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Probing Biological Bonds
by Means of Rotation of Superparamagnetic Beads

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ABSTRACT

The ability of superparamagnetic beads to rotate in a rotating magnetic field yields the possibility for probing the rotational properties of biological interactions with these beads. Torques can be applied to the beads, depending on the magnitude of a permanent magnetisation present in the bead. This research focuses on developing tools to easily determine the bead’s permanent magnetisation and derive functional information by investigating the beads’ rotational behaviour in rotating or static magnetic fields.

Homogeneous magnetic fields are generated with a newly designed magnetic quadrupole and its current regulator. Magnetic fields of 37 mT can be reached for currents of 1 A. The field has a virtually absent horizontal gradient and a vertical gradient that yields forces on the order of magnitude of the gravitational force that acts on the bead. An image analysis toolbox for Matlab has been developed to derive the orientation of the bead as function of time. This is done by image correlation with respect to the rotation of the bead.

The image analysis toolbox enables the development of techniques to quickly measure the permanent magnetic moment of an unbound bead in a rotating magnetic field. These techniques have an accuracy of 10% for the determination of the permanent magnetic moment. For bound beads a harmonic potential well is introduced to describe the bond’s behaviour under stress. A method to simultaneously measure the bead’s permanent magnetic moment and the torsion spring constant, involved in the harmonic potential, is presented. This method, which does not require the bead to continuously rotate, was shown to have an accuracy of 10% for the determination of permanent magnetisation of an unbound bead.

It was shown that a bead, in a field that instantaneously switches its direction, turns over an angle that increases for increasing field strength. The corresponding spring torsion constant was found to be about $4 \times 10^{-18}$ Nm. The typical error on this measurement is 35%. Experiments with bound beads are done with Streptavidin-coated beads that bind to Biotin-BSA incubated on the solid phase. This system showed 2 different kinds of behaviour for bound beads in a rotating field. Beads are restricted to an oscillating movement for low fields, while for higher fields an energy barrier can be overcome and beads can rotate.
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1 INTRODUCTION

Biosensors are devices that are developed to measure concentrations of specific biological target molecules in a fluid. A common type of biosensor is the sandwich immuno-assay (figure 1.1). In this device, the target molecules is sandwiched between two antibodies. The primary antibody is immobilised on the surface. The secondary antibody carries a label, e.g. a fluorescent dye. When unbound secondary antibodies are removed, the number of remaining labels is related to the concentration of target molecules.

![Figure 1.1: Working principle of a sandwich immuno-assay](image)

Because of its low background in complex biological fluids, superparamagnetic beads are very promising for this purpose. A second advantage of superparamagnetic beads is that, besides being used as label, they can be manipulated by means of an externally applied magnetic field. The research group Molecular Biosensors for Medical Diagnostics (MBx) at Eindhoven University of Technology (TU/e) aims for a biosensor that is also capable of actively probing functional information of the molecules, based on the actuation of superparamagnetic beads.[1]

It is shown that it is possible to let a bead rotate in a rotating magnetic field.[2]. For frequencies below 20Hz, this is due to a permanent magnetisation of the bead. When this magnetisation is not aligned with the external field, a torque is applied on the bead and the bead rotates. The magnitude of the torque depends on the external field strength, the bead magnetic moment and their mutual angle. Since the magnetic moments of beads in a single batch show a variation in magnitude, it has to be measured during the experiment. The fact that torques of a controllable magnitude can be applied to a superparamagnetic bead yields the possibility to probe for the rotational properties of biological molecules or the bond between biological molecules.
If a magnetic torque is applied to a bound bead, the bead will tend to rotate in the direction of the applied torque. This rotation can happen over only a small angle, because the bond, due to its stiffness, will apply an additional torque on the bead, opposed to the magnetic torque. For small angles, the energy needed to rotate a bead over a certain angle can be approximated by a harmonic potential well (figure 1.2).

![Figure 1.2: Harmonic potential well around an equilibrium angle $\theta_0$.](image)

Such a harmonic potential well is defined by the equilibrium orientation of the bound bead, $\theta_0$, and a torsion spring constant, $k$. The energy, with respect to a certain orientation $\theta$ is given by:

$$U = \frac{1}{2} k (\theta - \theta_0)^2$$

If such a harmonic potential around the equilibrium orientation of a bead can be defined, rotational bond force measurements are possible by measuring at which angle the bond breaks. This research focuses on measuring the torsion spring constant of the biological bonds, together with the determination of the permanent magnetic moment of the bead.
2 BEAD ROTATION

2.1 Superparamagnetic Beads

Superparamagnetic beads exist of a polystyrene shell that contains grains of magnetite (Fe$_3$O$_4$). These grains typically have sizes of about 6nm to 12nm. Figure 2.1 schematically shows such a superparamagnetic bead. The beads used in this research (Dynabeads M-280, Dynal Biotech ASA) have a diameter of 2.83µm.

![Figure 2.1: Sketch of superparamagnetic beads.](image)

The magnetisation of such a grain has a preferential direction, called the easy axis, determined by the orientation of the crystal lattice and the shape of the grain. This dependence of magnetic properties with respect to the easy axis is called magnetic anisotropy. A grain can flip its direction of magnetisation along the easy axis, when an energy barrier $KV$ can be overcome, where $K$ is the magnetic anisotropy energy density, maximally $1.8 \times 10^8$ J/m$^3$ [3] for magnetite, and $V$ is the volume of the grain. When, in the absence of an external magnetic field, this energy barrier is overcome by means of thermal energy, the typical flipping time, $\tau_{flip}$, is given by a Boltzmann factor:

$$\tau_{flip} = \tau_0 \exp \left( \frac{KV}{k_B T} \right)$$

(2.1)

where $\tau_0 = 10^{-9} – 10^{-10}$ s [4]. Due to the volume dependence the flipping time is longer for larger grains. Depending on the time for a grain to flip its direction of magnetisation, $\tau_{flip}$, a superparamagnetic limit for the grain size can be defined. A maximal flipping time of 1s, yields a maximal diameter for a magnetite grain to be superparamagnetic at room temperature of 21nm (figure 2.2).
In the absence of an external magnetic field, the magnetic moment of each grain points in a random direction; hence the net magnetisation of all grains in a superparamagnetic bead will average to zero. The same goes for the time average of a single grain, because the direction of the magnetisation changes continuously.

In the presence of an external field, with magnetic induction $B$, the interaction of the grain’s magnetic dipole moment, $\mu_{\text{grain}}$, with the field has to be taken into account as well.

$$\tau_s = \tau_0 \exp \left( \frac{KV \pm \mu_{\text{grain}} B}{k_B T} \right)$$  \hspace{1cm} (2.2)

The magnetic moment of the bead is the vector sum of all the magnetic moments of the grains, present in the bead. Since in the presence of and external magnetic field the flipping time depends on whether the grain’s magnetisation is aligned or opposed to the external field, a net induced magnetisation in the direction of and proportional to the external field will exist in the bead. This is the paramagnetic behaviour of a bead, which is expressed as:

$$\vec{\mu}_\text{bead} = \frac{\chi_{\text{bead}}}{\mu_0} \vec{B}$$  \hspace{1cm} (2.3)

where $\mu_0$ is the magnetic permeability of vacuum and $\chi_{\text{bead}}$ is the magnetic susceptibility of the bead. The susceptibility of a bead, $\chi_{\text{bead}}$, is found by multiplying the volume susceptibility of the bead, $\chi_{\text{vol}}$, by the volume of the bead. The volume susceptibility of Dynabeads M280 is 0.756 $[^3]$. Note that an ideal superparamagnetic bead that consists of small grains in a polystyrene shell does not have permanent magnetic moment.
2.2 Existence of a Permanent Magnetic Moment

The behaviour of a superparamagnetic bead in a rotating field has been investigated by Schellekens in 2007 [2]. The magnetic field was generated by means of two perpendicularly crossing wires that carry alternating currents with a 90° phase difference (figure 2.3). This way a rotating magnetic field is generated at the position where both wires cross each other. Since the wires lie at a different height, currents are calculated to have an equal field strength at the centre height of the bead. Images of a rotating bead have been acquired using a microscope with a water immersion objective and a high-speed camera.

![Figure 2.3: Two perpendicularly crossing wires, each carrying an alternating current with a mutual phase difference of 90° can be used to generate a rotating magnetic field.](image)

Rotation of a free bead was studied in a field with different field frequencies. For each field frequency the average rotation frequency of a bead was determined by measuring the time it takes for a bead to rotate over 360°. Figure 2.4 shows the average rotation frequency of a bead as function of the field frequency.

![Figure 2.4: Average rotation frequency of a bead as function of the field frequency.](image)
The bead rotates with a frequency equal to the field frequency, up to a certain maximum rotation frequency for the bead, called the breakdown frequency. For field frequencies higher than the breakdown frequency, the average rotation frequency of the bead decreases for increasing field frequency. The rotation of the bead implies that there must be a torque that acts on the bead that is given by [2]:

\[ \vec{\tau}_{\text{magnetic}} = \vec{\mu}_{\text{bead}} \times \vec{B} \]  

(2.4)

where \( \vec{\mu}_{\text{bead}} \) is the bead’s magnetic moment and \( \vec{B} \) the magnetic induction. Equation 2.4 implies that the bead’s magnetisation is not parallel to the magnetic field. This yields that the magnetisation that is responsible for the rotation of the bead is not induced. If this would be the case, then equation 2.3 and equation 2.4 together yield that there is no torque applied on the bead; hence the bead would not rotate.

\[ \vec{\tau}_{\text{magnetic}} = \vec{\mu}_{\text{bead}} \times \vec{B} = \frac{\chi_{\text{bead}}}{\mu_0} \left( \vec{B} \times \vec{B} \right) = 0 \]  

(2.5)

This is in contrast with the observation that the bead does rotate. The induced magnetic moment only contributes to rotation for field frequencies in the MHz-range [2]. For these field frequencies, the induced magnetic moment lag behind on the magnetic field, because of the finite relaxation time of the induced magnetisation. For low field frequencies the torque due to the induced magnetic moment is negligible with respect to the torque due to the permanent magnetic moment. Therefore in the rest of this work, \( \mu_{\text{bead}} \) refers to the permanent magnetic moment of the bead.

