter 3. After full immersion, the Q-factor partially recovers\(^1\) to a value of around 60. This value is determined by the most important factor in the damping: the liquid. With this value, it is still possible to detect the topography on some samples. However, for fragile samples like living cells, the sensitivity is generally not high enough to prevent damage to tip or sample. In 1998, Anczykowski et al. introduced a scheme in the field of Atomic Force Microscopy (AFM), to increase the effective Q-factor of the AFM cantilever.\(^2\) This scheme was quickly adapted by other groups.\(^3-9\) In this Chapter, this scheme is adapted for application to tuning fork-based shear-force detection. To understand the implications of such a scheme on the fundamental limits of tuning-fork-based force detection, a numerical simulation is carried out to study the effects of both thermal noise and amplifier noise, as added by the circuit.

4.2 Principle of Q-factor enhancement

4.2.1 Harmonic oscillator

Figure 4-1 shows a damped harmonic oscillator. This oscillator is already introduced in Chapter 2. It consists of three elements, a mass \(m\), a spring \(k\) and a damper \(\gamma\). The position \(x(t)\) of the oscillator moves as a function of time \(t\), when the oscillator is driven through the spring with a displacement \(d(t)\).

The equation of motion for this damped harmonic oscillator is:

\[
mx + \gamma x + k(x - d) = 0
\]  

(4-1)

This damped harmonic oscillator is a model for the tuning fork as a force sensor, where \(m\) is the effective mass of the tuning fork with attached fiber, \(k\) is the spring constant of one of the tuning fork prongs and \(\gamma\) is the sum of the internal damping in the tuning fork and the damping from the outside world. The tuning fork is excited by displacing its base with \(d(t)\). The resulting
deflection of the end of one prong is equal to $x(t)$. The only variables in the equation of
motion with time dependence are $x$ and $d$. Assuming only harmonic time dependence,
\textit{i.e.} $x(t) = x \cdot e^{i\omega t}$, the ratio between the amplitude of the harmonic oscillator, $x$ and the
driving amplitude $d$ as a function of radial frequency $\omega$ can easily be found.

$$\frac{x}{d} = \frac{k}{m} \frac{1}{k \omega \gamma - \omega^2}$$  \hspace{1cm} (4-2)

At resonance, the amplitude $x$ has a maximum and the $\omega^2$ term cancels the $k/m$ term,
provided that the damping $\gamma$ is low. The $Q$-factor is now defined as:

$$Q \equiv \frac{\omega_{res}}{\Delta \omega_{FWHM}} = \left. \frac{x}{d} \right|_{\omega = \omega_{res}} = \frac{\sqrt{km}}{\gamma}$$  \hspace{1cm} (4-3)

Where $Q$ is the $Q$-factor, $\omega_{res}$ is the resonance frequency and $\Delta \omega_{FWHM}$ is the full width
of the resonance curve at half the maximum amplitude. The amplitude of the oscillator
depends inversely on the damping. For the harmonic oscillator described here with a
$Q$-factor much larger than 1, the $Q$-factor is equal to the ratio in amplitude of the
deflection and the drive. Changing the damping would result in a different amplitude of the
oscillator and thus in a different $Q$-factor.

Normally, it is difficult to lower the damping, as it is limited by the environment of the
tuning fork. Fortunately, there is an alternative, electronic, method to compensate for
the damping of the harmonic oscillator. Basically, this method consists of adding a
term proportional to the time derivative of $x$, \textit{i.e.} $g \dot{x}$, to the drive function which
changes the equation of motion to:

$$m \ddot{x} + (\gamma - g) \dot{x} + k(x - d) = 0$$  \hspace{1cm} (4-4)

From the equation of motion a new effective damping constant follows, equal to $\gamma - g$.
The effective $Q$-factor, for $Q > 1$, can now be defined:

$$Q_{eff} \equiv \frac{\omega_{res}}{\Delta \omega_{FWHM}} = \left. \frac{x}{d} \right|_{\omega = \omega_{res}} = \frac{\sqrt{km}}{|\gamma - g|}$$  \hspace{1cm} (4-5)

Increasing the constant $g$ to a value closer to $\gamma$, results in an larger effective $Q$-factor
and a negative $g$ results in a lower effective $Q$-factor.
4.2.2 Q-enhancement circuit

For tuning forks, compensation for the damping can be achieved using an electronic Q-factor enhancement circuit (Q-box). As the tuning fork is made of quartz, a piezo-electric material, the deflection is directly converted to an electric signal proportional to $x$. When the sinusoidal tuning fork signal is $90^\circ$ phase-shifted, the resulting signal is proportional to the time derivative of the original tuning fork signal. Amplifying the phase-shifted signal with an adjustable gain and adding this to the original drive signal allows to compensate for the damping. Figure 4-2 shows the circuit. The drive signal is applied to the dither piezo that mechanically excites the tuning fork. The tuning fork signal is amplified by a pre-amplifier. Apart from being used for the shear-force feedback electronics, the tuning fork signal is also phase-shifted, amplified and added to the drive signal.

As mentioned before, this circuit could be useful in situations where large damping prevents sensitive operation of tuning forks in a shear force detection scheme. This is the case when the tuning fork is immersed in water. The viscosity of water is $1 \cdot 10^{-3}$ Pa·s compared to a value of $1.3 \cdot 10^{-5}$ Pa·s in air. The viscosity of water is the dominant damping source when a tuning fork is immersed in water. The large damping caused by water prohibits a high value of the Q-factor. As a consequence, the sensitivity of the shear-force detection is impaired. The circuit described above, can restore the Q-factor to a value normal in air and should as a consequence enable a sensitive shear-force detection system.

Enhancing the Q-factor is only the first step to obtain a more sensitive operation of the shear force detection. Normally a relative change in amplitude or an absolute phase-shift is chosen as a setpoint for probe-to-sample interaction. The interaction force between the probe and sample scales linearly with the amplitude of the tip divided by the Q-factor. Enhancing the Q-factor without changing the drive amplitude will increase the tip amplitude with the same factor as the Q-factor and without changing the relative setpoint, Q-factor enhancement will leave the interaction force unaltered. Only after reducing the driving amplitude, or adjusting the setpoint, the circuit helps to reduce probe-to-sample interaction forces.
4.2.3 Q-factor enhancement experiment

To prove that the Q-factor can indeed be enhanced, the circuit is implemented and the resonance curve of a tuning fork immersed in water is obtained.

A tuning fork is mounted on a dither piezo to mechanically excite the tuning fork at a constant amplitude. The driving frequency is swept, while the piezoelectric tuning fork signal is recorded, resulting in the resonance curve of the tuning fork.

Figure 4-3 shows two resonance curves for the same tuning fork in water. The drive amplitude was the same in both cases. The first resonance curve, where the circuit was disabled, indicates a Q-factor of 60, the normal situation in water. The second resonance curve shows the effect of the Q-enhancement circuit, where the same tuning fork shows an effective Q-factor close to 1500. This value is arbitrarily chosen and could be higher or lower at will.

4.3 Thermal noise considerations

The model used so far does not incorporate the effects of noise. To obtain a better understanding of the fundamental limits of the Q-factor enhancement circuit, its behavior is simulated in the presence of thermal noise and detector noise. The circuit in Figure 4-2 will not only add a signal to the drive signal to compensate for the damping in the system, also thermal noise of the tuning fork and noise in the detection and amplification of the tuning fork signal will be added to the drive signal. Noise with a frequency close to the resonance of the tuning fork will be amplified in this system and could limit the performance of the circuit.
4.3.1 Thermal noise simulation model

The thermal noise of a tuning fork has been simulated, following the method described by De Grooth.\textsuperscript{10} A relative heavy particle, in this case a tuning fork prong, can move in one dimension. This particle is subject to many random collisions with surrounding molecules. The collisions have two effects. First, as there are more collisions from the direction in which the tuning fork moves, the collisions damp the motion of the tuning fork prong. This is the physical background of the damping term in the harmonic oscillator equation of motion (4-1). Second, as the number of collisions is finite in a finite time interval and the collisions are random, the net force on a stationary tuning fork will not be zero in a finite time interval. Because collisions occur from both sides of a tuning fork, the net result of the collisions in a time interval follows a binomial distribution, with zero average and a half width equal to the square root of half the collisions in that time interval. For a large number of collisions, the binomial distribution is approximated by a Gaussian distribution. In a given time interval $\Delta t$, large enough to have many collisions, but too short for a large change in velocity of the tuning fork prong, a random net force caused by thermal noise will act on the tuning fork.

$$F_{\text{thermal noise}} = w_n \sqrt{\frac{\gamma k_B T}{m \Delta t}}$$

(4-6)

Here $w_n$ is a random number representing the stochastic character of thermal noise with a Gaussian distribution with a zero average and a variance of 2, $k_B$ is Boltzmann’s constant and $T$ is the temperature. Now the numerical model is straightforward, the speed after $n$ time steps $\Delta t$ will be:

$$v_n = v_{n-1} + \frac{F_{\text{ext}}}{m} \Delta t + \frac{w_n}{m} \sqrt{\frac{\gamma k_B T}{\Delta t}}$$

(4-7)

Two contributions cause a change in speed. The first contribution is the external force corresponding to the harmonic oscillator model, including the force resulting from the drive function, spring and damper:

$$F_{\text{ext}} = k (x_{n-1} - d_{n-1}) + \gamma v_{n-1}$$

(4-8)

The second contribution to the change in speed is the force caused by the thermal noise that is acting on the tuning fork, described in equation (4-6). Now it is possible to calculate the new position of the tuning fork:
\[ x_n = x_{n-1} + \nu_n \Delta t \]  

(4-9)

The new position and velocity can be used again in equation (4-7) and (4-8) to calculate the velocity for the next time step. The choice of the size of the time step is a compromise between accuracy and calculation time. The time step should be small compared to the time of one cycle of the harmonic drive function.

The iterative process described in equations (4-7), (4-8) and (4-9) is implemented in LabView.\textsuperscript{12} For every cycle of the drive function, the RMS amplitude of the simulated tuning fork motion is calculated. The phase lag between the harmonic drive function and the tuning fork motion is calculated for every cycle of the drive function, by calculating the time difference in zero crossings of the harmonic drive function \( \Delta \) and the zero crossings of the position \( x \) of the tuning fork. Obviously, the phase lag can only be calculated if the tuning fork is driven with a periodic function.

### 4.3.2 Simulation parameters

A harmonic oscillator corresponding to a 100 kHz tuning fork is simulated with thermal noise corresponding to a temperature of 293 K according to the model described in § 4.3.1.

Table 4-1 gives an overview of parameters of the harmonic oscillator representing the tuning fork for four situations.

<table>
<thead>
<tr>
<th>Table 4-1. 100 kHz tuning fork thermal noise simulation parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k ) [10^3 N/m]</td>
</tr>
<tr>
<td>( m ) [10^-7 kg]</td>
</tr>
<tr>
<td>( \gamma ) [10^-4 Ns/( m )]</td>
</tr>
<tr>
<td>( g ) [10^-4 Ns/( m )]</td>
</tr>
<tr>
<td>( d ) [10^-14 m]</td>
</tr>
<tr>
<td>( Q_{\text{eff}} )</td>
</tr>
<tr>
<td>( x ) [10^-12 m]</td>
</tr>
</tbody>
</table>
4 Q-factor enhancement

1. $Q_{100}$: this harmonic oscillator represents a tuning fork with a natural Q-factor of 100 and a drive of 0.07 pm.

2. $Q_{10,10\times drive}$: this harmonic oscillator represents a tuning fork with a natural Q-factor of 10, however with a drive amplitude that is 10 times larger compared to the first case to obtain the same tip amplitude as a tuning fork with a natural Q-factor of 100.

3. $Q_{100, enh}$: this harmonic oscillator represents a tuning fork with a natural Q-factor of 10 that is enhanced to an effective Q-factor of 100. The drive amplitude is 0.07 pm, the same as for $Q_{100}$.

4. $Q_{10}$: this harmonic oscillator represents a tuning fork with a natural Q-factor of 10. The drive amplitude is 0.07 pm, the same as for $Q_{100}$ and $Q_{100, enh}$.

The spring constant $k$ and the mass $m$ are chosen to match a 100 kHz tuning fork, as described in § 2.3.1. The damping $\gamma$ and the compensation for the damping $g$ are chosen to realize the desired effective Q-factor of the model. A small driving amplitude is necessary to determine the phase, that is the phase lag between the position of the oscillator and the drive function. The drive amplitude is chosen small enough to ensure a noticeable effect of thermal noise on the motion of the harmonic oscillator. As a result, the amplitude of the oscillator $x$, is small compared to a normal tuning fork prong amplitude of 0.1-1 nm. The time step is chosen such that each cycle of the oscillator is simulated in 100 time steps. For a 100 kHz tuning fork, this implies a time step of 100 ns. After each cycle, the RMS amplitude and the phase of the oscillator are determined.

The simulation is run for $2^{15}$ (32768) cycles for the four situations described in Table 4-1, corresponding to $3.2768 \times 10^6$ time steps for each situation. Because the simulation method, described in § 4.3.1 is a finite time difference method, and because the simulation starts with the oscillator at zero position and zero velocity, the oscillator has to start up in the beginning of the simulation. The first 500 points of the data set are not used for further analysis, to prevent the start-up of the oscillator from influencing the statistics.

4.3.3 Harmonic oscillator amplitude signal with thermal noise

The harmonic oscillator model representing a tuning fork as described in § 4.3.1, is simulated for $Q_{100}$, $Q_{10,10\times drive}$, $Q_{100, enh}$ and $Q_{10}$. The resulting data set contains the amplitude and the phase of the simulated harmonic oscillator. For tuning-fork-based force detection, both the amplitude and the phase can be used as a measure for interaction force between probe and sample. In this paragraph, the influence of thermal noise on the amplitude of the simulated oscillator is discussed. In the next paragraph, the influence on the phase of the oscillator is discussed.
Figure 4-4 presents the amplitude of the simulated harmonic oscillator in three ways. Figure 4-4(a) shows the amplitude of the oscillator versus time. This is how the amplitude of a tuning fork is observed in an experiment. The feedback loop to control the probe-to-sample distance has a limited bandwidth in the order of 100 Hz to 1 kHz. Therefore, the lower frequency components of the amplitude signal are more important than the higher frequency components that are ignored by the feedback loop. The effect of thermal noise on the lower frequency components in the amplitude signal is illustrated by filtering the oscillator amplitude through a first order lowpass filter with a cutoff frequency of 100 Hz in Figure 4-4(b). Finally, Figure 4-4(c) shows a histogram of the distribution of all 32,268 amplitude points in Figure 4-4(a) using bins with a width of $1 \times 10^{-13}$ m.

![Graphical representation of the amplitude of a harmonic oscillator](image.png)

Figure 4-4. (a) Amplitude signal versus time of a harmonic oscillator representing a tuning fork experiencing thermal noise at 293 K for $Q_{100}$, $Q_{10,10xdrive}$, $Q_{100,\text{enh}}$ and $Q_{10}$. (b) 100 Hz lowpass filtered amplitude signal versus time. (c) Histogram for amplitude data in (a).

In Figure 4-4, it is clearly visible that the influence of thermal noise on the amplitude of the harmonic oscillator is different for different $Q$-factors:

- For $Q_{10}$, the average amplitude is 0.73 pm. In all other cases ($Q_{100}$, $Q_{10,10xdrive}$ and $Q_{100,\text{enh}}$) the average amplitude is about 7 pm. The RMS noise on the amplitude is $\sim 0.2$ pm for oscillators where the $Q$-factor is not enhanced, independent of the drive amplitude. $Q_{100,\text{enh}}$ has a larger RMS noise of 0.7 pm, despite the same effective $Q$-factor and drive amplitude as for $Q_{100}$.

- All oscillators associated with a natural $Q$-factor, *i.e.* where the $Q$-factor is not enhanced, have approximately the same RMS amplitude. Because the thermal energy in a harmonic oscillator is coupled via the spring constant to the RMS
amplitude of the oscillator: \( \frac{1}{2} k <x^2>^{1/2} = \frac{1}{2} k_B T \), where \( k_B \) is Boltzmann’s constant, it can be expected that thermal noise of the oscillator is associated with a typical amplitude of the oscillator. The RMS value of the noise, equal to the standard deviation of the simulated amplitude values, is \( \sim 0.2 \) pm. This is in reasonable agreement with the value of \( \sim 0.3 \) pm for a tuning fork with a spring constant of 47 kN/m, found by Grober et al.\(^{1,13}\)

- \( Q_{100, \text{enh}} \) shows a broader amplitude distribution compared to oscillators where the Q-factor is not enhanced. An explanation for the increase in noise for the Q-factor enhanced oscillator can be found in equation (4-6). The force acting on the system by thermal fluctuations depends on \( \gamma \). The compensation for the damping \( g \) does not change the number of collisions and the stochastic term associated with it. The stochastic thermal noise term has no phase relation with the driving function and this term will be amplified by the Q-factor enhancement circuit.

- From Figure 4-4(b), it is clear that filtering eliminates most of the noise. However, more noise is filtered out for a harmonic oscillator without Q-factor enhancement. This can be explained by the fact that the harmonic oscillator has a response time in the order of \( Q/\omega_{\text{res}} \), where \( \omega_{\text{res}} \) is the resonance frequency of the oscillator. Thermal noise experienced by the oscillator with frequencies lower than \( \omega_{\text{res}}/Q \) will be amplified by the Q-factor enhancement circuit, while higher frequencies will not. Another difference with the unfiltered data is that in Figure 4-4(a) the width of the distribution for \( Q_{100} \) and \( Q_{10} \) was the same. After passing the signals through a lowpass filter, the width of the amplitude distribution in Figure 4-4(b) is smaller for \( Q_{10} \). A closer look at the amplitude signals in the frequency domain can explain this.

\[ \text{Figure 4-5. The gray line corresponds to a typical power spectrum of harmonic oscillator amplitude signal of a harmonic oscillator representing a tuning fork experiencing thermal noise at 293 K. The black line is a fit corresponding to a second order system.} \]

Figure 4-5 shows a typical noise spectrum of the amplitude signal of a harmonic oscillator. The spectrum is calculated by taking the Fourier transform of the amplitude signal with the average value of the amplitude subtracted. The nonzero average does not contribute to the spectrum, however due to the limited simulated time (0.33 s), it causes a windowing effect. Another effect of limited simulation time is that the spectrum appears ‘noisy.’ If the simulation would have been run
for a longer time, the spectrum would have better resembled the presented fit of the spectrum. For clarity, only similar fits are shown for the spectra in the rest of this Chapter.

The power spectra of the amplitude signals in Figure 4-4(a) are given in Figure 4-6. As thermal noise has a uniform (white) spectrum it can be used to probe the frequency response of the amplitude signal of the harmonic oscillator to stochastic force. The amplitude signal of a harmonic oscillator behaves like an integrator, with a cutoff frequency of \( f_{\text{res}}/Q \) and a slope of -2 above the cutoff frequency. When the different oscillators \( Q_{100}, Q_{10,10\times\text{drive}} \), \( Q_{100, \text{enh}} \), and \( Q_{10} \) are compared, a number of differences become clear:

- For systems without Q-factor enhancement, the energy content of the oscillator should be equal to \( \frac{1}{2} k_B T \), independent of the Q-factor. For lower Q-factors this means that the energy will be distributed over a larger part of the amplitude spectrum compared to systems with a higher Q-factor. This explains why a lowpass filter blocks more thermal noise for systems with a low Q-factor. At the same time, these systems have a larger bandwidth and a lowpass filter narrows the available bandwidth.

- The power spectra of the amplitude signal of the oscillators is independent of the drive amplitude. The spectra for \( Q_{10,10\times\text{drive}} \) and \( Q_{10} \) overlap. The drive amplitude only changes the average amplitude of a harmonic oscillator, which only changes the spectrum at zero frequency.

- \( Q_{100, \text{enh}} \) shows the same cutoff frequency as \( Q_{100} \), indicating that the effective Q-factor of \( Q_{100, \text{enh}} \) is indeed 100. However, the noise is at all frequencies a factor of 10 higher than for \( Q_{100} \), the oscillator with a natural Q-factor of 100. For frequencies above the cutoff frequency, the performance of \( Q_{100, \text{enh}} \) is the same as the performance of \( Q_{10} \), the not enhanced oscillator. For frequencies below the cutoff frequency, the thermal noise is up to 100 times amplified compared to \( Q_{10} \).
Another way to look at thermal noise is calculating the noise equivalent dissipative force. As described in Chapter 2, a change in amplitude can be associated with a dissipative force on the oscillator:

$$F_{int} = \frac{k}{Q} \Delta x$$  \hspace{1cm} (4-10)

$F_{int}$ is the dissipative interaction force between the oscillator and the external world. $\Delta x$ is the change in amplitude. Thermal noise results in a change in amplitude. With relation (4-10) the thermal noise can be converted into an equivalent force. Table 4-2 gives an overview of the effects of thermal noise on harmonic oscillators with and without Q-factor enhancement.

<table>
<thead>
<tr>
<th></th>
<th>1 $Q_{100}$</th>
<th>2 $Q_{10,10\text{drive}}$</th>
<th>3 $Q_{100,\text{enh}}$</th>
<th>4 $Q_{10}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q-factor</td>
<td>100</td>
<td>10</td>
<td>10 (100)$^a$</td>
<td>10</td>
</tr>
<tr>
<td>Drive amplitude[10$^{-14}$ m]</td>
<td>7.07</td>
<td>70.71</td>
<td>7.07</td>
<td>7.07</td>
</tr>
<tr>
<td>Average amplitude[10$^{-12}$ m]</td>
<td>6.95</td>
<td>6.94</td>
<td>6.94</td>
<td>0.73</td>
</tr>
<tr>
<td>RMS amplitude noise</td>
<td>0.21</td>
<td>0.20</td>
<td>0.67</td>
<td>0.19</td>
</tr>
<tr>
<td>Signal-to-noise ratio</td>
<td>33.1</td>
<td>34.7</td>
<td>10.4</td>
<td>3.8</td>
</tr>
<tr>
<td>Equivalent force[10$^{-12}$ N]</td>
<td>95.0</td>
<td>907</td>
<td>300</td>
<td>867</td>
</tr>
<tr>
<td>Average amplitude[10$^{-12}$ m]</td>
<td>6.95</td>
<td>6.94</td>
<td>6.94</td>
<td>0.73</td>
</tr>
<tr>
<td>RMS amplitude noise</td>
<td>0.022</td>
<td>0.24</td>
<td>0.020</td>
<td>0.020</td>
</tr>
<tr>
<td>Signal-to-noise ratio</td>
<td>93.9</td>
<td>315</td>
<td>28.9</td>
<td>36.5</td>
</tr>
<tr>
<td>Equivalent force[10$^{-12}$ N]</td>
<td>33.1</td>
<td>98.2</td>
<td>109</td>
<td>90.2</td>
</tr>
</tbody>
</table>

$^a$ With Q-factor enhancement

To judge the sensitivity of a harmonic oscillator with and without Q-factor enhancement, the noise equivalent force should be compared. In the ultimate tuning-fork-based shear force detection system, the force detection is limited by thermal noise. Over the full bandwidth, the Q-factor enhancement improves on the noise equivalent force. This force is with 300 pN still higher compared to a natural Q-factor of 100 with a noise equivalent force of 95 pN, but lower than the original force of 867 pN. In the frequency band that is used for shear-force feedback however, the Q-factor enhancement circuit results in a slightly larger noise equivalent force compared to
Thermal noise considerations

when the Q-factor is not enhanced. This indicates that there is no gain in sensitivity by enhancing the Q-factor, when the amplitude of a harmonic oscillator is used to control the shear-force interaction in a feedback loop.

4.3.4 Harmonic oscillator phase signal with thermal noise

In § 4.3.3, only the influence of thermal noise on the amplitude of a harmonic oscillator was studied. The amplitude of the tuning fork signal can be used as a measure of the probe to sample distance. However, the phase of the tuning fork signal with respect to the drive signal has a faster response to a change in probe-to-sample distance.\textsuperscript{14,15} For virtually all experiments described in this thesis, the phase of the tuning fork signal was used. Analogous to § 4.3.3, the behavior of the phase with respect to the drive of a harmonic oscillator experiencing thermal noise will be explored and compared for $Q_{100}$, $Q_{10,10,\text{drive}}$, $Q_{100,\text{enh}}$ and $Q_{10}$.

![Figure 4-7](image_url)

Figure 4-7. (a) Phase signal versus time of a harmonic oscillator representing a tuning fork experiencing thermal noise at 293 K for $Q_{100}$, $Q_{10,10,\text{drive}}$, $Q_{100,\text{enh}}$ and $Q_{10}$. (b) 100 Hz lowpass filtered phase signal versus time. (c) Histogram for phase data in (a).

Figure 4-7(a) shows time traces of the phase of the simulated harmonic oscillators, Figure 4-7(b) shows the time traces of the phase, filtered through a 100 Hz lowpass filter and the histogram in Figure 4-7(c) shows the distributions of the phase signals, where now all four situations can be distinguished.

The following differences for the behavior of the phase signals of the different harmonic oscillators can be noticed:
The noise in the phase of $Q_{10}$ in Figure 4-7(a) is by far larger than the noise of oscillators with a higher Q-factor or larger drive amplitude. The reason for this difference is that the phase is determined by timing the zero-crossings of the position of the oscillator with respect to the zero-crossings of the drive function. At small oscillator amplitudes, the noise can cause zero-crossings that are inadvertently used by the phase detector resulting in a large noise.

The histograms for $Q_{100}$ and $Q_{10,10\times\text{drive}}$ in Figure 4-7(c) are similar. They have approximately the same height and width, however their peaks are slightly shifted. This can be attributed to the fact that the resonance frequency of a harmonic oscillator changes slightly with a change in the damping. Around resonance, the phase has its strongest dependence on frequency and a small variation in resonance frequency causes a large variation in the phase. The shifts observed here are smaller than the width of the distribution and have no influence on the conclusions.

The histogram of $Q_{10}$ has a wide distribution of values for the phase. The histogram of $Q_{100,\text{enh}}$ has a narrower distribution, but this distribution is still wider than the distribution for $Q_{100}$ and $Q_{10,10\times\text{drive}}$ due to the amplitude of thermal noise.