The magnetic torque is opposed by the friction torque, which originates from the drag in the viscous fluid above the chip surface. For bulk medium, the friction torque is given by:

\[ \vec{\tau}_{\text{friction}} = -8\pi\eta R^3 \frac{d\dot{\theta}}{dt} \]  

(2.6)

where \( \eta \) is the dynamic viscosity of the medium and \( R \) is the radius of the bead. Figure 2.5 shows the different torques acting on a bead with a magnetisation that is not aligned with the field.
The inertia of the bead is negligible with respect to the viscous drag. The differential equation that governs the movement of the bead is found by expressing that the total torque on the bead is zero:

\[ 8\pi \eta R^3 \frac{d\dot{\theta}}{dt} = \vec{\mu}_{\text{bead}} \times \vec{B} \]  

(2.7)

Below the breakdown frequency, there is a constant phase lag between the magnetic field and the bead’s magnetisation. This results in a constant torque and hence a constant angular velocity of the bead.
For field frequencies higher than the breakdown frequency the bead cannot follow the magnetic field, hence the phase difference is not constant in time. The movement of the bead exists of two periods. There is the forward period, when the bead moves in the same direction as the field. The phase difference increases from 0 to π/2 while the magnetic torque increases and the bead starts rotating faster. Then the phase difference further increases from π/2 to π, while the magnetic torque decreases and the bead slows down again, until it stops for a phase difference of π, when the magnetic torque is zero. After that, the backward period starts. From -π to -π/2 the magnetic torque is opposed to the rotation direction of the magnetic field and the bead is accelerated in the opposite direction. From -π/2 to 0 the magnetic torque decreases again and the bead slows down, until for a phase difference of 0 the bead stops because the magnetic torque is zero again. After that the forward period starts again. This results in an oscillating movement of the bead with an average rotation frequency that is lower than the breakdown frequency.

Figure 2.6 shows the result of a simulation of a bead in water \[^2\]. On the left the average bead frequency is plotted as function of the field frequency. On the right, the
orientation of the bead as function of time is plotted for a field frequency a little below the breakdown frequency and few higher frequencies. It shows the oscillating movement of a bead for field frequencies above the breakdown frequency and how the oscillation frequency increases for increasing field frequency.

![Figure 2.6 Simulation of a bead rotating in water](image)

The breakdown frequency is the maximum rotation frequency that a bead can reach. It is reached when the magnetic torque is maximal. The maximum magnetic torque is given by $\mu_{\text{bead}}B$, so that the breakdown angular frequency, $\omega_{BD}$, is given by:

$$\omega_{BD} = \frac{\mu_{\text{bead}}B}{8\pi \eta R^3}$$  \hspace{1cm} (2.8)

It was shown by Schellekens that the breakdown frequency is proportional to the field strength \cite{2} (figure 2.7), which confirms that beads have a permanent magnetic moment.

![Figure 2.7 Breakdown frequency of a superparamagnetic bead as function of the field strength](image)

Schellekens showed a spread in the permanent magnetic moment for Dynabeads in a single batch of a factor of 5. For that reason the magnetic moment of each bead in particular has to be measured during an experiment in order to calculate the torque applied to a bead. First the breakdown frequency is measured, from which the permanent magnetic moment is calculated by means of equation 2.7. The method as
used by Schellekes, based on finding the maximum rotation frequency of the bead, requires several measurements at different field frequencies, which is a time consuming procedure. In order to do experiments on several beads at a time, a faster way to determine the breakdown frequency is needed.

2.3 Oscillations of a Bead Above Breakdown Frequency

When a magnetic field rotates in a plane perpendicular to a given axis of rotation, the magnetisation of a bead will be aligned in the plane perpendicular to this axis. In this plane, the magnetic torque can be expressed as:

$$\tau_{\text{magnetic}} = \mu B \sin(\omega_{\text{field}} t - \theta)$$  \hspace{1cm} (2.9)

where $\mu$ is the permanent magnetic moment of the bead, $\omega_{\text{field}}$ is the angular rotation frequency of the field and $\theta$ is the time dependent orientation of the bead. Then the differential equation 2.6 reads:

$$8 \pi \eta R^3 \frac{d\theta}{dt} = \mu B \sin(\omega_{\text{field}} t - \theta)$$  \hspace{1cm} (2.10)

Or by applying equation 2.7:

$$\frac{d\theta}{dt} = \omega_{\text{BD}} \sin(\omega_{\text{field}} t - \theta)$$  \hspace{1cm} (2.11)

For field frequencies below the breakdown frequency, the bead’s rotation frequency (left hand side in equation 2.11) equals the field frequency. This yields that the phase difference between the bead and the magnetic field is given by:

$$\omega_{\text{field}} t - \theta = \arcsin \left( \frac{\omega_{\text{field}}}{\omega_{\text{BD}}} \right)$$  \hspace{1cm} (2.12)

Note that the phase difference is limited between $-\pi/2$ and $\pi/2$ and has the same sign as $\omega_{\text{field}}$. This implies that the bead always lags behind on the field, unregarded the rotation direction of the field. For field frequencies above the breakdown frequency, the phase difference is not constant, resulting in an oscillating movement, as is shown in figure 2.8. During the forward period the phase difference lies between 0 and $\pi$, while during the backward period the phase difference lies between $-\pi$ and 0.
Figure 2.8: Oscillating movement above breakdown frequency. The forward period and backward period are marked.

In order to calculate the duration of the forward and backward period, the phase difference, \( \alpha \), is introduced as new variable in equation 2.11.

\[
\alpha = \omega_{\text{field}} t - \theta \iff \theta = \omega_{\text{field}} t - \alpha
\]  

(2.13)

And equation 2.11 reads:

\[
\omega_{\text{field}} - \frac{d\alpha}{dt} = \omega_{\text{BD}} \sin(\alpha)
\]

(2.14)

From equation 2.14, the duration of the forward period, \( \Delta t_{fw} \), and the backward period, \( \Delta t_{bw} \), can be calculated by separation of variables and integrating over \([0, \pi]\) and \([\pi, 0]\):

\[
\Delta t_{fw} = \int_{\alpha=0}^{\alpha=\pi} \frac{d\alpha}{\omega_{\text{field}} - \omega_{\text{BD}} \sin(\alpha)} = \frac{2}{\omega_{\text{field}}^2 - \omega_{\text{BD}}^2} \left[ \frac{\pi}{2} + \arctan \left( \frac{\omega_{\text{BD}}}{\sqrt{\omega_{\text{field}}^2 - \omega_{\text{BD}}^2}} \right) \right]
\]

\[
\Delta t_{bw} = \int_{\alpha=\pi}^{\alpha=0} \frac{d\alpha}{\omega_{\text{field}} - \omega_{\text{BD}} \sin(\alpha)} = \frac{2}{\omega_{\text{field}}^2 - \omega_{\text{BD}}^2} \left[ \frac{\pi}{2} - \arctan \left( \frac{\omega_{\text{BD}}}{\sqrt{\omega_{\text{field}}^2 - \omega_{\text{BD}}^2}} \right) \right]
\]

(2.15)

Because the forward period takes longer than the backward period, the bead moves on average in the same direction as the field, but with an oscillating movement (figure 2.8). The oscillation frequency, \( \omega_{\text{osci}} \), can be calculated from the sum of \( \tau_{fw} \) and \( \tau_{bw} \):

\[
\omega_{\text{osci}} = \left( \frac{d\alpha}{dt} \right)_{t = \Delta t_{fw} + \Delta t_{bw}} = \frac{2\pi}{\Delta t_{fw} + \Delta t_{bw}} = \frac{2\pi}{\omega_{\text{field}}^2 - \omega_{\text{BD}}^2}
\]

(2.16)

By means of equation 2.13 the average angular velocity of the bead can also be derived:
The average rotation frequency of the bead is lower than the field frequency, and depends on the breakdown frequency. For field frequencies below the breakdown frequency, the argument of the square root is negative and \( \omega_{oscil} \) is undefined. In this case the bead does not oscillate but has a constant rotation frequency that equals the field frequency.

### 2.4 Bound Beads

In contrast to a free bead, a bound bead can rotate only a finite number of times. In this case the concern goes out to the equilibrium orientation of a bound bead in a static magnetic field and the movement of the bead towards equilibrium. In the absence of a magnetic field, a bead that is bound to the surface has a certain equilibrium orientation, \( \theta_0 \). Energy has to be delivered to the bond in order to orient the bead in a different direction. For small angles, this energy can be described by a harmonic potential:

\[
U_{\text{harmonic}} = \frac{1}{2} k (\theta - \theta_0)^2
\]  

where \( k \) is the torsion spring constant of the bond. This harmonic potential causes an additional torque to be applied to the bead:

\[
\tau_{\text{harmonic}} = -\frac{dU_{\text{harmonic}}}{d\theta} = -k (\theta - \theta_0)
\]  

which has to be added to equation 2.10 to find the new differential equation of motion for bound beads:

\[
8\pi\eta R^3 \frac{d\theta}{dt} = \mu B \sin(\theta_{\text{field}} - \theta) - k (\theta - \theta_0)
\]  

where \( \theta_{\text{field}} \) is the orientation of the static magnetic field (\( d\theta_{\text{field}}/dt = 0 \)). The sine function in equation 2.20 does not allow an analytical solution for this equation, but when the bead’s orientation is near the field’s orientation, \( \theta_{\text{field}} - \theta \ll 1 \), a first order approximation of the sine function can be applied and an analytical solution exists. In figure 2.9 the relative error due to the linear approximation is given as function of \( \theta \). It shows an error of less than 10% for angles smaller than 1.
Equation 2.20 now reads:

$$8\pi\eta R^3 \frac{d\theta}{dt} = -(\mu B + k)\theta + \mu B \Theta_{\text{field}} + k \Theta_0$$

(2.21)

From this equation the equilibrium orientation in the presence of a magnetic field, $\Theta_{\text{equil}}$, can directly be found by expressing that $d\theta/dt = 0$ at equilibrium:

$$\Theta_{\text{equil}} = \frac{\mu B}{\mu B + k} \Theta_{\text{field}} + \frac{k}{\mu B + k} \Theta_0$$

(2.22)

For a bound bead in the presence of a magnetic field, the equilibrium orientation lies between $\Theta_0$ and $\Theta_{\text{field}}$, at the point where $\tau_{\text{harmonic}} = -\tau_{\text{magnetic}}$. Note that for a free bead ($k = 0$) in a magnetic field ($B \neq 0$) $\Theta_{\text{equil}} = \Theta_{\text{field}}$, which confirms that the magnetic moment of a free bead aligns with the field. For a bound bead ($k \neq 0$) in the absence of a magnetic field ($B = 0$) $\Theta_{\text{equil}} = \Theta_0$, which is consistent with the definition of $\Theta_0$ as the bead’s equilibrium position in the absence of a magnetic field.