As with noise on the amplitude of a harmonic oscillator used as a shear-force detector, the frequency content of the thermal noise in the phase signal is important. Therefore, Figure 4-7(b) shows the phase signals passed through a lowpass filter with a cutoff frequency of 100 Hz. The distribution of the phase signal of $Q_{10}$ has become comparably narrow as the distribution of phase signal of $Q_{100}$ and $Q_{10,10\times\text{drive}}$. Now, the phase signal of $Q_{100,\text{enh}}$ has the widest distribution.

As in § 4.3.3, the power spectra of the phase signals explain the dependence of the observed noise on the frequency band that is used to detect the noise. The power spectra are plotted in Figure 4-8.

Figure 4-8 shows that when the phase signal is considered to be a dynamic signal, the phase of a harmonic oscillator has the response of a second order system to a stochastic force. For frequencies below $f_{\text{res}}/Q$, the power density
does not depend on the frequency. At higher frequencies, the power density drops with a slope of -2. As there is no thermal energy associated with the phase signal, the total amount of noise does not have to be the same for different systems. This is indeed not the case in Figure 4-8. The phase signal of $Q_{100}$ and $Q_{10,10^{x}}$ have similar signal energies, but distributed differently over the spectrum: $Q_{10,10^{x}}$ has a broader frequency distribution. The spectral power density of the noise on the phase signal of $Q_{10}$ is up to two orders of magnitude larger than the noise on the phase signal of oscillators with a larger amplitude, especially for frequencies above the cutoff frequency.

Although the phase signals cannot be associated with a thermal energy, it is possible to calculate the conservative force necessary to change the phase of the oscillator with respect to the drive with the same value as the thermal noise on the phase:

$$F_{m}=\frac{k_{x}}{Q}\Delta \phi$$  \hspace{1cm} (4-11)

Where $\Delta \phi$ is the change in phase in radians. It is now possible to compare the behavior of the harmonic oscillators not only in terms of noise on the phase signal, but also the noise in terms of equivalent forces. The comparison can be found in Table 4-3.

**Table 4-3. Thermal noise data for the phase of harmonic oscillators with and without Q-factor enhancement**

<table>
<thead>
<tr>
<th></th>
<th>1 $Q_{100}$</th>
<th>2 $Q_{10,10^{x}}$</th>
<th>3 $Q_{100,\text{enh}}$</th>
<th>4 $Q_{10}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q-factor</td>
<td>100</td>
<td>10</td>
<td>10 (100)$^a$</td>
<td>10</td>
</tr>
<tr>
<td>Drive amplitude[10$^{-14}$ m]</td>
<td>7.07</td>
<td>70.71</td>
<td>7.07</td>
<td>7.07</td>
</tr>
<tr>
<td>Average phase[deg]</td>
<td>-90.0</td>
<td>-88.4</td>
<td>-89.4</td>
<td>-87.9</td>
</tr>
<tr>
<td>RMS phase noise[deg]</td>
<td>1.7</td>
<td>1.8</td>
<td>5.4</td>
<td>19</td>
</tr>
<tr>
<td>Equivalent force[10$^{-12}$ N]</td>
<td>91.2</td>
<td>955</td>
<td>295</td>
<td>1077</td>
</tr>
<tr>
<td>Filtered</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average phase[deg]</td>
<td>-90.0</td>
<td>-88.4</td>
<td>-89.4</td>
<td>-87.9</td>
</tr>
<tr>
<td>RMS phase noise[deg]</td>
<td>0.58</td>
<td>0.18</td>
<td>1.8</td>
<td>2.0</td>
</tr>
<tr>
<td>Equivalent force[10$^{-12}$ N]</td>
<td>31.7</td>
<td>99.1</td>
<td>98.2</td>
<td>114</td>
</tr>
</tbody>
</table>

$a$. With Q-factor enhancement

For the full frequency spectrum, the Q-factor enhancement improves the sensitivity of a harmonic oscillator. Yet, a natural Q-factor with the same value still results in a higher sensitivity. The increased sensitivity can be explained by the fact that at a larger
amplitude, it is easier to accurately determine the zero-crossing of the harmonic oscillator signal. In the frequency range used by the feedback loop, the enhancement does not improve the sensitivity.

The thermal noise equivalent force is approximately the same for both the thermal noise on the amplitude signal and the thermal noise on the phase signal of the harmonic oscillator. When tuning-fork-based shear force detection is limited by thermal noise, the sensitivity does not depend on the choice between amplitude and phase of the tuning fork as a measure for probe-to-sample interaction force.

For a harmonic oscillator used as a force sensor in SPM, where the amplitude or the phase of the oscillator is a measure for the probe-to-sample interaction force, Q-factor enhancement helps reducing the noise equivalent force over the full frequency spectrum. However, for low frequencies, important for probe-to-sample distance control, Q-factor enhancement does not improve the sensitivity, because the positive effect of a higher Q-factor is eliminated by the amplification of thermal noise at frequencies below the cutoff frequency $f_{\text{res}}/Q$.

4.4 Detector noise considerations

The tuning fork signal is amplified by a pre-amplifier. Besides the thermal noise acting on the tuning fork, the wires connecting to the pre-amplifier and the pre-amplifier itself add noise to the tuning fork signal. This type of noise is referred to as the detector noise. The fundamental difference between this type of noise and the thermal noise is that the thermal noise is caused by a force acting on the tuning fork and the properties of the tuning fork change the way the it responds to this force. In contrast, the detector noise is added to the signal as obtained from the tuning fork. For situations where the Q-factor is not enhanced, the noise is independent of the Q-factor or the drive amplitude. With Q-factor enhancement, the noise passes through the phase-shifter and amplifier and is added to the drive signal. This will have consequences for the tuning fork behavior.

4.4.1 Detector noise simulation model

To simulate a harmonic oscillator representing a tuning fork with detector noise present, the model developed in § 4.3.1 will be modified. To study solely the effects of detector noise, the temperature in the simulation will be set to zero. As detector noise acts on the signal representing the position of the harmonic oscillator, equation (4-9) will change to:
where $s$ is a random number with a Gaussian distribution, zero average and a variance of 1 and $N_{\text{det}}$ is the RMS noise in meters added by the amplifier. In the calculations $N_{\text{det}} = 1.0 \cdot 10^{-11}\text{m}$ is chosen for the detector noise. This type of detector noise will have a uniform spectral density (shot noise). Generally, for amplifiers used to amplify tuning fork signals, the assumption that the amplifier noise has a uniform spectral density for the frequency band of interest is valid. As the exact spectral content of the noise depends on the sources of the noise, i.e. the specific properties of the amplifiers and wires used, the outcome in an actual experiment can be slightly different from the simulated results presented here, however, the conclusions drawn will still be valid.

Four different situations are simulated:

1. $Q_{100}$: With a natural $Q$-factor of 100 and a RMS drive amplitude of $7.07 \cdot 10^{-13}\text{m}$.
2. $Q_{10,10\times \text{drive}}$: With a natural $Q$-factor of 10 and a RMS drive amplitude of $7.07 \cdot 10^{-12}\text{m}$.
3. $Q_{100, \text{enh}}$: With a natural $Q$-factor of 10, enhanced to a $Q$-factor of 100 and a RMS drive amplitude of $7.07 \cdot 10^{-11}\text{m}$.
4. $Q_{10}$: With a natural $Q$-factor of 10 and a RMS drive amplitude of $7.07 \cdot 10^{-13}\text{m}$.

All other simulation parameters can be found in § 4.3.1 and § 4.3.2. These are the same parameters as in § 4.3, except for a 10 times larger drive. The larger drive is chosen to match the amplitude of the oscillator with the chosen amplitude of the noise.

The choice for both amplitude of the noise and amplitude of the drive are somewhat arbitrary. However, the conclusions for the comparison of harmonic oscillators with and without $Q$-factor enhancement are independent of the amplitude of the noise and of the drive.

### 4.4.2 Harmonic oscillator amplitude signal with detector noise

The simulation is run for the four different situations described in the previous paragraph. To eliminate undesired effects of the start-up of the harmonic oscillator, the first 500 points of the simulation are omitted. In this paragraph, the amplitude signal as obtained from the harmonic oscillator simulation is analyzed. Figure 4-9 shows the amplitude versus time of a harmonic oscillator experiencing detector noise for $Q_{100}$, $Q_{10,10\times \text{drive}}$, $Q_{100, \text{enh}}$ and $Q_{10}$. 

\begin{equation}
x_n = x_{n-1} + v_n \Delta t + s_n N_{\text{det}}
\end{equation}
Figure 4-9(a) shows the time trace of the amplitude of the harmonic oscillators experiencing detector noise. Figure 4-9(b) shows the same time traces, but now filtered through a first order 100 Hz lowpass filter. A histogram of the amplitudes in Figure 4-9(a) is presented in Figure 4-9(c).

When the different oscillators, $Q_{10,10\times\text{drive}}$, $Q_{100,\text{enh}}$ and $Q_{100}$ are compared, a number of differences become clear:

- The RMS noise of $Q_{100,\text{enh}}$ is larger compared to situations where the Q-factor is not enhanced, because some of the detector noise is amplified by the Q-factor enhancement circuit.

- As there is no kinetic energy associated with this type of noise, there is no limitation for higher frequency components in the detector noise. These higher frequency components are more prominently present in the time traces compared to the time traces for thermal noise in Figure 4-4. This is somewhat different for $Q_{100,\text{enh}}$, where the time trace resembles the corresponding time trace in Figure 4-4 more. The more prominent presence of low frequency components can be explained by the amplification of frequency components of the detector noise below the cutoff frequency of the oscillator.

- The RMS noise shown in Figure 4-9(a) and (c) for $Q_{10}$ is slightly smaller compared to $Q_{10,10\times\text{drive}}$. This can be explained by the fact that the RMS noise is comparable to the average amplitude of the oscillator. For most of the time, the position of the oscillator is close to zero. The noise causes fluctuations around the zero position of
the oscillator. When calculating the RMS values of the position, the negative positions, become positive, narrowing the distribution of positions of the oscillator.

- The large difference in detector noise before and after filtering through a 100 Hz lowpass filter in Figure 4-9 is a confirmation of the earlier observation that detector noise adds more high frequency components to the harmonic oscillator amplitude signal than the thermal noise does. Most of these high frequency components can be filtered out by a lowpass filter. After filtering, the relative difference between harmonic oscillators with and without Q-factor enhancement has become larger. This can be explained by the fact that only low frequency components will be amplified by the Q-factor enhancement circuit in combination with the harmonic oscillator.

The amplification of low frequency components by Q-factor enhancement will even be more obvious in the power spectrum of the amplitude signals in Figure 4-9(a). The power spectrum is shown in Figure 4-10.

Figure 4-10 confirms the previous observations. Power spectra of amplitude signals from harmonic oscillators without Q-factor enhancement show a uniform (flat) spectrum. This can be explained by the fact that detector noise is added to the harmonic oscillator position afterwards and there is no energy associated with this type of noise. For a harmonic oscillator with Q-factor enhancement, the spectrum is the same for frequencies higher than $f_{\text{res}}/Q$, while for lower frequencies the detector noise is amplified by the Q-factor enhancement circuit. As for thermal noise, the noise terms can be calculated in terms of equivalent dissipative force. Table 4-4 summarizes the results.

As in § 4.3.3, the Q-factor enhancement gives a better force resolution for the full frequency range. The noise equivalent force of 0.693 nN for $Q_{100,\text{enh}}$ is almost an order of magnitude smaller than for $Q_{10}$. However, for $Q_{100}$, the noise equivalent force is still lower (0.464 nN). In the frequency range of importance for probe-to-
4 Q-factor enhancement

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q-factor</td>
<td>100</td>
<td>10</td>
<td>10 (100)(^a)</td>
<td>10</td>
</tr>
<tr>
<td>Drive amplitude([10^{-13} \text{ m}])</td>
<td>7.07</td>
<td>70.71</td>
<td>7.07</td>
<td>7.07</td>
</tr>
<tr>
<td>Average amplitude([10^{-12} \text{ m}])</td>
<td>70.13</td>
<td>70.10</td>
<td>71.22</td>
<td>12.14</td>
</tr>
<tr>
<td>RMS amplitude noise ([10^{-12} \text{ m}])</td>
<td>1.03</td>
<td>1.03</td>
<td>1.54</td>
<td>0.808</td>
</tr>
<tr>
<td>Signal-to-noise ratio</td>
<td>68.1</td>
<td>68.0</td>
<td>46.2</td>
<td>15.2</td>
</tr>
<tr>
<td>Equivalent force([10^{-9} \text{ N}])</td>
<td>0.464</td>
<td>4.64</td>
<td>0.693</td>
<td>3.64</td>
</tr>
</tbody>
</table>

\(a\). With Q-factor enhancement

Sample distance control, the noise equivalent force is approximately the same for \(Q_{100, \text{enh}}\) and \(Q_{10}\). The performance of the Q-factor enhancement circuit does not improve the noise equivalent force. In this frequency range, the difference in noise equivalent force between \(Q_{100}\) and \(Q_{100, \text{enh}}\) is more dramatic with 22.2 pN and 147 pN respectively.

4.4.3 Harmonic oscillator phase signal with detector noise

Detector noise will also affect the detection of the phase lag between the drive signal and the tuning fork signal. The detection of this phase lag, is based on timing the zero crossings of the tuning fork and the zero crossing of the drive signal. Noise results in incorrect detection of a zero crossing. As the phase lag between tuning fork signal and drive signal is normally used as a measure for the probe to sample interaction, the importance of understanding the influence of detector noise on the phase detection is evident. Figure 4-11 shows the phase lag versus time for the four situations already analyzed for amplitude signals in the previous paragraph.