In order to investigate the temporal behaviour of a bead during relaxation towards its equilibrium position, equation 2.21 has to be solved. The general solution to this equation reads:

$$\Theta(t) = C \cdot \exp\left(\frac{-(\mu B + k) t}{8\pi\eta R^3}\right) + \Theta_{\text{equil}}$$

(2.23)

where C depends on the initial orientation of the bead. Write for this initial orientation $\Theta_{\text{init}} = \Theta(t = 0)$, then:

$$\Theta_{\text{init}} = C + \Theta_{\text{equil}} \iff C = \Theta_{\text{init}} - \Theta_{\text{equil}}$$

(2.24)

so that the final solution of equation 2.21 is given by:
\[ \theta(t) = \left( \theta_{\text{init}} - \theta_{\text{equil}} \right) \exp \left( -\frac{\mu B + k}{8\pi \eta R^3} t \right) + \theta_{\text{equil}} \]  

This is the equation of a mono-exponential relaxation from \( \theta_{\text{init}} \) to \( \theta_{\text{equil}} \), with a relaxation rate constant \( \lambda = (\mu B + k) / 8\pi \eta R^3 \).
3 IMAGE ANALYSIS

The procedure to determine the permanent magnetic moment of a bead by measuring the breakdown, is based on measuring the average rotation frequency of the bead as function of the field frequency. The average rotation frequency is determined from a time-stack of images, acquired with a high-speed camera, by means of the correlation coefficient between subsequent images. A fixed region of interest in the first image is chosen and the correlation is calculated with the same region of interest in every other image. The periodicity of the array correlation coefficients obtained this way, yields the average rotation frequency of the bead. In order to investigate the oscillating movement of a bead at field frequencies above the breakdown frequency or the relaxation towards an equilibrium position in a static field, as described in the former chapter, it is necessary to measure the orientation of the bead with respect to a reference orientation as function of time. A different image analysis algorithm is needed for this goal and has been developed. This chapter gives a detailed description of the steps in this algorithm. Basically, the angle of the bead in every image with respect to the orientation in the first image is calculated. The first image in the time-stack plays the role of reference and is per definition rotated over 0°. The angle is calculated by converting the image to polar coordinates and next calculating the spatial correlation with respect to the polar angle. The maximum in the correlation spectrum then yields the mutual angle.

3.1 Polar Coordinates

If a Cartesian image of a bead is converted to polar coordinates, with the centre of the bead as origin of the polar coordinates system, a rotation of the bead about its centre in a Cartesian image, corresponds to a shift in the polar image. For this reason rotation is investigated in polar coordinates.

First of all the centre of the bead has to be derived. This is done by comparing the intensity of each pixel with a reference intensity. The pixel intensities are normalised; the most intense pixel is set to 1 and the least intense pixel is set to 0. The reference height is defined as a number between 0 and 1, usually 0.4. The coordinates of all pixels that are less intense than the reference intensity are collected (figure 3.1). The centre between the minimal and maximal $x$-coordinate is defined as the $x$-coordinate of the centre of the bead. The $y$-coordinate of the centre is found analogously. With this procedure an accuracy of half a pixel is reached.
Now if the centre is known, the image can be converted to polar coordinates. For radius $r$ and polar angle $\theta$, and for $(x_0, y_0)$ being the centre of the bead, the corresponding coordinates in the Cartesian image $(x, y)$ are found as:

$$\begin{align*}
x &= x_0 + r \cos(\theta) \\
y &= y_0 + r \sin(\theta)
\end{align*}$$

(3.1)

The polar angle $\theta$ goes from $0^\circ$ to $359^\circ$ in steps of $1^\circ$. The radius $r$ goes from a given start-value to a given end-value in steps of one pixel, to select a ring on the bead. The start-value is typically 3 pixels because smaller radii contain too little pixels to considerably contribute to the correlation function. The end-value is typically 20 pixels. Images are acquired with a high-speed camera (MotionPro HS-3) and a total magnification of $1260 \times$. For this setup the physical pixel size is 91 nm. Since a bead has a radius of 1.4 µm, 20 pixels is little more than the bead’s radius.

Except for some trivial cases, $x$- and $y$-coordinates that correspond to given polar coordinates, are not integer numbers, hence the intensity cannot directly be taken from the Cartesian image. Instead, the corresponding intensity is calculated as the weighted average of the four nearest neighbours with integer coordinates (figure 3.2). The point with non-integer coordinates defines four rectangles in the square defined by the four nearest neighbours with integer coordinates. The area of each rectangle is used as weight for the opposing pixel value.

Figure 3.2: Rectangles defined by a point with non-integer coordinates
\[ I(r, \theta) = \sum_{i=1}^{4} I_i A_i \] (3.2)

Note that the sum of the area’s of the four rectangles equals unity, which is necessary for the weighted average.

### 3.2 Image Correlation

The correlation function of an image rotated with respect to a reference image can be used to determine the angle of rotation when the correlation is calculated as function of the polar angle. Consequently, since the first reference image is per definition rotated over 0°, the maximum in the correlation spectrum of an image indicates the angle over which the bead in that image has rotated.

In figure 3.2 the reference image, of a sketched bead, and the actual image of the bead are shown on the left. The rotation of the bead is represented in the images in polar coordinates (figure 3.2, right) by a horizontal shift. The correlation function with respect to the pole-angle is shown in figure 3.3. The maximum correlation yields that the bead rotated over 60°.

![Image](image.png)

Figure 3.2: Reference (top) and actual image (bottom) in Cartesian (left) and polar (right) coordinates.
Figure 3.3: Correlation function with respect to the pole-angle. The maximum in the correlation spectrum indicates their mutual rotation angle.

The correlation as function of the polar angle is calculated in 3 basic steps. First the average image is subtracted in order to remove reflections that do not rotate with the bead. Next, the correlation is calculated for each radius in particular. Finally the correlation functions are normalised and summed to obtain the correlation function independent of the radius. These steps are described in detail in the following paragraphs.

3.2.1 Subtraction of Average Image

The intensity distribution over the image has two contributions. The first one originates from inhomogeneities in the bead. These inhomogeneities that rotate with the bead are interesting for tracing rotation. In figure 3.2, they are represented by the round spots on the bead in the cartesian image. On the other hand there are also inhomogeneities that originate from differences in angle between the bead’s surface and the incoming light beam. These inhomogeneities do not rotate with the bead, but remain the same orientation throughout the whole measurement. This is represented by the bright spot on the beads in figure 3.2. Since they do however correlate, they have to be removed before the correlation is calculated.

In the polar image the inhomogeneities that do not rotate are present in every image on the same place, hence they will be represented in an average image. Homogeneities that do rotate with the bead, are averaged out as more images are averaged. It is possible to remove the non-rotating inhomogeneities by subtracting the average image. Finally a constant intensity is added to the image in order the keep the mean intensity of the image constant and prevent pixel intensities from becoming negative.
3.2.2 Correlation Function and Fourier Transforms

The correlation function for a particular radius $r_0$, with respect to the polar angle, of an image with the reference image is defined as:

$$g(r_0, \theta) = \langle I(r_0, \phi + \theta)I_1(r_0, \phi) \rangle_\phi = \frac{1}{N} \sum_\phi \langle I(r_0, \phi + \theta)I_1(r_0, \phi) \rangle$$  \hspace{1cm} (3.3)

where $g$ is the correlation function, $N$ is the number of angles, $I$ and $I_1$ are the intensities of the actual and reference image and $\phi$ and $\theta$ are polar angles. Calculating the correlation function by its definition (equation 3.3) is a very time consuming procedure. On the other hand, this same function can also be expressed by means of Fourier transforms.

$$g(r_0, \theta) = \frac{1}{N} F^{-1}\left( F(I(r_0, \theta))F^*(I_1(r_0, \theta)) \right)$$  \hspace{1cm} (3.4)

where $F$ and $F^{-1}$ are Fourier transform operator with respect to the polar angle and its inverse and $*$ is the complex conjugate operator. When this formula is implemented by means of Fast Fourier Transform (FFT) algorithms, several orders of magnitude of calculation time can be saved.

Equation 3.4 can easily be obtained from equation 3.3 in the continuous case. Then the sum in equation 3.3 becomes an integral and the number of angles becomes the length of the interval:

$$g(r_0, \theta) = \frac{1}{2\pi} \int_0^{2\pi} I(r_0, \phi + \theta)I_1(r_0, \phi) d\phi$$  \hspace{1cm} (3.5)

Now by substituting $\phi$ with $\phi - \theta$ this can be rewritten as a convolution product. Note that $d(\phi - \theta) = d\phi$ and that because of rotation symmetry, integration boundaries are arbitrary, as long as integration happens over an interval of length $2\pi$.

$$g(r_0, \theta) = \frac{1}{2\pi} \int_0^{2\pi} I(r_0, \phi)I_1(r_0, \phi - \theta) d\phi = \frac{1}{2\pi} I(r_0, \theta) \otimes I_1(r_0, -\theta)$$  \hspace{1cm} (3.6)

where $\otimes$ denotes the convolution product. The convolution product becomes an ordinary product in Fourier space:

$$g(r_0, \theta) = \frac{1}{2\pi} I(r_0, \theta) \otimes I_1(r_0, -\theta) = \frac{1}{2\pi} F^{-1}\left( F(I(r_0, \theta))F(I_1(r_0, -\theta)) \right)$$  \hspace{1cm} (3.7)
Now substitute \( F(I_1(r_0,-\theta)) = F^\ast(I_1(r_0,\theta)) \) in equation 3.7 and the continuous analogon of 3.4 arises. For this last step, note that images contain real data and hence equal their complex conjugate.

\[
F(I(r_0,-\theta)) = \frac{1}{2\pi} \int_{0}^{2\pi} I(r_0,-\theta)e^{-i\omega\theta} d\theta = \frac{1}{2\pi} \int_{-2\pi}^{0} I(r_0,\theta)e^{i\omega\theta} d\theta \\
= \frac{1}{2\pi} \int_{-2\pi}^{0} I^\ast(r_0,\theta)e^{i\omega\theta} d\theta = \frac{1}{2\pi} \int_{0}^{2\pi} (I(r_0,\theta)e^{-i\omega\theta})^\ast d\theta = F^\ast(I(r_0,\theta))
\]

(3.8)

3.2.3 Normalised Correlation Function

The correlation calculated until now yields a new function for every radius in one image. To gain the overall correlation function that only depends on the pole angle, correlation functions are normalised and summed. The correlation function is normalised by dividing it by the mean intensity of both images and 1 is subtracted to remove the (normalised) offset.

\[
G(\theta) = \sum_{r_0} \left( \frac{g(r_0,\theta)}{\langle I(r_0,\theta) \rangle_\theta \langle I_1(r_0,\theta) \rangle_\theta} - 1 \right) 
\]

(3.9)

3.3 Angle Tracking

When the correlation functions are calculated for every image, it is possible to find the rotation angle as a function of time by following the maximum in the correlation spectrum. Because of artefacts, the angle is not always given by the global maximum of the correlation function, therefore a local maximum is followed. It is certain that the first image is rotated over 0°. For the other images, the local maximum of the correlation function of image \( n \) is sought in the interval \( [\theta_{n-1} - d\theta, \theta_{n-1} + d\theta] \), where \( \theta_{n-1} \) is the angle found in the correlation function of image \( n-1 \) and \( d\theta \) is the upper boundary for the maximum angle over which the bead can rotate between the recordings of two subsequent images.

A second item to take care of is the periodicity of the movement. The image analysis procedure described above yields an angle between 0° and 360°. Therefore 360° is added or subtracted until the difference with the preceding angle is smaller than 180°, in order to find the cumulative angle. This limits the angle over which a bead can rotate between the recordings of two subsequent images to halve a cycle, according to Nyquist’s sampling theorem.
Figure 3.4 shows the time-dependent spatial correlation function of a bead that rotates with a constant frequency of 2Hz. Figure 3.5 right shows the cumulative angle as function of time, found by tracking a local maximum in the correlation function 3.9. On the left, the residuals of a linear fit are shown. Since the bead rotates with a constant angular velocity, this is a measure for the accuracy of the algorithm. The theoretical angular accuracy that can be reached is equal to the step-size of the angle, 1° in this case. In practice the accuracy is only 10° due to noise in the images (figure 3.5 left). The accuracy of 10° is enough for fitting the angle as function of time with the theoretical models described in chapter 2. Statistical error can be decreased by averaging repeated measurements.
As mentioned before, Schellekens [2] used 2 crossing wires to generate a rotating magnetic field. This causes the bead to be pulled strongly, in horizontal as well as in vertical direction, to the point where both wires cross, because of the large gradients that exist around a current wire. The forces that originate from these gradients can influence the outcome of a measurement of the spring torsion constant. A second disadvantage is that only a one bead at a time can be rotate, because the field only rotates over a few square micrometers. To overcome these two disadvantages a new setup has been designed in order to have as small as possible gradients and have a homogenous magnetic field over the entire field of view of the microscope. Consequently, more beads can be investigated simultaneously. The new setup exists of a quadrupole of electromagnets, together with a regulator to drive the currents trough the coils. The quadrupole and current regulator are shown in figure 4.1.

Figure 4.1: Magnetic quadrupole (on top) and its 4 channel current regulator (below).
4.1 Current Regulator

The current regulator is made of a push-pull amplifier that is controlled by an operational amplifier (opamp). The push-pull amplifier exists of a PNP as well as an NPN power transistor, in order to enable the current regulator to source (push) as well as to drain (pull) currents. A scheme of the home built current regulator is shown in figure 4.2. The complete current regulator in figure 4.1 exists of four push-pull amplifiers. The inputs, $V_{in}$, of the amplifiers are connected to four function generators (Agilent 33250A). The power supply connections, $V_{CC}$ and $V_{EE}$, are shared by all amplifiers and connected to the outputs of a symmetric power supply (Agilent E3631A) that delivers a voltage of ±5 V. This voltage is high enough to drive a current up to 1 A through the coils and low enough to not overheat the transistors in the current source for lower currents.

![Figure 4.2: Scheme of the current regulator.](image)

The opamp measures the voltage across the reference resistor, $R_{ref}$, and keeps it equal to the input voltage $V_{in}$. Hence, the output current, $I_{out}$, which is the current through the coil, is proportional to the input voltage, $V_{in}$, with the reference resistance, $R_{ref}$, as proportionality constant. Because the feedback circuit directly measures the current through the coil, non-linear behaviour of the transistors or a change in impedance of the coils due to heating up, are detected and compensated. Measurements showed that the feedback works well for input signals up to 100 Hz. For higher frequencies, the period of the signal becomes comparable to the reaction time of the feedback circuit.

In general, an opamp in a feedback circuit keeps the positive and negative input voltage equal. If, for example, the voltage across $R_{ref}$ is lower than the input voltage, $V_{in}$, the output voltage of the opamp will increase and the output current $I_{out}$ will increase until the voltage across $R_{ref}$ equals $V_{in}$. An analogous situation occurs if the voltage across $R_{ref}$ is higher than the input voltage. A function generator is connected to $V_{in}$ in order to define the shape of the current as a function of time.
4.2 Magnetic Quadrupole

4.2.1 Design

The magnetic quadrupole exists of 4 pole tips, with a coil around it, pointing to the inside and a flux guiding square on the outside (115mm×115mm), as is shown in figure 4.1. All bars are made of soft iron. The bars on the outside measure 10mm×10mm, while the bars of the pole tips and the cores of the coils measure 5mm×5mm. Opposing pole tips are separated 10 mm. In order to have a homogenous field above the pole tips, rather than in between, pole tips are sloped under an angle of 45°. The sample is located at the centre of the quadrupole, 2mm above the top of the poles, at the origin figure 4.3. For that reason, all following measurements of the magnetic field strength are done in this point, unless indicated otherwise.

Simulations (Comsol Multiphysics 3.4) show that pole tips sloped under 45° yield approximately the smallest horizontal gradient. Figure 4.4 shows a comparison of the simulated gradient for pole tips under angle of 30°, 45° and 60°. The horizontal gradient for pole tips under 45° is clearly smaller than in the other cases.

Figure 4.3: Location of the sample in the magnetic quadrupole

Figure 4.4: Horizontal gradient for different slopes of the pole tips. Apparently there is a minimum for about 45°.
Figure 4.5 shows a simulation of a vertical cross section of the quadrupole. It shows how the field lines are curved upward because of the sloped pole tips. The rectangle in the centre of the quadrupole represents the sample under investigation.

Simulations of a horizontal cross-section are shown in figure 4.6. On the left a field from pole to pole is simulated and on the right a field under an angle of 45°. It shows that in the centre the field lines are parallel in the field of view of the microscope, which measures approximately $100\mu m \times 100\mu m$ (top panels). It also shows how the iron square on the outside works as a flux guide (bottom panels). The white rectangles represent the coils.
4.2.2 Characterisation: Linearity and Hysteresis

In this and the next paragraph, the static and dynamic properties of the quadrupole are measured. Before each measurement the coils are demagnetised. Therefore a sinusoidal current of 1 Hz is applied with an initial amplitude that is larger than the highest current applied in the foregoing measurement. The amplitude decreases linearly to zero in 20 seconds.

To see how fast a sudden change of the magnetic field can be applied, the step response has been measured. The response time is defined as the time when the magnetisation reached $1 - e^{-1}$ of its final value. With the GMR-sensor of a Philips biosensor the relative field strength has been measured in time. Figure 4.7 shows the results without feedback together with the result with feedback control by means of the current regulator. From the graph it can be seen that with the current regulator a sudden change of the magnetic field can reached for over 95% in 0.01s. It is also clear that feedback control accelerates this process 3.9 times.

![Figure 4.7: Step response of the current in the coils in de the quadrupole, without (lower curve) and with (upper curve) feedback control. Without feedback the current reaches $1 - e^{-1}$ of its final value in 3.3 ms, while with feedback, this only takes 0.85 ms.](image)

With the same GMR-sensor, the field strength has been measured dynamically while a sinusoidal current with an amplitude of 45.5mA and frequency of 0.2Hz is applied. These are rather low currents in order to stay in the linear regime of the GMR-sensor. The frequency is low enough to avoid distortions due to the reaction time of the current regulator. The signal was sampled with a sampling rate of 100Hz during 20 cycles (National Instruments, BNC-2110 adaptor + LabVIEW 7.1), and averaged. In figure 4.8 the relative field strength is plotted as a function of time (noisy line), together with the relative current (thin line). In figure 4.9, the same data is plotted as relative field strength vs. current.
In figure 4.8 can be seen that the field strength in time is a distorted sine function, but the shape is still adequate for a rotating magnetic field. The graph in figure 4.9 shows the result of hysteresis of the soft-iron poles. Another hysteresis loop has been measured statically with a Hall-sensor (F.W. Bell, STH57-0404) connected to an electronic control unit (F.W. Bell, Model 5070 Gauss/Teslameter). With this self-calibrating sensor, the absolute field strength can be measured. The current has been swept from -400mA to +400mA, in steps of 50mA. The result is shown in the graph in Fig. 4.10.
Within this range no saturation occurs, hence the field strength as function of the current can be linearly approximated (dashed lines) with a proportionality constant of 37 mT/A and an error of 1 mT.

4.2.3 Characterisation: Gradients

In order to determine the magnetic field gradients, the field strength is measured as function of the horizontal coordinate, $x$, and vertical coordinate, $z$, (Figure 4.3). The field strength is measured with the Hall-sensor, while DC-currents of $+277$ mA and $-277$ mA are applied through two opposite coils. The results of the measurements (straight lines) are shown in figure 4.11 and 4.12, for the horizontal gradient, together with simulations (dash-dotted lines).

Figure 4.11: Field strength as function of the horizontal coordinate. The dash-dotted lines are the results from the simulations.
The small asymmetry in the field strength in the \( x \)-direction is because the setup was not exactly level, and hence the Hall-probe is closer to the pole on one side than on the other side. The measured field strength is 20\% lower than predicted by the simulations because of the non-ideal properties of the real quadrupole, compared to the simulations. Figure 4.13 and 4.14 show the measurements and simulations for field strength and the vertical gradient as function of the vertical coordinate, \( z \).
In order to estimate the importance of the measured field gradients on a superparamagnetic bead, the magnetic force that acts on the bead is compared to the gravitational force on the bead. When the permanent magnetisation is negligible with respect to the induced magnetisation, the magnetic force that acts on a paramagnetic bead is given by:

\[ F = \frac{4\pi}{3} R^3 \chi^\prime \left( \frac{B^2}{2\mu_0} \right) = \frac{4\pi}{3} R^3 \chi B \nabla B \]  

(4.1)

where \( R \) is the bead’s radius (\( R = 1.4 \mu m \) for Dynabeads M-280), \( \chi \) is the magnetic susceptibility of the bead (\( \chi = 0.756 \) \[3\]), \( B \) is the magnetic induction and \( \mu_0 \) is the magnetic permeability of vacuum (\( \mu_0 = 4\pi \times 10^{-7} \) T.m/A). The right hand side of the equation allows the force to be calculated when the gradient is know for a given magnetic induction. For a field of 10mT, the horizontal and vertical gradients are smaller than 1T/m in the field of view. This implies that the horizontal and vertical component of the magnetic force acting on a bead are smaller than \( 6.91 \times 10^{-14} \) N. The gravitational force on a dry bead is \( 5.02 \times 10^{-14} \) N and of a bead in water is \( 1.43 \times 10^{-14} \) N. The maximum magnetic force is 1.4 times the gravitational force of a bead, hence they are of the same order of magnitude. In contrast with this is the crossed wires chip discussed before. Simulations showed that for a current of 50mA a field of 4.3mT is present at the centre of the bead, i.e. 2\( \mu m \) above the wire. Since the vertical gradient a this point is 1645 T/m, the bead is pulled down with a force of \( 4.89 \times 10^{-11} \) N, which is orders of magnitude larger than the gravitational force on the bead. Because of the homogeneous field with small gradients, the quadrupole is suitable for bead rotation at every point in the field of view of the microscope.
5 EXPERIMENTS

The procedures to measure the permanent magnetic moment of beads and the equipment developed to generate rotating magnetic fields over large area’s have been experimentally tested on both free and bound beads. Rotating fields for the experiments with free beads are generated with two crossing, current carrying wires and the magnetic quadrupole, described in chapter 4. Experiments on bound beads are only done with the magnetic quadrupole. Obviously, for each measurement a different bead is used.

The permanent magnetic moment of free beads is determined by means of three different methods. Two methods use a rotating field, the third method measures relaxation in a static field.

The first method measures the breakdown frequency by the maximum rotation frequency a bead can reach. The breakdown frequency is related to the permanent magnetic moment, which is calculated subsequently. For field frequencies higher than the breakdown frequency, the bead has a oscillation movement superimposed on the rotation. The frequency of these oscillations is related to the breakdown frequency. Therefore the second method is based on measuring the oscillation frequency, from which the breakdown frequency and the permanent magnetic moment can be derived. The applied field frequency must be chosen higher than the breakdown frequency, before the breakdown frequency is known exactly. This does not lead to an inconsistency of the procedure, since an oscillating moment of the bead arguments that the field frequency high enough.