Figure 4-11(a) shows time traces of the phase for \(Q_{100}\), \(Q_{10, \text{10xdrive}}\), \(Q_{100, \text{enh}}\), and \(Q_{10}\). Figure 4-11(b) is the 100 Hz lowpass filtered and Figure 4-11(c) shows a histogram of the phase data in Figure 4-11(a).
Detector noise considerations

For the time traces of the phase, the following observations can be made:

- All but one of the time traces that are plotted in Figure 4-11(a) have approximately the same RMS noise on the phase signal. These belong to harmonic oscillators with the same amplitude of \(7 \times 10^{-11} m\). The RMS noise on the phase signal is higher for \(Q_{10}\). This can be explained by the effect that phase detection is based on measuring the zero-crossings and for lower amplitudes it is more difficult to determine a zero crossing. The histograms of the phase signals plotted in Figure 4-11(c) provide a better insight in the amount of noise on these signals.

- The histograms in Figure 4-11(c), show the distributions of detector noise in Figure 4-11(a) more clearly. Except for \(Q_{10}\), the width of the histograms are the same, independent on the \(Q\)-factor and the use of the \(Q\)-factor enhancement circuit. The only important factor in the amount of noise, is the amplitude of the oscillator.

- In Figure 4-11(b), the RMS noise on the phase signals is substantially lower after filtering. The phase signal of the harmonic oscillator with a low drive amplitude still has the largest RMS noise. However, the RMS noise on the phase of the harmonic oscillator with \(Q\)-factor enhancement is now of the same order.

Figure 4-11. (a) Phase signal versus time of a harmonic oscillator representing a tuning fork experiencing detector noise for \(Q_{100}\), \(Q_{10, 10}\) driver, \(Q_{100, enh}\) and \(Q_{10}\). (b) 100 Hz lowpass filtered phase signal versus time. (c) Histogram for phase data in (a).
Taking the Fourier transforms of the phase signals give the power spectra shown in Figure 4-11. The power spectra confirm that the phase signal power density is independent of the frequency. The power density of the phase signal depends only on the amplitude of the harmonic oscillator.

To make a fair comparison between the various situations, the noise equivalent conservative force can again be calculated. Table 4-5 gives an overview of the effects of detector noise on the phase lag between the harmonic oscillator signal and the drive signal.

Table 4-5. Detector noise data for harmonic oscillators with and without Q-factor enhancement

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3 (100)(^a)</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q-factor</td>
<td>100</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Drive amplitude([10^{-13} \text{ m}])</td>
<td>7.07</td>
<td>70.71</td>
<td>7.07</td>
<td>7.07</td>
</tr>
<tr>
<td>Average phase([\text{deg}])</td>
<td>-90.0</td>
<td>-88.4</td>
<td>-89.2</td>
<td>-88.9</td>
</tr>
<tr>
<td>RMS phase noise([\text{deg}])</td>
<td>3.1</td>
<td>3.1</td>
<td>3.2</td>
<td>11</td>
</tr>
<tr>
<td>Equivalent force([10^{-9} \text{ N}])</td>
<td>1.73</td>
<td>17.3</td>
<td>1.81</td>
<td>10.6</td>
</tr>
<tr>
<td>Average phase([\text{deg}])</td>
<td>-90.0</td>
<td>-88.4</td>
<td>-89.2</td>
<td>-88.9</td>
</tr>
<tr>
<td>RMS phase noise([\text{deg}])</td>
<td>0.14</td>
<td>0.12</td>
<td>0.37</td>
<td>0.49</td>
</tr>
<tr>
<td>Equivalent force([10^{-9} \text{ N}])</td>
<td>0.0774</td>
<td>0.685</td>
<td>0.208</td>
<td>0.467</td>
</tr>
</tbody>
</table>

\(\text{a. With Q-factor enhancement}\)

From the noise equivalent forces in Table 4-5 it follows that Q-factor enhancement helps in improving the sensitivity. For the full frequency range, the noise equivalent force for \(Q_{100}\) and \(Q_{100, \text{enh}}\) is about the same. After filtering, the phase signal of \(Q_{100}\) has a lower noise equivalent force. Still, \(Q_{100, \text{enh}}\) has a lower noise equivalent force.
compared to $Q_{10}$. This is because the detection of a zero crossing becomes more accurate at a higher amplitude. It should be noted that the noise equivalent force would be lower if the phase would be determined with a more sophisticated technique, for example a lock-in amplifier, where the measurement of the phase is not solely dependent on the zero crossing of the harmonic oscillator signal.

When limited by detector noise, $Q$-factor enhancement does not improve the sensitivity in the frequency range used for probe-to-sample distance control, if the amplitude of a harmonic oscillator is used as a measure for probe-to-sample interaction. When the phase of the oscillator is used, $Q$-factor enhancement improves the sensitivity because of a larger oscillator amplitude. However, using a more sophisticated method to detect the phase eliminates the advantage of $Q$-factor enhancement.

### 4.5 Noise measurements

When the noise on the amplitude or phase signal is measured for a real system, it becomes hard to discriminate thermal noise from detector noise, as the observed noise will be a combination of the two. In most experimental cases, the detector noise is the dominant source of noise. To verify the results obtained from the simulations, a tuning fork with a fiber attached and a $Q$-factor of 100 is excited at resonance. The tuning fork amplitude is recorded for 2 s with time steps of 4 ms. The amplitude was chosen to be of the same order as the observed noise, which was dominated by detector noise.

![Detector noise as observed from a tuning fork with a natural Q-factor of 100. The amplitude versus time is shown for the tuning fork without Q-factor enhancement, with the Q-factor enhanced to 300 and 2000.](image)

Figure 4-13. Detector noise as observed from a tuning fork with a natural Q-factor of 100. The amplitude versus time is shown for the tuning fork without Q-factor enhancement, with the Q-factor enhanced to 300 and 2000.
After obtaining the amplitude for the tuning fork with a natural Q-factor of 100, the Q-factor is subsequently enhanced to a value of 300 and 2000. Figure 4-13 shows the effects of the Q-factor enhancement circuit. If the Q-factor is enhanced to 2000, the noise becomes large, especially the low frequency components. This is better visible in the power spectra of the amplitude signals in Figure 4-14.

The spectra of the signals are uniform, which would confirm that the dominant type of noise is detector noise. However, only the cutoff frequency of the tuning fork with an enhanced Q-factor of 2000 falls within the frequency range of the spectrum. To analyze if the extra noise for the higher effective Q-factors is compensated by the extra sensitivity associated with a higher Q-factor, the noise equivalent forces are calculated in Table 4-6.

**Table 4-6. Detector noise data for harmonic oscillators with and without Q-factor enhancement**

<table>
<thead>
<tr>
<th></th>
<th>1</th>
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</thead>
<tbody>
<tr>
<td>Q-factor</td>
<td>100</td>
<td>300</td>
<td>2000</td>
</tr>
<tr>
<td>Average amplitude[V]</td>
<td>0.1440</td>
<td>0.4459</td>
<td>2.915</td>
</tr>
<tr>
<td>RMS amplitude noise[V]</td>
<td>0.00030</td>
<td>0.00087</td>
<td>0.026</td>
</tr>
<tr>
<td>Signal-to-noise ratio</td>
<td>480</td>
<td>512</td>
<td>112</td>
</tr>
<tr>
<td>Equivalent force[nN]</td>
<td>0.135</td>
<td>0.131</td>
<td>0.585</td>
</tr>
</tbody>
</table>

Table 4-6, shows that the Q-factor enhancement does not improve the sensitivity of the tuning fork based shear force detection in terms of noise equivalent force. Enhancing the Q-factor from 100 to 300, the noise equivalent force does not change. Enhancing the Q-factor to a value of 2000 results in a larger noise equivalent force than for the
tuning fork with a natural Q-factor of 100. This can be explained by the fact that for large enhancements of the Q-factor, the Q-factor enhancement circuit is more likely to become instable.

Table 4.7. Sensitivity of Q-factor enhancement to deviations in the compensation for the damping.

<table>
<thead>
<tr>
<th>Q-factor enhancement</th>
<th>1% extra compensation</th>
<th>1% extra compensation</th>
<th>extra compensation resulting in instability (Q-factor→∞)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q-factor: 100→300</td>
<td>Q = 306</td>
<td>2%</td>
<td>50.0%</td>
</tr>
<tr>
<td>Q-factor: 100→2000</td>
<td>Q = 2469</td>
<td>23%</td>
<td>5.3%</td>
</tr>
</tbody>
</table>

The effective Q-factor is inversely dependent on the difference between the damping $\gamma$ and the compensation for the damping $g$. When the difference ($\gamma - g$) is small compared to the damping $\gamma$, a small change in the compensation $g$ results in a large change in effective Q-factor. This is the case when the effective Q-factor is enhanced to high values. Table 4.7 shows the effect of a small, 1%, change in compensation for the damping, for the effective Q-factors used in the measurements. The Table also shows how much extra compensation is necessary to make the compensation equal to the damping, resulting in an infinite effective Q-factor and an instable system. For strongly enhanced Q-factors, this noise and drift in the Q-factor enhancement circuit will limit the performance and sensitivity of the shear force detection system.

4.6 Conclusions

The simulations are done for harmonic oscillators as force sensors. The results are valid for any force sensor that can be described by a harmonic oscillator. From simulations and measurements, it follows that the Q-factor enhancement circuit does increase the effective Q-factor. The sensitivity however, is still limited by thermal noise or detector noise. The Q-factor enhancement does in principle not improve the performance or sensitivity of a harmonic oscillator used for force detection, such as shear force detection with tuning forks. Effectively, the same noise is present at a higher amplitude of the tuning fork.

There can be a practical advantage, if the amplitude of the tuning fork signal is detected using a RMS detector, with a large bandwidth, the higher amplitude of the Q-factor enhanced tuning fork signal results in a better signal to noise ratio. When the phase lag between the tuning fork signal and the drive function is used as a measure for the shear force interaction between probe and sample, the Q-factor enhancement can increase the signal-to-noise ratio, if the phase detection is based on timing the zero-crossing of the tuning fork signal. With more advanced detection electronics, e.g. a
lock-in amplifier, the signal can be discriminated better from the noise. Increasing the time constant of a lock-in amplifier allows a more sensitive operation of the shear force detection compared to the use of Q-factor enhancement, as it eliminates more high frequency noise and it does not amplify low frequency noise, as the Q-factor enhancement circuit does. Q-factor enhancement is not useful for increasing the sensitivity of a thermal noise, or detector noise limited force detector.

Q-factor enhancement could be useful in situations where a high Q-factor is needed for other reasons than just sensitivity. Obtaining force distance curves in atomic force microscopy might be a good application. When the effects of noise can be neglected, Q-factor enhancement has the same effect as a natural Q-factor of the same value.
4.7 References

12. LabView, National Instruments Corporation, 11500 N Mopac Expwy, Austin, TX 78759-3504, USA
4 Q-factor enhancement
Near-field optical microscopy

In this Chapter, a newly designed Near-field Scanning Optical Microscope (NSOM) for biological studies with single molecule sensitivity is employed. With the new microscope, a study is made of DNA samples. Long DNA molecules, 16 μm and 300 nm respectively, are used to obtain topographic images. Fluorescence images of single Cy3.5 molecules attached to short strands of ‘bubble’ DNA are used to calculate the distances between these molecules. Repetitive imaging shows that the single molecule localization accuracy is 3 to 4 nm, which is an order of magnitude better than the localization accuracy obtained by far-field techniques. The observed localization accuracy is close to the calculated, shot noise limited accuracy of 0.8 nm.
5.1 Introduction

Near-field Scanning Optical Microscopy (NSOM), already introduced in Chapter 1, offers a number of advantages over more conventional types of optical microscopy, e.g. confocal and wide-field microscopy. Increased resolution, only limited by the aperture size of the near-field probe, simultaneous detection of topography and near-field effects of the electric field at the aperture can be used for a more comprehensive study of the sample under investigation. As the glass fiber probes are extremely fragile, NSOM is an elaborate technique. Despite all efforts on the development of a more robust probe, fiber probes are still the most successful. The complicated operation limits the practical use of NSOM to applications where the advantages of this technique are crucial.

An example of such an application is a study of fluorescently labeled proteins interacting with DNA. The acquired topography of the DNA can be used to localize strands of DNA. The optical signal from the fluorescent molecules attached to the proteins can be used to distinguish different types of proteins and to resolve the relative position of the proteins with respect to the DNA. Knowledge about the position of and interaction between specific proteins and DNA can lead to a better understanding of, for example, DNA damage repair mechanisms. Localization of fluorescent probes using far-field techniques is already widely used. However, a better localization accuracy is expected for NSOM, thanks to the better optical resolution.

This Chapter demonstrates the possibility to obtain topographical images of DNA with the newly designed instrument. Furthermore, as a demonstration of the achievable localization accuracy of single fluorescent molecules using NSOM, the relative position of Cy3.5 molecules attached to DNA is determined.

5.2 Sample preparation

The sample preparation can be divided into two steps. In the first step, a suitable substrate for DNA and single molecule detection with NSOM is prepared. The second step consists of the deposition of the DNA on this substrate.