Instead of in a rotating magnetic field, the permanent magnetic moment of beads is also determined in a static magnetic field. Although this procedure is developed for bound beads, it is applied to a free bead to compare the outcome for the permanent magnetic moment found with the former measurements on the same bead. The experiment is done by instantaneously switching the direction of a quasi-static magnetic field over 90° and back. Immediately after such a switch, the initial position of the bead’s magnetic moment makes an angle of 90° with the magnetic field, since is was aligned in the previous direction of the field. Then the bead starts to align its magnetic moment in the new direction of the magnetic field. Switching the field is done at a frequency of 1 Hz, in order to allow the bead to align with the new direction of the field.

Finally, the behaviour of bound beads is investigated with a rotating field, to determine the properties of a molecular bond upon rotation. The Biotin-Sterptavidin system is used as a model system. This is done with Streptavidin coated beads and Biotin immobilized on the surface. Bound beads are also investigated in a quasi-static field, with different magnetic inductions. Bound beads will not completely align with
the magnetic field. The field switches its direction from $+90^\circ$ to $-90^\circ$ with respect to the orientation of the bead’s permanent magnetic moment, and back (figure 5.1). When the field switches from $\theta_{\text{field}}$ to $\theta'_{\text{field}}$, the bead turns from $\theta_{\text{equil}}$ to $\theta'_{\text{equil}}$.

![Figure 5.1: Relevant angles for the experiment with a bound bead in a quasi-static magnetic field.](image)

5.1 Experimental Setup

Rotating magnetic fields for the determination of the permanent magnetic moment are generated by means of the Philips crossing wires chip, described in paragraph 2.2, or the magnetic quadrupole, described in chapter 4. The rotational behaviour of beads is observed with a Leica DM6000M microscope with a $63\times$ water immersion objective. An additional $10\times$ magnifying lens and a $2\times$ magnifying lens in front of the camera yield a total magnification of $1260\times$. On the crossed wires chip beads are investigated in reflection modus. On the magnetic quadrupole beads are investigated in transmission modus. Images have been recorded with a high-speed camera (MotionPro HS-3) at 100 fps. Since the step-response of the magnetic quadrupole is $10$ ms (figure 4.7), the field can be switched within the time between the recording of 2 subsequent images. Each measurement with the crossed wires exists of a series of $3272$ images, for measurements with the quadrupole series of $2000$ images are recorded. Image series are analysed with the algorithm described in chapter 3, which has been implemented with Matlab 7.4 (The Mathworks), the code can be found in the appendix. A marker for the centre of the bead and one for its orientation are drawn on the image to check the reliability of the outcome of the analysis with the eye.

The crossed wires chip contains a grid of $8 \times 8$ wires, from which only one wire in each direction carries a current. Figure 5.2 shows a cross section and a top-view the chip.
To be able to make images that are bright enough at a high frame rate, the chip has been polished and coated with a gold surface that increases reflection. Since both wires are at a different distance to the bead, the current through the top wire should only be 0.762 times the current through the lower wire $^{[2]}$. The resistance of the bottom wire was $13.2 \, \Omega$ and the resistance of the top wire was $17.2 \, \Omega$. Since the ratio of the resistances is 0.767, the same voltage has been applied to both wires. For experiments in a rotating field, alternating currents that are $90^\circ$ out of phase have been applied with a peak-to-peak voltage of 0.8 V, resulting in a field of 1.87 mT. Also a voltage of 1.2 V was used resulting in a field of 2.80 mT. Experiments in a quasi-static field have been done with DC-voltages of 0.4 V and 0.6 V; hence the same magnetic fields of 1.87 mT and 2.80 mT have been applied. Magnetic beads are introduced from a solution of Dynabeads M280 that has been diluted 2000× with deionised water. Typically a 200 µl drop is put on the chip, which serves as medium for the beads and as immersion medium.

The sample that is placed on the quadrupole exists of a closed cylinindric cell with a diameter of 8.4 mm and a height of 180 µm, sandwiched between two glass coverslides (Menzel Gläzer #1). Inside the cell is a 10 µl drop of a 100× diluted Dynabeads M280 solution in dionised water. For a rotating magnetic field generated with the magnetic quadrupole, the function generators connected to the current regulator are controlled by a LabVIEW 7.1 (National Instruments) program that enables the choice between a rotating field or a field that switches between two given angles. In both cases the frequency and field strength can be chosen. Besides easy application of magnetic fields, the program also offers a built-in demagnetisation procedure for the poles. Therefore a sinusoidal current is simultaneously applied through all four coils. The amplitude decreases linearly in time to zero. The initial field...
strength, the frequency of the current and the number of cycles until the amplitude reaches zero can be chosen.

Biological interactions are investigated on the Biotin-Streptavidin system. This system is chosen as a model-system, because it involves the strongest non-covalent biological bond known in literature. Streptavidin is a protein that has 4 binding sites for Biotin. These binding sites are wells in which the relatively small Biotin molecule fits (figure 5.3).

Biotin is immobilised on the surface by means of Biotinylated Bovine Serum Albumin (Biotin-BSA), which is a protein that typically contains 8 Biotin molecules on its surface. Therefore a solution of 1 mg/ml BSA, from which 0.05% of the molecules are Biotin-BSA, in Phosphate Buffered Saline (PBS) is incubated on a glass coverslide (Menzel Gläzer #1) for 24 hours. Negative controls, on which only non-specific binding can occur are made with BSA only. After incubation, the coverslides are washed in PBS and dried in a nitrogen flow. A closed cylindrical cell is made, with the biologically activated surface on top. The cell contains a 10 µl drop of 100× diluted Dynabeads M280 solution in PBS with 0.05% Tween20 added. In order to let the Streptavidin coated on the beads bind to the Biotin on the activated surface, the sample is turned upside down while gravity pulls the beads towards the activated surface. Meanwhile a static magnetic field of 1 mT is applied by means of the magnetic quadrupole, to align the permanent magnetic moments of all the beads in a sample. After one minute, the sample is turned over again (figure 5.4). Only bound beads can stay on the activated coverslide on top, because unbound beads will fall onto the non-activated coverslide on the bottom of the sample.
5.2 A Free Bead Measured on the Crossed Wires Chip

5.2.1 Results

Figure 5.5 shows an image of a bead on the crossed wires chip, polished and coated with gold. The high reflection of the gold surface results in a bright image with good contrast for analysing rotation.

![Image of a bead on a polished crossed-wire setup, coated with gold.]

Figure 5.5: Image of a bead on a polished crossed-wire setup, coated with gold.

Method 1

For the first method, the average rotation frequency of the bead is measured for different field frequencies. Figure 5.6 shows the first 10 seconds of an analysed measurement, performed with a field frequency of 2.5 Hz and a magnetic field of 2.80 mT. The slope of the line fitted through the data points yields the average rotation frequency of the bead.

![Linear fit of the orientation of a bead as function of time. The slope of the line yields the average rotation frequency of the bead.]

Figure 5.6: Linear fit of the orientation of a bead as function of time. The slope of the line yields the average rotation frequency of the bead.

The average rotation frequency of the bead for two field strengths and as function of the field frequency is given in the graph in figure 5.7. From the graph the breakdown frequency can be estimated:
\[
 f_{BD} = (1.2 \pm 0.1) \text{ Hz} \quad (B = 1.87 \text{ mT}) \quad (5.1)
\]
\[
 f_{BD} = (1.8 \pm 0.1) \text{ Hz} \quad (B = 2.80 \text{ mT}) \quad (5.2)
\]

Figure 5.7: Average rotation frequency as function of the field frequency. For the points on the black line, the bead rotates with the field frequency, hence for the field frequency is lower than the breakdown frequency.

The permanent magnetic moment of the M280 bead was calculated with equation 2.8 using the dynamic viscosity of water at room temperature. \((\eta_{\text{water}} = 10^{-3} \text{ Pa.s})\):

\[
 \mu_{\text{bead}} = (2.8 \pm 0.3) \times 10^{-16} \text{ J/T} \quad (B = 1.87 \text{ mT}) \quad (5.3)
\]
\[
 \mu_{\text{bead}} = (2.9 \pm 0.3) \times 10^{-16} \text{ J/T} \quad (B = 2.80 \text{ mT}) \quad (5.4)
\]

The accuracy of this method depends on the frequency step. A higher accuracy can be obtained by smaller steps in frequency. Due to the amount of measurements that have to be made, this is a very time consuming procedure.

Method 2a

The second method, which measures the frequency of the oscillations that occur when a magnetic field that rotates with a frequency higher than the breakdown frequency, is quicker because only a single measurement has to be performed. The breakdown frequency is related to the oscillation frequency and the field frequency (equation 2.16). From the measurements of the angle versus time that are recorded in the previous experiment with a field frequency higher than the breakdown frequency, the linear fit has been subtracted and only the oscillations remain. Two examples of oscillations without slope are shown in figure 5.8. Panel A shows the oscillations for a field of strength 1.87 mT at 2Hz, panel B for the same field strength and a frequency of 2.25Hz. In figure 5.8 C and D their respective Fourier-spectra are shown.
Figure 5.8: Oscillations that remain when the slope of a measurement, performed with a field frequency above the breakdown frequency, is removed. Measurements are done with a field strength of 1.87 mT and a field frequency of 2Hz (A) and 2.25Hz (B). Their Fourier spectra are shown below (C+D).

The oscillation frequency can be read from the Fourier-spectrum. The minimum error for these measurements is the frequency step in the spectrum, $1 / 32 \text{s} = 0.03 \text{ Hz}$. However, in the Fourier spectra in figure 5.8 C and D the peaks are significantly broader than the frequency step, about 0.1 Hz in panel D and even 0.2 Hz in panel C. The full width at half maximum (FWHM) of the peak has been taken as error on the oscillation frequency. Next, breakdown frequencies have been calculated using $\omega_{\text{field}}$ and $\omega_{\text{oscill}}$ by means of equation 2.16. The results are shown in figure 5.9 for different field frequencies and magnetic inductions.

![Graph](image)

Figure 5.9: Measured breakdown frequency as function of applied field frequency

The determined breakdown frequency appears to increase for increasing field frequency. This is most probably due to the induced magnetisation of the bead that yields a small additional torque on the bead $^2$. The torque due to the induced magnetic moment increases for increasing field frequency; hence the maximum rotation frequency that a bead can reach increases as well. In order to find the bead’s permanent magnetic moment as accurate as possible one should measure at a field frequency as low as possible, i.e. a little above the breakdown frequency. The
permanent magnetic moment has been calculated for a field frequency of 1.25 Hz at 1.87 mT and for 2.5 Hz at 2.80 mT.

\[ \mu_{\text{bead}} = (2.7 \pm 0.5) \times 10^{-16} \text{ J/T} \quad (B = 1.87 \text{ mT}) \quad (5.5) \]

\[ \mu_{\text{bead}} = (3.4 \pm 0.4) \times 10^{-16} \text{ J/T} \quad (B = 2.80 \text{ mT}) \quad (5.6) \]

These values are within the error margins of the values found earlier (equation 5.3 and 5.4). The mean contribution to the errors originates from the fact that in practice, oscillations are not always well pronounced. Figure 5.10 shows a part of a measurement with a field frequency slightly above the breakdown frequency \( f_{\text{field}} = 2 \) Hz for \( B = 2.80 \text{ mT} \). The oscillations are difficult to distinguish from the errors in the image analysis. An oscillation starts when the angle between the magnetic field and the bead’s magnetic moment is 90°. For angles in this order, deviations due to rotational Brownian motion have a strong influence on the moment an oscillation set in and thereby on the measured oscillation frequency.

![Figure 5.10: Oscillations above the breakdown frequency are not always well pronounced.](image)

**Method 2b**

Since a single oscillation of a bead implies that the field rotates \( 2\pi \) more than the bead, the oscillation frequency is given by the difference of the field frequency and the average bead frequency (equation 2.17). Therefore the oscillation frequency can also be calculated from the average rotation frequency, instead of determined by means of Fourier methods. Figure 5.11 shows the breakdown frequency, determined via the average rotation frequency of the bead, for different field frequencies.
Note that the graph in figure 5.11 contains more data points than the graph in figure 5.9. In these measurements the oscillations are not enough pronounced to be analysed by means of Fourier methods, although the average rotation frequency of the bead could have been determined. The values of the breakdown frequency are comparable to the value obtained via the Fourier spectrum (figure 5.7), but the error bars are typically a factor 2 smaller. Again an increase of the breakdown frequency occurs for increasing field frequency. Significantly smaller errors are also found when the permanent magnetic moment is calculated from the breakdown frequency.

\[
\mu_{\text{bead}} = (2.7 \pm 0.2) \times 10^{-16} \text{ J/T} \quad (B = 1.87 \text{ mT}) \quad (5.7)
\]
\[
\mu_{\text{bead}} = (3.0 \pm 0.2) \times 10^{-16} \text{ J/T} \quad (B = 2.80 \text{ mT}) \quad (5.8)
\]

**Method 3**

The third method to determine the permanent magnetic moment of the bead is based on measuring the angle as function of time for a bead while it moves its magnetic moment to its equilibrium orientation in a quasi-static field. For unbound beads, the equilibrium orientation equals the direction of the magnetic field. Since the bead is not bound, \( k = 0 \), the relaxation rate constant, \( \lambda \), only depends on \( \mu_{\text{bead}} \) (equation 2.25):

\[
\lambda = \frac{\mu_{\text{bead}} B}{8 \pi \eta R^3}
\]  
(5.9)

The relaxation rate \( \lambda \) appears to be equal to the breakdown angular frequency for a rotating field (equation 2.8). Using the periodic movement of the bead, 32 periods of one up going and one down going movement have been averaged for both field
strengths. The up going and down going movement are analysed separately. The standard deviation of the angle is taken as the measurement error. Figure 5.12 shows the fits for \( B = 1.87 \text{ mT} \) while figure 5.13 shows the fits for \( B = 2.80 \text{ mT} \). The resulting magnetic moments are given in table 5.1. Note that the first points that are taken into account are at most 40° away from the equilibrium angle, which equals the direction of the magnetic field. This is done in order not to violate the linear approximation of the sine function that is involved in the model (equation 2.21).

![Figure 5.12: Exponential relaxation in a static field of 1.87 mT](image1)

![Figure 5.13: Exponential relaxation in a static field of 2.80 mT](image2)

<table>
<thead>
<tr>
<th>( B ) [mT]</th>
<th>1.87</th>
<th>2.80</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \mu_{\text{up}} ) ([10^{-16} \text{ J/T}])</td>
<td>3.1 ± 0.3</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>( \mu_{\text{down}} ) ([10^{-16} \text{ J/T}])</td>
<td>3.6 ± 0.4</td>
<td>3.2 ± 0.4</td>
</tr>
</tbody>
</table>

Although the errors are comparable to the measurements based on the Fourier transform of the oscillations, this method has the additional advantage that it can also be applied on bound beads to determine the bond’s torsion spring constant and the bead’s permanent magnetic moment in a single step.
5.2.2 Discussion

In this paragraph 3 different methods to determine the permanent magnetic moment of a bead are used. An overview of the results yielded by the different methods is given in table 5.2.

<table>
<thead>
<tr>
<th>Method</th>
<th>( \mu_{\text{bead}} \right[10^{-16} \text{ J/T}] ) ((B = 1.87 \text{ mT}))</th>
<th>( \mu_{\text{bead}} \right[10^{-16} \text{ J/T}] ) ((B = 2.80 \text{ mT}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: ( f_{\text{max}} )</td>
<td>( 2.8 \pm 0.3 )</td>
<td>( 2.9 \pm 0.3 )</td>
</tr>
<tr>
<td>2a: ( f_{\text{oscil}} )</td>
<td>( 2.7 \pm 0.5 )</td>
<td>( 3.4 \pm 0.4 )</td>
</tr>
<tr>
<td>2b: ( f_{\text{avg}} )</td>
<td>( 2.7 \pm 0.2 )</td>
<td>( 3.0 \pm 0.2 )</td>
</tr>
<tr>
<td>3: quasi-static</td>
<td>( 3.1 \pm 0.3 )</td>
<td>( 2.8 \pm 0.4 )</td>
</tr>
<tr>
<td></td>
<td>( 3.6 \pm 0.4 )</td>
<td>( 3.2 \pm 0.4 )</td>
</tr>
</tbody>
</table>

All methods yield within the error bars the same value for the permanent magnetic moment, although with different accuracies.

The first method, based on finding the maximum rotation frequency that a bead can reach, is not practical in use: it uses several measurements at different field frequencies, which makes it time consuming. The accuracy can be increased, but this will make it only more time consuming. Method 2a and 2b and method 3 are of more practical use, since they require only a single measurement. The most accurate single measurement method is 2b, which is based on the average rotation frequency of the bead. Method 2a, based on the Fourier transform of the oscillations, is less accurate because it uses oscillations that are not always well pronounced and suffers from deviations in the oscillation frequency during measurement. Method 3, which is applied in a quasi-static field, has accuracy comparable to method 2a and offers the advantage to be applicable to bound beads.

5.3 A Free Bead Measured with the Magnetic Quadrupole

5.3.1 Results

An image of a bead on the magnetic quadrupole recorded in transmission modus is shown in figure 5.14A. Figure 5.14B shows an image of a bead that is recorded in reflection modus, after a sputtered gold surface has been placed under the sample. The image recorded in transmission modus is brighter and shows more contrast over a broader ring along the bead. Therefore datasets to be analysed are recorded in transmission modus. The bead itself is still dark as compared to a bead on the crossed wires chip (figure 5.5). Therefore the aperture’s diameter cannot be decreased in order
to increase the contrast in the image, since this will make the bead even darker. Consequently the analysis of the images is more difficult.

Figure 5.14: A: Image of a bead on the magnetic quadrupole in transmission modus. B: Another bead in reflection modus.

A second effect that makes the analysis of the images more difficult is the translational movement of the bead. On the crossed wires chip, large gradients pull the bead towards the centre of the crossing point of both wires. These gradients are not present on the magnetic quadrupole; hence Brownian motion and thermal convection in the sample lead to a bead that drifts during the measurement. Figure 5.15 shows this difference in movement; on the left the $x$-coordinate of a bead on the crossed wires chip is plotted as function of time, while on the right the $x$-coordinate is plotted for a bead on the magnetic quadrupole. Both measurements are done with a field of 3mT.

Figure 5.15: $x$-coordinate as function of time of a bead on the crossed wires chip (left) and on the magnetic quadrupole (right).

Apart from the larger freedom of the bead’s centre, the bead on the magnetic quadrupole easily rotates about the axis of its own magnetic moment. Since the movement of the bead and the lower contrast in the image make the analysis more difficult, only time series limited to maximally 4 seconds could be analysed. By looking through the microscope, the breakdown frequency is estimated to be about 6 Hz. A measurement with a field frequency of the 7 Hz is done in order to derive the breakdown frequency as accurate as possible (method 2). Figure 5.16 shows the linear fit of the measurement (A), the oscillations superposed on it (B) and the Fourier spectrum of the oscillations (C).
Figure 5.16: Linear fit (left), oscillations superposed on the linear movement (top, right) and Fourier spectrum of the oscillations.

The oscillation frequency, read from the Fourier spectrum is 3.75 Hz. This implies for the breakdown frequency and the permanent magnetic moment (method 2a):

\[ f_{BD} = (6 \pm 1) \text{ Hz} \]  \hspace{1cm} (5.10)
\[ \mu_{\text{bead}} = (9 \pm 2) \times 10^{-16} \text{ J/T} \]  \hspace{1cm} (5.11)

The breakdown frequency calculated from the slope of the linear fit and the corresponding permanent magnetic moment are given by (method 2b):

\[ f_{BD} = (5.9 \pm 0.6) \text{ Hz} \]  \hspace{1cm} (5.12)
\[ \mu_{\text{bead}} = (8.5 \pm 0.9) \times 10^{-16} \text{ J/T} \]  \hspace{1cm} (5.13)

These calculations confirm the first rough estimation of the breakdown frequency to be of about 6 Hz.

Relaxation towards equilibrium in a quasi-static field has also been done to find the permanent magnetic moment of the bead (method 3). Since only a limited time sequence can be analysed a new reference image is used each time the magnetic field is switched. The first image recorded after the switch is used as reference. In total 8 up going and 8 down going movements are analysed and averaged. Figure 5.17 shows the data points that are taken into account for the exponential fit, together with their standard deviation.
The permanent magnetic moments yielded from these measurements are (method 3):

\[
\mu_{\text{bead}} = (9.0 \pm 0.9) \times 10^{-16} \text{ J/T} \quad \text{(up)} \quad (5.14)
\]

\[
\mu_{\text{bead}} = (9 \pm 1) \times 10^{-16} \text{ J/T} \quad \text{(down)} \quad (5.15)
\]

5.3.2 Discussion

Images on the magnetic quadrupole are darker than on the crossed wires chip, coated with gold, which yields less contrast in the images. Furthermore unbound beads are able to drift on the magnetic quadrupole, while on the crossed wires chip they are pulled towards the crossing point of the wires. Because of these difficulties, only a limited time series can be analysed if the first image is used as a reference to determine the permanent magnetic moment of beads.

In spite of the limitation of the analysis with respect to the duration of the experiment, the results yield the same accuracy as the measurements on the crossed wires chip. For the Fourier-method this is because for shorter measurements the frequency step in the spectrum increases, but still remains smaller than the error yielded by the FWHM-value of the peak in the Fourier-spectrum. For the average rotation frequency, the accuracy is insensitive for the duration of the measurement, as long as several times the oscillation period is analysed.