5.2.1 Substrate preparation

For topographic imaging of DNA, the substrate needs to be extremely flat. Freshly cleaved mica provides an atomically flat substrate. However, the optical properties are far from ideal as mica is birefringent and has a relatively high absorption of light. Cover glasses have good optical properties, but have a surface roughness that would prevent the topographic detection of DNA.
As a solution, a piece of mica is glued to a coverglass (Menzel Gläser, Ø24 mm), using an UV-curing glue (Norland optical adhesive no. 73). After curing, the mica is cleaved until only a thin layer of mica is left on the coverglass. Just before depositing the DNA on the substrate, the mica is cleaved once more. The total thickness of the glue and the mica has to be thin compared to the thickness of the coverglass of 170 μm, so that the mica surface is within the working distance of the microscope objective collecting the light.

### 5.2.2 DNA deposition

Three types of DNA were used. For verification of the achievable resolution, 16 μm long λ-DNA and 1kbp (300 nm) double stranded DNA were imaged. For single molecule fluorescence experiments, a 90 bp (30 nm) DNA construct was investigated, where the ends of the two strands match to form double stranded DNA, but where the middle part does not match. A ‘bubble’ of two single stranded pieces of DNA is formed between the matching ends. A Cy3.5 molecule is attached to one end of the DNA construct. In all cases, the DNA was diluted to a concentration of 3 ng/μl in the following solution: 20 mM Tris buffer, pH 7.4 and 10 mM MgCl₂. About 2 μl of the DNA solution was deposited on a freshly cleaved mica surface. After several minutes of incubation, the sample was rinsed with HPLC-grade water and dried with nitrogen.

A conventional cantilever-based atomic force microscope was used to verify the presence of DNA in the correct concentration on the sample.

### 5.3 Topography of DNA

Figure 5-1 shows some shear force images of DNA. The images were obtained on the newly designed near-field optical microscope, described in § 1.5, using a pulled, tapered fiber, coated with aluminum as probe. For these shear force images, the optics of the near-field microscope are not used and the oil immersion objective is exchanged with a long working distance air objective to facilitate the coarse approach of the probe to the sample. All images show unfiltered data, except for the subtraction of a parabola at each line.

The images were acquired with a scan speed of 1 line/s. Figure 5-1(a), (b) and (c) show the topography of 16 μm long λ-DNA molecules. Figure 5-1(d) shows the topography of 300 nm long double stranded DNA molecules. The RMS noise in the images is 0.3 nm. DNA has a persistence length of 50 nm. As a consequence, the 16 μm long DNA molecules tend to cluster, where the 300 nm long DNA molecules do not cluster.
The topographic resolution of NSOM is limited by three factors:

**Electronic Noise.** With an instrument designed to work with intact cells, detecting DNA is not trivial. The scan range is 32 μm × 32 μm × 26 μm in x, y, and z direction. The least-significant bit of the 12-bit DAC in the electronics corresponds to a height difference of 6.4 nm. This is more than four times the height of the DNA itself. The scan range of the scanner in the z-direction can be reduced by dividing the output voltage of the high-voltage amplifier by a constant. Ultimately the resolution of the scanner is determined by the electronic noise. As a voltage of 6 mV is enough for a height change of the scanner equivalent to the height of DNA, care should be taken to minimize the electronic noise.
PROBE CONVOLUTION. The topographic resolution in SPM is always limited by the convolution with the probe. In Figure 5-2, a drawing of a Focused Ion Beam (FIB) milled NSOM probe,\textsuperscript{11} is shown, with on top a 90 bp ‘bubble’ DNA molecule to scale. The use of FIB-milled probes is desirable for their excellent optical properties in terms of throughput and preservation of polarization, however the convolution of the flat endface with the sample will severely limit the topographic resolution. Even though there will always be some protruding part of the probe that determines the topographic resolution, it is clear that from such a probe hardly any topography can be expected for ‘bubble’ DNA.

Figure 5-2. FIB milled NSOM probe, the dark spot in the center is the aperture, the surrounding material is the aluminum coating. On top of the probe, in the center of the aperture is a DNA molecule, drawn to scale.

MECHANICAL VIBRATIONS. For NSOM measurements on single fluorescent molecules, the fluorescence is collected by a 1.4 NA oil immersion objective. An index matching oil layer is present between the glass cover slip (sample) that is scanned and the stationary microscope objective. The oil impedes the motion of the cover slip and introduces mechanical vibrations in the cover slip. For an objective with a lower NA, the impact of the oil layer between cover slip and objective is less, however at the cost of a lower photon collection efficiency.\textsuperscript{24-26}

Tuning-fork-based shear force detection allows topographic imaging of single DNA molecules. However, in NSOM experiments, the topographic resolution can be limited by mainly mechanical vibrations induced by the objective and probe convolution. The topographic images will only show an indistinct spot for small ‘bubble’ DNA molecules and is not very useful.
5.4 Localization accuracy of single molecules: theory

As discussed before, finding the relative positions of specific proteins can help answering questions on the interaction mechanisms of these proteins with DNA. Labeling specific proteins with fluorescent labels allows optical detection of the position of the proteins. The accuracy of localization can be better than the resolution of the optical system used to find the position of the labeled proteins. As the terms localization accuracy and resolution are easily confused, it is important to distinguish both terms.

5.4.1 Localization accuracy versus resolution

With far-field techniques, the resolution of an optical imaging system is defined as the minimum distance between two identical point sources, where the images of the two point sources still can be discriminated. Using the Rayleigh criterion, the resolution is defined as the distance from the maximum in intensity of the image of the first point source to the first minimum in intensity in the image of the second point source. In a diffraction limited system used to image the fluorescence of point-like single molecules, the resolution according to the Rayleigh criterion is:

\[ R = \frac{0.61\lambda}{NA} \]  

(5-1)

where \( R \) is the resolution, \( \lambda \) is the wavelength of the fluorescent light and \( NA \) is the numerical aperture of the microscope objective. For typical fluorophores imaged with a confocal microscope, the resolution is in the order of 300 nm, where the aperture-size-limited resolution of NSOM is in the order of 70 nm to 100 nm. The localization accuracy however, can be better than the resolution. When individual molecules can be discriminated based on optical parameters characteristic for each molecule, e.g. polarization and wavelength, distances between molecules smaller than the resolution can be measured.

In the case of confocal microscopy, the position of a fluorescent molecule will be in the center of the diffraction limited spot. In the case of NSOM, the image of a fluorescent molecule depends on the dipole-dipole interaction between probe and molecule. For circularly polarized light and molecules with an in plane dipole orientation, the position is in the center of the fluorescent spot. The accuracy of localization is a complex function of the number of photons collected, the fluorescence background, the shape and the resolution of the image of the molecule and the dynamic behavior of the molecule. In ambient conditions, localization accuracies of 30 nm have been obtained using confocal microscopy.\textsuperscript{13-16,18-21} The localization accuracy can be improved in confocal or wide-field microscopy by increasing the number of photons collected from the molecule. Cooling the sample to
cryogenic temperatures, for example, improves the localization accuracy to 3.4 nm.\textsuperscript{17,22} Still, the localization accuracy is expected to be better using NSOM, due to the narrow response function (<100 nm).

### 5.4.2 Shot noise limited localization accuracy

A fit of the image of a single molecule with the response function of the imaging system results in a specific location with a certain accuracy. Under the following assumptions, the achievable accuracy can be calculated:

- Molecular fluorescence intensity fluctuations are determined by shot noise
- Background fluorescence fluctuations are limited by shot noise
- Background fluorescence is low compared to the molecular fluorescence signal
- The response of the imaging system to a point-like input is known

For NSOM, the first three assumptions are usually valid. The shape of the response function however, is different from the Gaussian response function in far-field optics with a Gaussian input field, that is normally assumed for localization of molecules.\textsuperscript{13-22,27-29}

The response function for a NSOM probe can be calculated from the electric field at the aperture, the interaction of the electric field with the fluorescent molecule and the emission pattern of the molecule. The model of the electric field at a subwavelength aperture in a thin, perfectly conducting sheet of metal, developed by Bethe\textsuperscript{30} and Bouwkamp,\textsuperscript{31} can be used to calculate the electric field at the aperture of a near-field optical probe. The response function of an image of a single molecule can then be found using the methods developed by Betzig\textsuperscript{32} and Gersen.\textsuperscript{33}

Because the aperture and alignment for every near-field probe is somewhat different and because the response of a single fluorescent molecule depends on the orientation of the dipole moment of that molecule and the dipole-dipole interaction with the

![Figure 5-3. The solid line indicates the approximated response function of NSOM probe to a point-like input at position $\bar{x}$. The aperture size is $L$, the background level $B$ and the signal above the background $S$. In a distance $\delta \Gamma$, the response rises from the $B$ to $S$. The dotted line indicates the measured response at a position $\bar{x}_m$ with an error $\sigma_\Gamma$.](image-url)
probe, the response function of the imaging system is generally not known. Here, the response is approximated by the function shown in Figure 5-3. The presented function is an approximation of the response function expected for molecules with an in-plane dipole moment, circularly polarized excitation and the probe close to the sample surface. The advantage of the approximated response function is that it can easily be implemented in a fit algorithm and that it is possible to calculate the achievable accuracy analytically. The approximated response function in Figure 5-3 is described by:

\[
R(S, \tilde{x}, x) \rightarrow \begin{cases} 
5 & \text{for } |x - \tilde{x}| \leq \frac{L}{2} - \delta \Gamma \\
5 - \frac{S}{\delta \Gamma} \left[ |x - \tilde{x}| - \left( \frac{L}{2} - \delta \Gamma \right) \right] & \text{for } \frac{L}{2} - \delta \Gamma < |x - \tilde{x}| \leq \frac{L}{2} \\
0 & \text{for } |x - \tilde{x}| > \frac{L}{2} 
\end{cases}
\] (5-2)

Where \( R \) is the response function of position \( x \), \( S \) is the signal level above the background level \( B \), \( \tilde{x} \) is the position of the point-like source, \( L \) is the size of the aperture and \( \delta \Gamma \) is the distance over which the response rises from \( B \) to \( S \).

A fit of the response function to the observed single molecule signal can be made by minimizing: \(^{28}\)

\[
\chi^2(S, \tilde{x}) = \sum_{i=1}^{N} \frac{1}{\sigma_i^2} [R_i(S, \tilde{x}) - M_i]^2
\] (5-3)

where \( \chi^2 \) is a measure for the quality of the fit, \( \sigma_i \) is the error on the \( i \)th measured value \( M_i \), \( R_i \) is the value of the response function at the \( i \)th point and \( N \) is the number of data points between positions \(-L/2\) and \( L/2\). The values of \( S \) and \( \tilde{x} \) where \( \chi^2 \) is minimized are \( S_m \) and \( \tilde{x}_m \) respectively. The error in the position that is found this way is:

\[
(\tilde{x} - \tilde{x}_m)^2 = \frac{\Delta(S_m, \tilde{x}_m)}{N} \left[ \frac{1}{L} \int_{-L/2}^{L/2} \frac{1}{\sigma_i^2} \left( \frac{\partial}{\partial \tilde{x}} R(S_m, \tilde{x}_m, x) \right)^2 \, dx \right]^{-1}
\] (5-4)

where \( \Delta \) is a constant that depends on the number of free fit parameters. For 1 fit parameter, with a 68\% (1\( \sigma \)) confidence limit, \( \Delta = 1 \), for 2 fit parameters, \( \Delta = 2.3 \) and for 3 fit parameters, \( \Delta = 3.5 \). The response function \( R_i \) can now be defined, according to Figure 5-3:
Localization accuracy of single molecules: theory

The square of the derivative of $R_i$ is:

$$\left[ \frac{\partial}{\partial \xi} R_i(S, \tilde{\xi}, x) \right]^2 \rightarrow \begin{cases} 0 & \text{for } |x - \tilde{\xi}| \leq \frac{L}{2} - \delta \Gamma \\ \left( \frac{S}{\delta \Gamma} \right)^2 & \text{for } \frac{L}{2} - \delta \Gamma < |x - \tilde{\xi}| \leq \frac{L}{2} \\ 0 & \text{for } \frac{L}{2} < |x - \tilde{\xi}| \end{cases}$$  \quad (5-5)$$

Substituting (5-5) in (5-4) leads to:

$$\frac{(\tilde{\xi} - \tilde{\xi}_m)^2}{L^2} = \frac{\Delta}{2NL} \left( \frac{\delta \Gamma}{S} \right)^2 \left[ \int_{L/2 - \delta \Gamma}^{L/2} \frac{1}{\sigma_i^2} \, dx \right]^{-1}$$  \quad (5-6)$$

Two terms contribute to the noise: the noise in the fluorescence signal and the noise in the background. Under the assumption that the noise in the background is independent of the position, $\sigma_i^2(x) = R_i(x) + B$, and using (5-2), the integral in (5-6) can be rewritten:

$$\int_{L/2 - \delta \Gamma}^{L/2} \frac{1}{\sigma_i^2} \, dx = \int_0^\infty \frac{1}{S_\delta \Gamma x + B} \, dx$$  \quad (5-7)$$