For the measurement in a static field, a new reference image is chosen each time the field is switched, in order to gain enough data that can be averaged. Apparently, the bead in the experiment on the quadrupole has a larger relaxation rate constant than the bead in the experiment on the crossed wires chip. The fact that the analysed time series is shorter than for the same experiment of the crossed wires chip, is compensated by the fact that the permanent magnetic moment reaches its equilibrium orientation faster. Therefore the same accuracy, as for the crossed wires chip, can be reached on the magnetic quadrupole.
5.4 A Bound Bead Measured with the Magnetic Quadrupole

5.4.1 Results

The feasibility to study molecular interactions by rotational actuation of beads was investigated using Streptavidin coated beads and a surface incubated with biotinylated BSA, diluted in BSA. The samples with 0.05% Biotin-BSA show beads attached to the functionalised top glass. In order to rule out non-specific binding, negative control experiments have been done on a surface only covered with BSA. In these experiments all beads were lying on the bottom surface after incubation. This implies that all beads that are bound to the top glass are specifically bound to the biotinylated BSA.

A field of 3 mT continuously rotating with a frequency of 1 Hz has been applied and two different categories of beads can be distinguished in rotational behaviour. Some bound beads were rotating continuously with the field, showing the same behaviour as a free bead, while others showed an oscillating movement, as is shown in figure 5.18. In the latter case, the bead apparently follows the magnetic field until the torque applied by the bond is opposed to the maximum magnetic torque that can be exerted on the bead and than the bead rotates back. The difference with the oscillating movement of a free bead in a rotating field above the breakdown frequency is that in the case of bound beads at frequencies below the breakdown frequency, the oscillation frequency equals the field frequency and the oscillations do not contain an average rotation.

![Figure 5.18: Oscillating movement of a bound bead in a rotating magnetic field.](image)

Several beads show an interesting behaviour for different field strengths. By increasing the field strength, beads that showed an oscillating movement at 3 mT could start to rotate. By decreasing the field strength, beads that were initially rotating could start oscillating.

A bead that showed an oscillating movement for field strengths from 3 mT to 30 mT is also measured in a field that instantaneously switches its direction over 180°. The angle is chosen large enough in order to make the bead rotate over a considerable angle. Figure 5.19 shows the movement of the bead when the field is switched, for a
field of 9 mT (A) and 22 mT (B). Both measurements are averaged 11 times; the error-bars give the standard deviation of the angle.

The bead does not rotate over 180° as the field does, but the bond pulls the bead back. The experiment is repeated for several different field strengths. The angle over which the bead turns is given as function of the magnetic induction in figure 5.20. The plot shows an increasing angle for increasing field strength, which is expected since the magnetic torque increases.

During the measurements at 15 mT and 26 mT the bead rotates over an angle smaller than the trend would predict. Apparently the rotation of the bead is hindered by an unknown effect.

For a linear approximation of a sine-function, the argument should typically be smaller than 1 radian. Although, the angle between the direction of the bead’s magnetisation and the magnetic field is larger than 1 radian, a quantitative analysis has been performed anyway. Therefore the time series, as shown in figure 5.19, are fitted with equation 2.23. Figure 5.21 shows the resulting magnetisation (A) and torsion spring constant (B), measured at different field strengths.
The values for the permanent magnetic moment lie between $10^{-16}$ J/T and $10^{-15}$ J/T, which is the same order of magnitude as the permanent magnetic moment measured on free beads. There is a trend to measure a decreasing permanent magnetic moment for increasing field strength. This is due to the induced magnetisation. When a bead rotates in a static magnetic field, the induced magnetisation aligns with the field on a finite timescale of typically 1 to 6 ns \cite{5}. Because of this finite relaxation time the induced moment turns in the direction in which the bead rotates (figure 5.22). The angles over which the induced magnetisation turns depends on the timescale of alignment and the angular velocity of the rotating bead. The interaction between the induced magnetisation and the magnetic field yields a torque that is opposed to the bead’s direction of rotation. The rotating bead is slowed down and the permanent magnetic moment is underestimated. Since the opposing torque increases for increasing field strength, the measured magnetic moment decreases for increasing field strength.

Note that the induced magnetisation causes an overestimation of the permanent magnetic moment in a rotating magnetic field and an underestimation in a static magnetic field.

For the spring torsion constant values of typically $4 \times 10^{-18}$ Nm are found. A typical torsional modulus of DNA, i.e. the product of the spring torsion constant and the length of the DNA-strand, is 46.5 pN·nm\textsuperscript{2} \cite{6}. Since DNA has a length of 0.34
nm/base-pair, the measured spring torsion constant on the Biotin-Steelptavidin system is 292 times the torsion spring constant of a 10 base-pairs DNA-strand.

5.4.2 Discussion

The negative controls showed that all bound beads are specifically bound. In PBS (pH 7.4), both Streptavidin and BSA are negatively charged. Therefore beads are electrostatically repelled by the surface. Beads are also pulled from the surface by gravity and a vertical gradient that yields a force of the same order of magnitude. Because of all these downward forces, the beads that continuously rotate have to be chemically bound to the surface.

The fact that there are beads that oscillate at low field strengths while they continuously rotate for higher fields, implies that there is an energy barrier that has to be overcome in order to let the bead rotate. Since beads have a permanent magnetic moment of typically $10^{-15}$ J/T, hence the energy involved in the magnetic torques applied on the bead is 180 kJ/mol, for a field of 3 mT under an angle of 90° with the bead’s magnetic moment. For higher energies, some oscillating beads can overcome the barrier that prevents full rotation and these beads start to rotate. Since the bond does not dissociate from the surface, bead stays in focus. Apparently there is a bond between the Biotin on the BSA-molecules and the Streptavidin molecule on the bead that allows free rotation about the bond.

An analogue model is found in organic chemistry. E.g. in propane ($C_3H_8$), rotation about the C-C bond is possible with an energy barrier of 14 kJ/mol (figure 5.23) [7].

![Figure 5.23: Potential energy of a C-C bond in propane as function of the rotation.](image)

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Since the energy barrier for the rotation of propane is smaller than in the energies of the magnetic torques in the experiments, it is unlikely that in these experiments rotation about one single bond took place. The Biotin-Streptavidin system is an interaction between a protein (Streptavidin) and a molecular Biotin group, that is characterised by a large interaction area. The 3D morphology of both surfaces is specific and barriers for rotation can be larger than the barriers involved in the rotation about a C-C bond, as in the case of propane.

On the other hand, it cannot be excluded that the energy barrier originates from breaking and rebinding Biotin-Streptavidin bonds. The formation of double bonds is plausible, since each Biotinylated BSA-molecule contains about eight Biotin molecules. Breaking these bonds is possible, since the Biotin-Streptavidin bond has an energy of 80 kJ/mol \cite{8}, which is smaller than the energy involves in the magnetic torques. Consequently, it cannot be excluded that the measured energy barrier originates from the need to break a bond, after which the bead rotates about the other remaining Biotin-Streptavidin bond.

Further research is needed in order to investigate these suggestions. In any case, the feasibility to measure molecular interactions by means of rotational actuation with superparamagnetic beads has been proven.
6 CONCLUSION

In this research, the goal was to develop methods for the determination of the permanent magnetic moment of superparamagnetic beads and to check for the possibility to investigate molecular interactions by means of the rotational excitation. Since the beads have a permanent magnetic moment, torques can be applied on the bead by means of an external magnetic field. The permanent magnetic moment of Dynabeads M280 show a spread of a factor 5, so that this has to be measured during the experiment in order to derive the torque applied to the bead.

A new quadrupole setup and its current regulator have been designed to create a homogeneous magnetic field over the field of view of the microscope. The current regulator exists of a push-pull amplifier with current feedback. This way non-linear response of the transistors and heating of the coils are corrected for. For currents of 1 A, magnetic fields of 37 mT can be applied, that can rotate with frequencies up to 100 Hz. Although the soft iron poles of the quadrupole have some hysteresis, input signals do not have to be corrected to generate a rotating field. Since the sample lies above the pole tips, the tips of the poles are sloped over 45° to direct the field lines upward. Also at about 45°, there is a low horizontal gradient present in the field. The vertical gradient applies forces on the bead that are of the order of magnitude of the gravitational force that acts on the bead. Compared to the crossed wires chip, the maximum field strength is 10 times larger, the vertical gradient is 100 smaller and the horizontal gradient is negligible. Due to the small horizontal gradients on the magnetic quadrupole, free beads can drift. This makes the analysis of the rotational movement of the bead more difficult.

An image analysis toolbox for Matlab has been developed to derive the orientation of the bead as a function of time. The correlation spectrum of the first image with every image in the time series is calculated with respect to the orientation of the bead, and the orientation of the bead as function of time is derived from the correlation spectra. For successful image analysis, contrast and light intensity in the images appeared to be crucial. A sputtered gold surface under the sample on the magnetic quadrupole did not reflect enough light, as the polished gold coating on the crossed wires chip did. Therefore images on the quadrupole were recorded in transmission modus. The results of the image analysis can be improved by labelling the beads with high contrast labels, e.g. fluorescent labels.

For the determination of the permanent magnetic moment of a free bead, it was shown that it is possible to do this with a single measurement in a rotating field, with a field frequency above the breakdown frequency. This requires a rough estimation of the breakdown frequency that is made by looking at a rotating bead and estimate at which
field frequency the oscillating movement of the bead sets in. The more accurate value of the breakdown frequency, from which the permanent magnetic moment can be derived, is calculated from the oscillation frequency. There are two different ways to determine the oscillation frequency of a bead: by means of the Fourier spectrum or calculate it as the difference between the field frequency and the average rotation frequency of the bead. Because oscillations are not always well pronounced, the latter method is more accurate. The average rotation frequency of the bead yields an accuracy of typically 10%, while the Fourier spectrum yields an accuracy of only 20%. It was also shown that it is possible to measure the permanent magnetic moment of a superparamagnetic bead without a rotating field, by measuring the relaxation rate of a bead that aligns its magnetic moment with a static field. This method, which yields an accuracy of about 10%, is also applicable for bound beads. If the rotation about the bond can be described by a harmonic potential, the torsion spring constant and the permanent magnetic moment can be measured at the same time.

Some bound beads in a rotating field oscillate for low magnetic inductions and rotate continuously for higher inductions. Apparently energy barriers for rotation exist for the interaction between the Streptavidin coated bead and the biotinylated BSA molecules on the surface. This behaviour demonstrates the feasibility to probe molecular interaction by rotational actuation. A simultaneous measurement of the permanent magnetic moment and the torsion spring constant yielded values of a plausible order of magnitude. The exact values might be affected by a linear approximation of a sine function for angles larger than 1 rad.
7 References


Appendix: Image Analysis Toolbox for Matlab

In this appendix the Matlab-code for the image analysis is given. The function AddDataSet collects all the information about the dataset to be analysed. This dataset contains:

- The location of the images
- The filename of the first image
- The number of the last image
- The location of the bead of interest in the first image
- The size of the region of interest (ROI). In this ROI, which is centred around the centre of the bead in the previous image, the centre of the bead in the actual image is sought.
- The reference intensity for the determination of the centre of the bead
- The minimum radius for polar coordinates
- The maximum radius for polar coordinates
- The total number of angles for polar coordinates
- The maximum angles over which a bead can rotate between the recordings of two subsequent images.