Provided that $S > 0$, the integral in (5-7) can be solved and (5-6) results in:

$$\frac{\tilde{\xi} - \tilde{\xi}_m}{L} = \frac{\Delta \delta \Gamma}{2NL} \frac{1}{S} \left( \frac{S}{B + 1} \right)$$  \quad (5-8)$$

As could be expected, the relative accuracy of the localization is depending on the shot noise in the signal, $\sqrt{S}$. The relative accuracy increases also with the number of data points $\sqrt{N}$, because with more data points, the noise is averaged over more points. The fact that the edges of the response function are more important for obtaining an accurate fit than the center part of the response function is reflected by the dependence of the accuracy on the edge sharpness $\sqrt{L/\delta \Gamma}$. The signal-to-background ratio has a high impact on the accuracy only for low values of the signal-to-background ratio. These effects can be compared to results for a Gaussian response function:28

$$R(S, \tilde{\xi}, x) = S \exp \left[ -\frac{(x - \tilde{\xi})^2}{2\Gamma^2} \right]$$  \quad (5-9)$$
A Gaussian response function results in a relative localization error:

\[
\frac{\bar{x} - \bar{x}_m}{\Gamma} = \frac{\Delta}{N_T S} \int_{-t}^{t} \frac{1}{ \left( e^{\frac{u^2}{2}} - \frac{B}{S} \right)} du
\]

(5-10)

where \( t = L/2\Gamma \) and \( N_T \) is the number of data points within \( \Gamma \), \( N_T = N/\Gamma \). If \( t \) is reasonably large, namely most of the Gaussian peak is used for the localization and the signal-to-background ratio is large, the first term in the integral in (5-10) reduces to: \( \sqrt{2\pi} \) and the second term reduces to: \( \frac{2B}{3S}t^3 \). The relative localization error (5-10) now reduces to:

\[
\frac{\bar{x} - \bar{x}_m}{\Gamma} = \frac{\Delta}{N_T S} \frac{1}{ \sqrt{2\pi} - \frac{2B}{3S}t^3 }
\]

(5-11)

Even though the response function is quite different, the same elements as in (5-8) can be found in (5-11). For the number of data points, the signal strength and the signal-to-background ratio, this is obvious. The edge sharpness is implicitly defined in the dimensionless variable \( t \).

5.5 Localization accuracy of single molecules: experiment

The distances between single Cy3.5 molecules have been measured to explore the potential of NSOM to determine the distances between single molecules in a DNA construct. The accuracy obtained for the distances between these molecules is an indication for the achievable accuracy using NSOM.

5.5.1 Experimental setup

The Cy3.5 molecules were excited at 568.2 nm wavelength. The excitation light was filtered with a 568.2 nm NB filter (Omega Optical) before coupling into the NSOM fiber, to eliminate auto fluorescence of the transportation fiber that brings the light from the laser to the setup. The excitation intensity was \(~5\text{ kW/cm}^2\). The fluorescence was collected with a 64x, 1.4 NA oil-immersion objective, then filtered using a 585 ALP long pass filter (Omega Optical) and focused on a photon counting Avalanche Photo Diode (APD) (Perkin Elmer, EG&G).
5.5.2 Single molecule imaging

In § 5.4, a method was developed for estimating the ultimate localization accuracy of single molecules in the presence of shot noise and a relative high signal-to-background ratio. Here the accuracy is limited by Poisson statistics. In reality, the accuracy can be constrained by a number of additional sources, such as photodynamics of single molecules, diffusion of molecules, scanner non-linearity, drift in the instrument etc.

![Fluorescence images of single Cy3.5 molecules obtained with NSOM.](image)

Figure 5-4. Fluorescence images of single Cy3.5 molecules obtained with NSOM. The discrete jumps in intensity are an indication that the observed molecules are indeed single molecules. (a) and (b) are the images obtained in forward and reverse scan direction respectively. (c) and (d) are a zoom in, again in the forward and reverse scan direction, on 4 molecules in (a) and (b). All images are 200 x 200 pixels and show unfiltered data. The integration time was for (a) and (b) 2.5 ms and for (c) and (d) 1 ms. The numbers in (a) indicate the molecules that will be used for localization. The arrows in (b) indicate the position of the line traces in the fast (f) and slow (s) scan direction that are shown in Figure 5-5 and Figure 5-6.

Figure 5-4 shows four consecutive NSOM images on a number of single fluorescent molecules. The molecules show behavior that is normal in single molecule detection. On a longer time scale in Figure 5-4 (a) and (b), dark periods can be observed in the order of 1 s. At shorter integration times in Figure 5-4 (c) and (d), the
dark periods are still present, however also faster fluctuations in the fluorescence intensity can be observed at a time scale in the order of 1 ms. These fast fluctuations can be attributed to excursions of the molecule to the non-fluorescent triplet state.\textsuperscript{35}

The fluorescence count rate can be calculated from the data in the images shown in Figure 5-4. The average intensity observed from the molecules in Figure 5-4 is 20 kcount/s. The background, consisting out of the background in the detection and the darkcounts of the APD, is 1.3 kcount/s. The signal-to-background ratio is then 15.

A line trace shows the counts per pixel as a function of position, and as NSOM is a scanning technique, also as a function of time.

A line trace of an image as shown in Figure 5-4, in the (fast) \( x \)-direction will have a shorter time scale than a line trace in the (slow) \( y \)-direction.

The line trace in the fast scan direction shown in Figure 5-5, is taken on a line where the molecule is not in a dark state. It shows the convolution of the molecule with the probe. The profile can be used to estimate the excitation profile of the NSOM probe. The trapezoidal shape, assumed in § 5.4 appears to be a remarkably reasonable approximation. The parameters as discussed in § 5.4 are: \( \delta r = 30 \) nm, for the width of the edge of the profile of the single molecule and the size of the aperture \( L = 140 \) nm.

The number of pixels, or data points, within the image of one molecule depends on the number of pixels in the image, the probe size and the scan range:

\[
N = L \cdot \frac{\# \text{ pixels}}{\text{scan range}} \tag{5-12}
\]

As the images are \( 200 \times 200 \) pixels, the number of data points that can be used to determine the position of a molecule is 28.0
The line trace in the slow scan direction presented in Figure 5-6, shows a more erratic behavior. This can be explained by the argument that NSOM is a scanning technique. In the slow scan direction there is a 1 s time gap between data points on this line trace.

For most molecular photodynamics, this is a long time interval and values between data points are not correlated for photodynamics. Dark periods are an exception and could extend for over more than one data point. The frequent dark periods, observed in the Figure 5-4 (a) and (b) cause the erratic behavior observed in Figure 5-6.

Summarizing, the following parameters can be found from the data presented in Figure 5-4:

Table 5-1. Parameters for calculation of potential single molecule localization accuracy in Figure 5-4.

<table>
<thead>
<tr>
<th>molecule</th>
<th>$\delta \Gamma$ [nm]</th>
<th>L [nm]</th>
<th>S [cnts]</th>
<th>B [cnts]</th>
<th>S/B</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) and (b)</td>
<td>30</td>
<td>140</td>
<td>50</td>
<td>3.2</td>
<td>15.6</td>
<td>28.0</td>
</tr>
<tr>
<td>(c) and (d)</td>
<td>30</td>
<td>140</td>
<td>20</td>
<td>1.3</td>
<td>15.4</td>
<td>55.4</td>
</tr>
</tbody>
</table>

With these data it is possible to calculate the shot-noise-limited accuracy for these parameters. In the case of the molecules in Figure 5-4 (a) and (b), the error in the position is 1.1 nm, in the case of the molecules in Figure 5-4 (c) and (d), the error in the position is 0.79 nm.

5.5.3 Intermolecular distance measurement

The locations of the molecules in Figure 5-4 can now be determined. The dark periods as observed in Figure 5-4 contribute strongly to the $\chi^2$ error in the fit as defined in § 5.4. A cross correlate of the image with an idealized response function should be less sensitive to the photodynamics of a single molecule, as the whole image of the
molecule is taken into account. A ‘cylindrical’ response function is chosen: a value of 1 within a disk with a radius that matches the size of the molecules in the image and a value of 0 outside that disk. For the maximum values in the cross correlate, the location of the disk corresponds to the locations of the molecules.

Using this method, the position of 8 molecules is determined in Figure 5-4 (a) and (b):

Table 5-2. Position of 8 molecules in Figure 5-4 (a) and (b)

<table>
<thead>
<tr>
<th>molecule</th>
<th>x [nm]</th>
<th>y [nm]</th>
<th>x [nm]</th>
<th>y [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>232.5</td>
<td>670.0</td>
<td>227.5</td>
<td>640.0</td>
</tr>
<tr>
<td>2</td>
<td>312.5</td>
<td>842.5</td>
<td>307.5</td>
<td>820.0</td>
</tr>
<tr>
<td>3</td>
<td>445.0</td>
<td>642.5</td>
<td>442.5</td>
<td>617.5</td>
</tr>
<tr>
<td>4</td>
<td>542.5</td>
<td>817.5</td>
<td>547.5</td>
<td>800.0</td>
</tr>
<tr>
<td>5</td>
<td>530.0</td>
<td>927.5</td>
<td>530.0</td>
<td>927.5</td>
</tr>
<tr>
<td>6</td>
<td>217.5</td>
<td>285.0</td>
<td>230.0</td>
<td>245.0</td>
</tr>
<tr>
<td>7</td>
<td>495.0</td>
<td>250.0</td>
<td>495.0</td>
<td>235.0</td>
</tr>
<tr>
<td>8</td>
<td>305.0</td>
<td>112.5</td>
<td>305.0</td>
<td>92.5</td>
</tr>
</tbody>
</table>

With the same method, the position of 5 molecules that are present in Figure 5-4 (c) and (d) can be calculated. Caution should be exercised when considering the position of molecule 9, that has a position close to molecule 1. In the larger scan range, the algorithm does not discriminate molecule 1 and 9.

Table 5-3. Position of 5 molecules in Figure 5-4 (c) and (d)

<table>
<thead>
<tr>
<th>molecule</th>
<th>x [nm]</th>
<th>y [nm]</th>
<th>x [nm]</th>
<th>y [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>101.63</td>
<td>208.31</td>
<td>106.68</td>
<td>212.10</td>
</tr>
<tr>
<td>2</td>
<td>209.58</td>
<td>397.69</td>
<td>214.63</td>
<td>401.48</td>
</tr>
<tr>
<td>3</td>
<td>338.35</td>
<td>231.04</td>
<td>348.45</td>
<td>225.99</td>
</tr>
<tr>
<td>4</td>
<td>409.05</td>
<td>376.23</td>
<td>416.63</td>
<td>363.60</td>
</tr>
<tr>
<td>9</td>
<td>173.88</td>
<td>207.90</td>
<td>171.36</td>
<td>210.42</td>
</tr>
</tbody>
</table>

To determine the accuracy in the localization, all distances between molecules are calculated for the four images in Figure 5-4. Due to inaccuracy in the position determination, different distances will be found between the same molecules in
different images. Three groups of images are evaluated. The first group contains the images of Figure 5-4(a) and (b), the second group contains the images of Figure 5-4(c) and (d) and the last group contains all images of Figure 5-4.

The distances, in x and y between the molecules that are present in all images in one group are calculated. For the same distance in the different images, the standard deviation in x and y is acquired. The average of all standard deviations is the accuracy of the calculated distance. Under the assumption that the localization has approximately the same accuracy for all molecules, the localization accuracy can then be found by dividing the accuracy in distance by $\sqrt{2}$.

Table 5-4. Single molecule localization accuracy

<table>
<thead>
<tr>
<th>Group</th>
<th>Figure 5-4a</th>
<th># molecules</th>
<th># distances</th>
<th>x[nm]</th>
<th>y [nm]</th>
<th>x[nm]</th>
<th>y [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a),(b)</td>
<td>8</td>
<td>28</td>
<td>4.4</td>
<td>9.6</td>
<td>3.1</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>(c),(d)</td>
<td>5</td>
<td>10</td>
<td>3.9</td>
<td>5.9</td>
<td>2.8</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>(a),(b),(c),(d)</td>
<td>4</td>
<td>6</td>
<td>13</td>
<td>12</td>
<td>9.2</td>
<td>8.3</td>
<td></td>
</tr>
</tbody>
</table>

a. The distances between molecules are compared for the measurements indicated in this column.

When the localization accuracy is calculated for a group consisting out of one measurement in the forward direction and one measurement in the reverse direction, there is striking difference between the accuracy in the x and in the y direction. Here the effect of the dark period becomes eminent. The time constant associated with these dark periods is large compared to the integration time and slow compared to the line frequency. In the fast scan direction, the dark period just lowers the amount of light, while in the slow scan direction, the apparent position of the molecule is biased.

Zooming in on a few molecules increases the achievable accuracy, because the number of data points $N$ is increased. As the integration time is chosen shorter for the smaller scan range, the effect of excursions to the triplet state becomes noticeable in the fast scan direction. The fluctuations in the fluorescence intensity caused by excursions to the triplet state, adversely influence the localization accuracy for the fast scan direction. Together, these effects increase the accuracy for measurements in Figure 5-4 (c) and (d), however lower the difference between the fast and the slow scan direction.

Comparing all measurements in Figure 5-4, i.e. (a), (b), (c) and (d), shows a worse localization accuracy than theoretically expected in § 5.5.2. Shot noise in the detection of the signal and background is not the limiting factor in determining the
position of the molecule. Blinking and excursions to the triplet state shift the estimated position away from the real position of the molecule. The large inaccuracy when all four images are taken into account, can simply be explained by the fact that the scanner did not have a feedback circuit to control the position of the scanner. Piezo non-linearity will decrease the accuracy in localization of single molecules.

The empirically found accuracy is about one order of magnitude better than the accuracy found with confocal microscopy\textsuperscript{17,19,21} or wide-field\textsuperscript{13-15,18} microscopy. Recently, Bloeß et al.\textsuperscript{22} reported a localization accuracy of 3.4 nm using far-field techniques. This localization accuracy is similar to the localization accuracies reported here, however Bloeß et al. worked at cryogenic temperatures, collected 2.2-10\textsuperscript{6} photons in a total integration time of 30 s, which is 70 times more photons and a 20 times longer integration time compared to the data presented here. The superior localization accuracy for NSOM is explained both by the higher resolution of NSOM and by the small excitation volume, resulting in a high signal-to-background ratio.

### 5.6 Conclusions

Near-field Scanning Optical Microscopy, is a suitable technique for single molecule fluorescence detection. Advantages are the high resolution, small excitation volume and the combined topography.

The topography of DNA molecules can be resolved using shear force detection with a near-field optical probe. The use of an oil immersion objective, introducing mechanical vibrations to the system and the flat endface of the probe, limiting the lateral resolution, reduce the quality of topographic imaging.

The high resolution and high signal-to-background ratio make NSOM the preferred technique for an application where the detection of distances between single fluorescent molecules is important. As long as the molecules have different optical parameters (dipole orientation or emission wavelength), the distances smaller than the resolution of the image between molecules can be determined with an accuracy of a few nm. At smaller distances Fluorescence Resonant Energy Transfer (FRET)\textsuperscript{36} becomes a potential candidate to acquire distance information.

The localization accuracy observed in the data presented in this Chapter is 3 nm in the fast scan direction and 4.2 nm in the slow scan direction, which is significantly better than results with alternative techniques.\textsuperscript{13-21} The observed accuracies are of the same order as the ultimate limit of about 1 nm, in case that the data would be limited by shot noise in the measurement.
5.7 References


Near-field optical microscopy

30. H.A. Bethe, Phys. Rev. 66, 163 (1944)
34. J.-A. Veerman, Single molecule detection with a near-field optical microscope, PhD thesis (University of Twente, The Netherlands, 1999)
General discussion and outlook

This Chapter discusses the implications of the work described in this thesis for the field of Scanning Probe Microscopy. An outlook is given on potential applications in the near future, with an emphasis on tuning-fork-based Atomic Force Microscopy and biological applications for Near-field Scanning Optical Microscopy (NSOM). Preliminary results demonstrate the feasibility of NSOM with single molecular sensitivity in a liquid environment.
6.1 Introduction

Soon after the invention of Scanning Tunneling Microscopy,\(^1\) a whole range of techniques came about, where a small sensor probes local properties of a sample on a nanometer scale.\(^2\) For most of these scanning probe techniques, an alternative method was necessary to detect the probe-to-sample distance. By far the most versatile method is to measure force interaction between probe and sample.\(^3\) Tuning forks, as described in this thesis provide one of the methods to measure interaction forces between probe and sample.

Tuning forks have their own characteristics in which they differ from cantilever-based force sensors used in Atomic Force Microscopy (AFM). A comparison can be made in terms of sensitivity, resolution, speed, ease-of-use and cost.

In Near-field Scanning Optical Microscopy (NSOM), tuning forks are the most popular force sensors. Alternative methods are also applied, such as modified AFM cantilevers and several optical force detection schemes.

In this Chapter, the prospective use of tuning forks is described, first in AFM-like applications and second in Near-field Scanning Optical Microscopy (NSOM) applications.

6.2 Tuning forks in AFM

Chapter 2 established tuning forks as an excellent alternative to cantilevers as force sensors in AFM. The distinct characteristics of tuning forks result in advantages and disadvantages compared to cantilevers on several aspects.

SENSITIVITY. Because of their high spring constant of 45 kN/m, tuning forks are unsuitable for contact mode applications and (static) force spectroscopy. In modes where the probe is oscillated perpendicular to the surface (noncontact mode), the performance in ambient conditions is comparable for tuning forks and cantilevers. The spring constant of tuning forks is still higher compared to the spring constant of regular noncontact mode cantilevers (~1 N/m), yet the higher Q-factor in air and the smaller probe amplitude compensate for this. However, with the development of ultra-thin cantilevers,\(^4,5\) having a spring constant in the order of 0.01 N/m, cantilevers have the best potential for detection of small forces. In a liquid environment, the high spring constant of tuning forks is not compensated by a high Q-factor and AFM cantilevers have the best performance.
RESOLUTION. The high stiffness of tuning forks, combined with the relatively high mass result in a small amplitude caused by thermal noise compared to cantilevers. As a result, the achievable height resolution is better for tuning forks, even allowing detection of sub-atomic features. The lateral resolution will in both cases be limited by the probe convolution. When a probe is mounted on a tuning fork, the choice of probe and the choice of detector are independent. For cantilevers, the probe and cantilever are produced simultaneously and the material of choice is a compromise to obtain a suitable probe on a suitable cantilever.

SPEED. In general, noncontact modes where the probe is oscillated with respect to the sample surface are slow compared to contact mode AFM. For noncontact mode, the force detection bandwidth is proportional to the resonance frequency of the tuning fork or cantilever divided by the Q-factor. The available bandwidth is in the same order for tuning forks and cantilevers, where the exact number depends on the tuning fork or cantilever of choice. A 100 kHz tuning fork with a Q-factor of 500 has a force detection bandwidth of 200 Hz, allowing a scan speed in the order of 1 line/s for a 200×200 pixel image.

EASE OF USE. The absence of optics in tuning-fork-based force detection, simplifies the use of the microscope. There is no need to align a laser beam on a small cantilever and the reflected beam on a position sensitive detector. Reflective samples do not induce a risk of interference between light reflected of the sample and light reflected of the cantilever. Furthermore, a circuit to automatically drive a tuning fork at its resonance frequency is easy to implement. A circuit that uses the tuning fork as an acoustic detector could be useful to automatically approach the sample, facilitating the operation of a tuning-fork-based AFM even more. On the other hand, tuning forks with a usable AFM probe mounted are not available commercially. Mounting a suitable probe could be elaborate. Another aspect is the occurrence of an uncontrolled contact between probe and sample (tip crash). Both a noncontact cantilever-based probe and a tuning-fork-based probe will be damaged by such an event, however the damage will be worse for the tuning fork due to its higher stiffness.

PRICE. Tuning forks are cheap force detectors. The price of a standard 32.768 kHz tuning fork is low (≈ 0.44 €) compared to the price of an AFM cantilever (≈ 40 €). For tuning forks, the probe is not included in the price. The labour cost to mount a probe to a tuning fork and the cost of the probe itself eliminates the advantage in price. Only when the probe is integrated with the tuning fork, there is a potential advantage in price. The cost of the microscope itself though, could be lower for a tuning-fork-based instrument, because of the lack of optics and alignment. The instrument can also be more compact for the same reasons.
The properties of tuning forks can make them the force detector of choice in applications where routine measurements have to be performed in an oscillated probe mode, where ease-of-use and size of the instrument are important parameters. Before such tuning-fork-based applications will emerge, tuning forks with integrated probe have to be developed.

6.3 Tuning forks in NSOM

6.3.1 Applications of NSOM

Before discussing the future of tuning forks in NSOM, it is necessary to consider potential applications, where NSOM is the preferred technique to study a sample.

Advantages of NSOM are:

- High resolution, only limited by the aperture size and penetration depth in the probe coating material.
- Small excitation volume, potentially reducing background in detection of fluorescence.
- Simultaneous detection of topography, that could lead to extra information on the sample.
- Additional optical information thanks to near-field effects at the aperture of the probe, such as a z-component of the electric field.

Disadvantages of NSOM are:

- Elaborate technique, due to extremely fragile probes.
- Slow imaging speed, because only information on one pixel is acquired at a time.

The disadvantages of NSOM have limited its use to applications, where the requirements of the application render NSOM the only suitable alternative. Most examples of such applications, where an improvement in resolution from \( \sim 300 \text{ nm} \) to \( \sim 70 \text{ nm} \), a small excitation volume and combined topography are useful, can be found in biology.\(^{10}\) These examples include the detection of proteins in cell membranes\(^{11}\) and the study of proteins interacting with DNA, as discussed in Chapter 5. One specific application, Photon Scanning Tunneling Microscopy\(^ {12}\) (PSTM), an unique tool to study integrated optics structures is left out of this discussion, because it is specific to integrated optics applications, although it is a similar technique to NSOM.
6.3.2 NSOM in liquid

An important requirement for most applications in biology, is the immersion of the sample and thus the probe in a buffer solution. The probe-to-sample distance control is more difficult in a liquid environment. A number of strategies have been developed to enable successful application of NSOM in liquid.

Scanning Ion Conductance Microscopy. An alternative development, where scanning in liquid is mandatory for the technique to work, is Scanning Ion Conductance Microscopy (SICM). Instead of an optical fiber, a glass micro pipette is pulled, coated with aluminum and filled with a buffer solution as electrolyte. With a voltage across an electrode in the pipette and a counter electrode in the buffer solution outside the pipette, the current through the electrodes is a measure for the probe-to-sample distance: at close proximity, the aperture is closed by the sample and the ion current is restricted.

This technique is well capable of keeping the probe sufficiently far away from the sample surface to prevent damage to probe or sample, even if the sample contains living cells. On the other hand, the probe-to-sample distance is with ~250 nm too large to allow high resolution images. Another reason for the resolution to be poor is that the optical aperture size is large, because the aperture for ion conductance of ~50 nm is surrounded by glass. A last disadvantage is that the reported brightfield optical signal-to-background ratio is only 10. This is too low to allow single molecular fluorescence studies.

Cantilever-Based NSOM. As discussed before, AFM cantilevers have a significantly lower spring constant than tuning forks. In a liquid environment, where the Q-factor is low, cantilevers provide lower interaction forces and a smaller chance of probe or sample damage. A considerable amount of work has been performed to develop cantilever-based or cantilever-like NSOM probes and recently progress has been made towards the production of such probes. However, at present time, cantilever-based NSOM probes are not readily available and more work has to be performed to reliably produce these probes in large enough quantities.

Optical Shear-Force Detection. Bare NSOM fiber probes also have a significantly lower spring constant compared to tuning-fork-based probes. Upon immersion in liquid, the resonance frequency and Q-factor of a NSOM fiber probe drop, however the probe is still usable for shear-force detection in liquid. Two types of optical shear-force detection are possible when immersing only the fiber: interferometric detection of the fiber oscillation and optical beam deflection. Both methods have proved to work in a liquid environment, however suffer from the disadvantage of elaborate beam alignment and a risk of introducing an additional source of background light.
Piezoelectric force detection with only the probe immersed. As discussed before, piezoelectric force detection is widely used in NSOM. The characteristic high spring constant of these techniques, is compensated by a high Q-factor. This Q-factor drops significantly upon immersion in liquid. To prevent a dramatic drop in Q-factor, it is possible to immerse only the probe in liquid.\textsuperscript{27-30} Using this method, care should be taken to distinguish probe-liquid interaction and probe-sample interaction. These methods are also characterized by a critical control of the fluid level. The approach by Naber et al.\textsuperscript{28} is less sensitive to the fluid level, as the fiber probe that is mounted on a tuning fork, oscillates perpendicular to the sample surface. With this method, the amount of displaced water is minimized, however, mounting the fiber probe and the optical alignment will be more elaborate.

Tuning-fork-based NSOM in liquid. Chapter 3 already discussed tuning-fork-based shear-force detection in liquid. It is clear that interaction forces in liquid are significantly higher than under ambient conditions. However, shear-force detection is still feasible, especially on flat, relative hard substrates and NSOM in liquid should be possible using tuning forks. For reliable imaging it is necessary to develop a closed, temperature controlled cell, as up to now, the stability is infringed by evaporation and temperature changes.

6.3.3 Tuning-fork-based NSOM in liquid
To test the potential for NSOM in liquid with tuning-fork-based shear-force detection, the DNA sample labeled with Cy3.5 molecules described in § 5.5, is imaged under ambient conditions and with the sample immersed in HPLC-grade water. Excitation wavelength, excitation power and all other experimental parameters, except for the probe, were the same as for the image in Figure 5-4 (a) and (b).

Figure 6-1 shows the resulting image. The image upon immersion in water has a different appearance. The fluorescence intensity, after background subtraction, is comparable before and after immersion of the sample and has a value of \( \sim 3.0 \) kcounts/s. The intensity of the background however, has risen from 50 counts/s to 4.8 kcounts/s.

The origin of the increase in background is unclear. An explanation could be that the DNA detaches from the surface and contaminates the water. Although this could explain some increase in background, it is unlikely that this argument is the sole explanation for the observed increase in background, as around two DNA molecules have to be in the excitation volume of the probe. Another, more likely, explanation could be contamination of the HPLC water with fluorescent molecules.
6.4 Conclusions

Two developments can be expected for the application of tuning forks in the near future. First is the application of tuning forks as force sensor in noncontact AFM. Replacing the cantilever, laser diode and position sensitive detector by a tuning fork, allows the design of a simple and cheap instrument for routine topographic measurements. Such instrument would have the advantages of easy probe replacement, small probe oscillation amplitudes, lack of optics and automated operation.

For NSOM under ambient conditions, tuning forks have already been the most used force detector for several years, thanks to the reliability, ease of use and flexibility of tuning forks. With the possibility to apply tuning forks in a liquid environment, it can be expected that a new range of applications will emerge in biology, where the high resolution and combined topography of NSOM are important advantages.
6.5 References


6 General discussion and outlook
Summary

Quartz tuning forks, originally designed for frequency control, are sensitive nonoptical force sensors in Scanning Probe Microscopy (SPM). Within a few years since the introduction in 1995, tuning forks have become the most used force sensor in Near-field Scanning Optical Microscopy (NSOM). The advantages of tuning forks as force sensor in SPM, such as the simplicity, compact size, the lack of optical detection and the low price have led to the application of tuning forks in other types of SPM.

This thesis explores new applications of tuning forks in SPM. These applications fall into two categories: the application as force detector in Atomic Force Microscopy (AFM) as alternative to common cantilever-type force sensors and the application as shear force detector in NSOM for biological applications.

Chapter 1 introduces quartz piezoelectric tuning forks as force sensor. A probe is mounted on a tuning fork such that either a force normal to the sample surface or a shear force parallel to the sample surface is detected. To detect an interaction force, a tuning fork is mechanically or electrically brought into resonance. Interaction between probe and sample changes the tuning fork motion and accordingly the piezoelectric signal. Chapter 1 concludes with a discussion on the physical origin of shear force interaction.

Chapter 2 describes tuning forks as force sensors in more detail. A finite element model is developed that demonstrates that a fiber probe introduces asymmetry in the tuning fork, increasing the drive efficiency of the mechanically driven tuning fork. A harmonic oscillator model enables calculation of the magnitude of the interaction forces between probe and sample, both when the amplitude of the piezoelectric tuning fork signal is used and when the phase of the tuning fork signal with respect to the drive is used as a measure for probe to sample interaction. Finally, a comparison is made between tuning-fork-based AFM and AFM based on conventional cantilevers.

To allow biological application of SPM with tuning forks as force sensor, it is necessary to immerse the sample and thus the tuning fork in a liquid environment. Chapter 3 studies the behavior of tuning forks in liquid. The resonance characteristics of a tuning fork, that change dramatically upon contact between tuning fork and liquid, recover partially upon complete immersion of the tuning fork. Because of this partial recovery, it is possible to use tuning forks for shear force microscopy in a liquid environment.
The Q-factor of a tuning fork, defined as the resonance frequency divided by the full width at half the maximum amplitude of the resonance spectrum of a tuning fork, is an important parameter for the sensitivity of a tuning fork. This Q-factor can be increased with an electronic circuit. Simulations and experiment presented in Chapter 4 show that the sensitivity of tuning forks, if limited by thermal noise, does not improve. If the sensitivity is limited by detector noise on the tuning fork signal, the detection of the phase of the tuning fork signal improves, if the phase detection is based on zero-crossings.

An important application of fluorescence microscopy with single molecular sensitivity is localization of single fluorescent molecules used to mark proteins. Chapter 5 explores the achievable localization accuracy with NSOM in theory and experiment. Thanks to the high resolution of NSOM, the observed localization accuracy of 3 nanometer is an order of magnitude better compared to far-field techniques. With far-field techniques comparable accuracies have only been obtained at cryogenic temperatures.

Chapter 6 discusses potential new applications of tuning forks in SPM. New applications can again be divided in AFM applications and NSOM applications. For NSOM, there is an emphasis on application of tuning forks and alternative force detectors in liquid environments. Preliminary results show that single fluorescent molecule detection in liquid is feasible using tuning-fork-based NSOM.

This thesis shows that tuning forks are sensitive, versatile force detectors usable from vacuum to liquid environments. The numerous advantages of tuning forks, such as ease-of-use, the lack of optics, the compact size, wide availability and the low price, enable new applications of tuning forks in SPM.
Samenvatting


In dit proefschrift worden nieuwe toepassingen van stemvorken in SPM verkend. Deze toepassingen vallen in twee categorieën: de toepassing als krachtsensor in atomaire krachtmicroscopie als alternatief voor de gangbare bladveersensor en de toepassing als shear force sensor in nabije-veld optische microscopie ten behoeve van biologische toepassingen.

Hoofdstuk 1 introduceert kwarts piezo-elektrische stemvorken als krachtsensor. Een probe wordt zo danig op de stemvork gemonteerd dat of een interactiekraft normaal op het preparaatoppervlak of een shear force interactiekraft parallel aan het preparaatoppervlak gedetecteerd kan worden. Om een kracht te meten wordt een stemvork elektrisch of mechanisch in resonantie gebracht. Interactie tussen probe en preparaat verandert de beweging van de stemvork en daarmee het piezo-elektrische signaal van de stemvork. Hoofdstuk 1 eindigt met een discussie over de fysische achtergrond van shear force interactie.

Hoofdstuk 2 gaat nader in op de stemvork als krachtsensor. Een eindige elementen model wordt ontwikkeld waarmee duidelijk wordt dat een fiber-probe de stemvork asymmetrisch maakt waardoor de efficiëntie van mechanische aanrijving groter wordt. Met behulp van een harmonische-oscillator-model is het mogelijk de grootte van de interactiekrachten tussen probe en preparaat te berekenen, zowel voor het gebruik van de amplitude van het piezo-elektrische stemvorksignaal als het faseverschil tussen het stemvorksignal en de aandrijving als maat voor interactie tussen probe en preparaat. Tot slot wordt een vergelijking gemaakt tussen atomaire krachtmicroscopie gebaseerd op stemvorken en gebaseerd op conventionele bladveertjes.

Om biologische toepassingen van SPM met stemvorken als krachtsensor mogelijk te maken, is het nodig om het preparaat en dus ook de stemvork in een vloeistofomgeving te plaatsen. Hoofdstuk 3 bestudeert het gedrag van stemvorken in
Samenvatting

vloestof. De resonantiekarakteristiek, die verandert als de stemvork in contact komt met vloestof herstelt zich gedeeltelijk als de stemvork helemaal wordt ongedoemd. Door dit gedeeltelijk herstel blijkt het mogelijk stemvorken voor shear force microscopie te gebruiken in een vloestofomgeving.

De Q-factor van een stemvork, gedefinieerd als de resonantiefrequentie gedeeld door de breedte van de resonantie curve van de stemvork, is een belangrijke parameter voor de gevoeligheid van de stemvork. Deze Q-factor kan met behulp van een elektronisch circuit vergroot worden. Uit simulaties en experiment gepresenteerd in Hoofdstuk 4 blijkt dat de gevoeligheid van de stemvork, wanneer deze beperkt wordt door thermische ruim, niet verbetert. Indien de gevoeligheid van de stemvork beperkt wordt door detectie van op het piezo-elektrische signaal, verbetert de fasedetectie als deze op bepaling van nuldoorgangen is gebaseerd.

Een belangrijke toepassing van fluorescentiemicroscopie met moleculaire gevoeligheid is de localisatie van fluorescerende moleculen die gebruikt worden om eiwitten te markeren. In Hoofdstuk 5 wordt de haalbare localisatiewerkelijkheid in theorie en praktijk uitgewerkt voor nabij veld optische microscopie. Door de hoge resolutie van nabij veld optische microscopie is de localisatiewerkelijkheid met 3 nanometer een orde beter in vergelijking met verre veld microscopie. Een vergelijkbare nauwkeurigheid kan met de laatste techniek alleen worden behaald bij cryogene temperaturen.

Hoofdstuk 6 bespreekt potentiële nieuwe toepassingen van stemvorken in SPM. Ook hier kan weer een tweeling gemaakt worden tussen toepassing in atomaire krachtmicroscopie en nabij veld optische microscopie. In de laatste categorie ligt de nadruk op toepassing van stemvorken en alternatieve krachtdetectie in een vloestofomgeving. Voorlopige resultaten laten zien dat detectie van individuele fluorescente moleculen in vloestof mogelijk is met behulp van op stemvorken gebaseerde nabij veld optische microscopie.

Dit proefschrift laat zien dat stemvorken gevoelige, veelzijdige krachtsensoren zijn, die inzetbaar zijn van vacuüm- tot vloestofomgevingen. De vele voordelen, zoals eenvoud in gebruik, het niet-optische karakter, het kleine formaat, de grote beschikbaarheid en de lage prijs, maken nieuwe toepassingen in SPM mogelijk.
Nawoord

‘Bij het lezen van een proefschrift besteedt de gemiddelde lezer de meeste tijd aan de dingen waaraan de auteur de minste tijd heeft besteed’.

Dit was een van de potentiële stellingen die ik op mijn lijstje had staan. Ik denk dat de stelling waar is. De meeste lezers bekijken de omslag en bladeren vervolgens snel door het proefschrift om het nawoord te lezen. Waarom gaan lezers in luttele seconden door de inhoud waaraan vier jaar hard is gewerkt, om uitgebreid een nawoord te lezen dat in een middagje wordt geschreven? Wellicht om te zien of hun naam genoemd wordt in een lijst namen van mensen die worden bedankt. Misschien ook wel in de hoop dat er iets grappigs staat, of om een glimp op te vangen van de mens achter het proefschrift. Dit zullen op zich wel drijfveren zijn, maar de bedanklijstjes zijn vaak voorspelbaar en voor humor kun je beter iets anders lezen dan een proefschrift. Bovendien zullen de meeste lezers de mens achter het proefschrift al beter kennen dan duidelijk wordt uit een nawoord. Ik denk dat de belangrijkste reden voor de interesse in het nawoord iets anders is. Zoals gezegd is een proefschrift gevuld met een aantal jaren werk. Daarvan vind je alleen de resultaten terug, maar je vindt niets over de inspanning om die resultaten te bereiken. Zelf vind ik juist de ervaringen van promotendi tijdens het onderzoek interessant om te lezen. Niet erg wetenschappelijk misschien, maar wel menselijk. Dat roept natuurlijk de vraag op hoe mijn ervaringen in de afgelopen jaren waren.

Volgens mij is een promotieonderzoek te vergelijken met een fietsvakantie. Voor vertrek zoek je al je bagage bij elkaar: je vooropleiding. Je stelt samen met je promotor het doel vast dat je wilt bereiken en vervolgens kan de route worden uitgestippeld. Het is duidelijk waar belangrijke mijlpalen staan en waar zware bergpassen zijn, maar je bent degelijk voorbereid en vol goede moed ga je op weg. In mijn geval was het doel enerzijds de ontwikkeling van een nabije-veld optische microscoop voor biologische toepassingen, anderzijds het meten van ‘iets’ biologisch met de nieuwe microscoop en dat alles in samenwerking met TopoMetrix, een fabrikant van dit type microscopen in Silicon Valley. De grootste uitdaging was het controleren van de afstand tussen fibertip en preparaat met een kwarts stemvork in een vloeistof omgeving. Ik had allerlei ideeën en bevond me in een enthousiaste omgeving. Ook het vele reizen dat ik in de eerste fase van mijn onderzoek mocht doen was erg leuk. Maar toen kreeg ik de eerste lekke band. Het bleek onverwacht te kostbaar om nieuwe stemvorken te laten maken en ik moest het maar gewoon eens zo proberen in vloeistof. Met een snel geplakte band ging de tocht weer vrolijk verder. Het is nog steeds niet helemaal duidelijk waarom, maar het meten in water bleek redelijk goed te gaan. Ondertussen was het duidelijk
geworden dat TopoMetrix niet veel langer zelfstandig door zou gaan. De vergelijking tussen stemvorken en de standaard bladvleermuizen tstumaar krachtsmicroscopie die ik heb uitgevoerd om het bedrijf gunstig te etaleren, resulteerde in mijn eerste publicatie. Het schrijven daarvan was een pittig klimmje, maar toen het eenmaal gepubliceerd was, volgde een mooie afslag. Kortom, het ging voor de wind en overmoedig begon ik aan de steilste klim van de reis. Ik wilde graag een hoogteplaatje maken van levende cellen. Dat viel niet mee. Ook niet nadat er een andere opstelling was gebouwd waarmee ik beter kon zien wat er gebeurde. De cellen kregen het te koud voor ik ze goed en wel kon meten. Ook een elektronische truc om de gevoeligheid te verbeteren bleek niet echt te helpen. Wel is veel duidelijk geworden over de zin en onzin van deze truc, ook in andere toepassingen. Ondertussen begon de tijd aardig op te raken: het werd nodig om de route te verleggen. In plaats van levende cellen was DNA ook een biologisch sample waar interessante dingen aan gemeten konden worden. Op de valreep bleek dat positiebepaling met nabije-veld optica goede resultaten opleverde in vergelijking met reguliere verre-veld optica. En tot slot lukte het nog om aan DNA gebonden fluorescente moleculen in water te meten. Dat leverde meer vragen op dan het oploste, maar voor mij was daarmee wel het eind van mijn tocht bereikt.

Terugkijkend is het een mooie tocht geweest met stevige beklimmingen, mooie afslagen en de nodige verassingen.

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De tocht gaat natuurlijk verder en ik wens Marjolein en Barbel daarbij veel succes.

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En nu op weg naar nieuwe avonturen!

Wouter