After that, the dataset is put into the function AnalyseBeadsRotation that handles the rest of the analysis. After the analysis, results are saved in a .mat-file, a log-file is made with the setting that were used for the analysis and markers are drawn on the images to check the reliability of the result with the eye.

```matlab
function Dataset = AddDataset(Dataset)
    go = 'y';
    while go(1) == 'y'
        % Specify the folder and the first image
        [ FileName1, Path ] = uigetfile( '*.tif', 'Open the first image ...' );
        Image = imread( strcat( Path, FileName1 ) );
        % Show the first image
        set( 0, 'Units', 'pixels')
        fig = figure( 'Position', get( 0, 'ScreenSize' ) );
        colormap( gray )
        imagesc( Image )
        axis image
        title 'Select the bead'
        % Select the bead
        dcm_obj = datacursormode( fig );
        set( dcm_obj, 'SnapToDataVertex', 'off', 'Enable', 'on')
        waitforbuttonpress
        info_struct = getCursorInfo( dcm_obj );
        C0 = round( info_struct.Position );
        close
        % Analysis parameters
        Nstop = input('Number of the last image: ');
        SizeROI = input('Size of Region of Interest: ');
        CrossSec = input('Rel. height centre: ');
        Rmin = input('Minimum radius: ');
        Rmax = input('Maximum radius: ');
        Na = input('Number of angles: ');
        dAngle = input('Max. increment of angle: ');
        Dataset(end+1,:) = {Path FileName1 C0 Nstop SizeROI CrossSec Rmin Rmax Na dAngle};
        go = lower(input( '\nAdd another set? (Y/N): ','s'));
    end
end
```
function [ Angles Centers Correlation ] = AnalyseBeadRotation( Dataset )
for i = 1:size(Dataset,1)
    % Prepare folder to save results
    [Folder ClkStart] = Init( Dataset(i,:) );
    % Analyse filenames
    FileName ImageNr Digits = InitFileName( Dataset(i,:) );
    % Initialise parameters
    NoImages = numel(ImageNr);
    Folder0 = Dataset{i,1};
    SizeROI = Dataset{i,5};
    RelInt = Dataset{i,6};
    Rmin = Dataset{i,7};
    Rmax = Dataset{i,8};
    NoAngles = Dataset{i,9};
    % Preallocate memory and initialise parameters
    [Images Centers C0] = InitLoad( Dataset(i,:) );
    % LOAD IMAGES FROM FILES
    h = waitbar( 0, 'Loading data...');
    for n = 1 : NoImages
        % Read the image
        Img = ReadFile( Folder0, FileName, ImageNr(n), Digits );
        % Cut out the region of interest and find the center
        [ImgROI delta] = GetROI( Img, C0, SizeROI );
        C0 = FindCenter( ImgROI, RelInt ) + delta;
        % Convert to polar coordinates
        Images(:,:,n) = Cart2Polar( Img, C0, Rmin, Rmax, NoAngles );
        % Center of bead is center of next ROI
        Centers(:,n) = C0; C0 = round(Centers(:,n));
        waitbar( n / NoImages, h )
    end
    close(h);
    % Calculate average image and references for correlation calculation
    [ImgAvg ImgRefFFT ImgRefSum] = InitCorr( Images );
    % Preallocate memory and initialise parameters
    [Correlation Angles dAngle] = InitAngle( Dataset(i,:) );
    Angle0 = 0; % initialise center of ROI (limited to 1 cycle)
    Angle1 = 0; % initialise old angle (unlimited)
    % CALCULATE CORRELATION AND DERIVE ANGLE AS FUNCTION OF TIME
    h = waitbar( 0, 'Calculating correlation...');
    for n = 1 : NoImages
        % Subtract average image
        Img = Images(:,:,n) - ImgAvg;
        % Calculate correlation with first image
        Correlation(:,n) = CalcCorr( Img, ImgRefFFT, ImgRefSum );
        % Angle found at maximum correlation in ROI (in angle steps)
        Angle0 = FindAngle( Correlation(:,n), Angle0, dAngle );
        % Add/subtract 360 degrees
        Angle1 = AddCycles( Angle0, Angle1, NoAngles );
        % Convert angle from angle steps to degrees
        Angles(n) = Angle1 * 360/NoAngles;
        waitbar( n / NoImages, h )
    end
    close(h);
    % Save results in .mat-file
    save (strcat(Folder,'/data.mat'),'Angles','Centers','Correlation');
end
% MARK IMAGES AND SAVE
h = waitbar( 0, 'Drawing marker on images...');
for n = 1 : NoImages
    % Read The file
    Img = ReadFile( Folder0, FileName, ImageNr(n), Digits );
    % Cut out the Region of interest
    ImgROI = GetROI( Img, Centers(:,n), SizeROI );
    % Draw a marker on the bead
    ImgROI = Mark( ImgROI, Rmax, Angles(n) );
    % Write the marked image to the disk
    WriteFile( ImgROI, [Folder '/images/'], FileName, ImageNr(n), Digits );
    % Update waitbar
    waitbar( n / NoImages, h )
end
% Make a log-file with the used parameters and save it
MakeLog( ClkStart, Dataset(i,:) );
end
function [Folder, ClkStart] = Init( Dataset )
ClkStart = ClockString; % start time
Folder = [Dataset{1}(1:end-1) ' (' ClkStart ')'];
mkdir(Folder); % folder for results
mkdir(strcat(Folder,'/images')); % folder for marked images
% Read image
Img = imread([Dataset{1} Dataset{2}]);
% Boundaries of ROI
xMin = round( Dataset{3}(1) - Dataset{5}/2 + 1 );
xMax = round( Dataset{3}(1) + Dataset{5}/2 );
yMin = round( Dataset{3}(2) - Dataset{5}/2 + 1 );
yMax = round( Dataset{3}(2) + Dataset{5}/2 );
% Cut out ROI
ImgROI = Img( yMin+1:yMax-1, xMin+1:xMax-1 );
Img( yMin:yMax, xMin:xMax ) = 255;
Img( yMin+1:yMax-1, xMin+1:xMax-1 ) = ImgROI;
% Write image with marked ROI in folder for results
imwrite(Img,[Folder '/' Dataset{2}]);
end

function [FileName, ImageNr, Digits] = InitFileName( Dataset )
% Number of digits in image number
Digits = numel(num2str( Dataset{4} ));
% FileName before image number
FileName = Dataset{2}( 1 : end-Digits-4 );
% Number of the first image and of all images
Nstart = str2double( Dataset{2}( end-Digits-3 : end-4 ) );
ImageNr = Nstart:Dataset{4};
end

function [Images, Centers, C0] = InitLoad( Dataset )
C0 = Dataset{3};
Digits = numel(num2str( Dataset{4} ));
Nstart = str2double( Dataset{2}( end-Digits-3 : end-4 ) );
NoImages = Dataset{4} - Nstart + 1;
NoRadii = (Dataset{8} - Dataset{7}) + 1;
Images = zeros( NoRadii, Dataset{9}, NoImages );
Centers = zeros( 2, NoImages );
end

function Img = ReadFile( Path, FileName, ImageNr, Digits )
FileNr = strcat( '00000000', num2str(ImageNr) );
FileNr = FileNr( end-Digits+1 : end );
Img = imread([ Path FileName FileNr '.tif' ]); end

function [ImgROI, delta] = GetROI( Img, Center, SizeROI )
xMin = round( Center(1) - SizeROI/2 + 1 );
xMax = round( Center(1) + SizeROI/2 );
yMin = round( Center(2) - SizeROI/2 + 1 );
yMax = round( Center(2) + SizeROI/2 );
ImgROI = Img( yMin:yMax, xMin:xMax );
delta(1) = xMin - 1;
delta(2) = yMin - 1;
end

function Center = FindCenter( Img, RelInt )
Img = double( Img );
Int = RelInt * max(max( Img )) + (1-RelInt) * min(min( Img ));
[Iy, Ix] = find( Img < Int ); % cross section
Center(1) = ( min(Ix) + max(Ix) )/2; % x-coordinate
Center(2) = ( min(Iy) + max(Iy) )/2; % y-coordinate
function ImgPol = Cart2Polar( ImgCart, C, Rmin, Rmax, Na )

R = Rmin:Rmax; % Radii
Nr = numel(R); % Number of radii
CosTheta = cos( 2*pi * (0:1/Na:(1-1/Na)) );
SinTheta = sin( 2*pi * (0:1/Na:(1-1/Na)) );
ImgPol = zeros( [ Nr Na ] ); % preallocate 'ImagesPolar'

for r = 1 : Nr
    for a = 1 : Na;
        x = C(1) + R(r)*CosTheta(a);
        y = C(2) + R(r)*SinTheta(a);
        x0 = floor(x); y0 = floor(y);
        value(1) = ImgCart( y0, x0 ) * (x0 - x+1) * (y0 - y+1);
        value(2) = ImgCart( y0, x0+1 ) * (x - x0) * (y0 - y+1);
        value(3) = ImgCart( y0+1, x0 ) * (x0 - x+1) * (y - y0);
        value(4) = ImgCart( y0+1, x0+1 ) * (x - x0) * (y - y0);
        ImgPol(r,a) = sum(value);
    end
end

function [ImgAvg ImgRefFFT ImgRefSum] = InitCorr( Images )

ImgAvg = mean( Images, 3 ) - mean(mean(mean( Images )));
Img = Images(:,:,1) - ImgAvg; % 1st image
ImgRefFFT = conj(fft( Img, [], 2 )); % fft of 1st image
ImgRefSum = sum( Img, 2 ); % sum of 1st image
end

function [Correlation Angles dAngle] = InitAngle( Dataset )
dAngle = ceil( Dataset{10} /360 * Dataset{9} );
Digits = numel(num2str( Dataset{4} ));
Nstart = str2double( Dataset{2}( end-Digits-3 : end-4 ) );
NoImages = Dataset{4} - Nstart + 1;
Correlation = zeros( Dataset{9}, NoImages );
Angles = zeros( 1, NoImages );
end

function Corr = CalcCorr( Img, ImgRefFFT, ImgRefSum )

[ Nr Na ] = size( Img ); % size of image
Coeff = Na ./ (sum( Img, 2 ) .* ImgRefSum); % coeff. for normalisation
CorrFFT = fft( Img, [], 2 ) .* ImgRefFFT; % FFT of correlation
g = real(ifft( CorrFFT, [], 2 )); % non-norm. correlation
Corr = g' * Coeff - Nr; % summed+norm. correlation
end

function Angle = FindAngle( Corr, AngleOld, dAngle )
% Number of angles
Na = numel(Corr);
% Minimum and maximum angle
Min = AngleOld - dAngle; if Min < 1 ; Min = Min + Na; end
Max = AngleOld + dAngle; if Max > Na; Max = Max - Na; end
% Region of interest
if Min > Max
    ROI = [Min:Na 1:Max];
else
    ROI = Min:Max;
end
% Angle is at maximum correlation
[MaxVal MaxInd] = max( Corr(ROI) );
Angle = ROI(MaxInd) - 1;
end

function Angle = AddCycles( Angle, AngleRef, Na )

while Angle < AngleRef - Na/2; Angle = Angle + Na; end
while Angle > AngleRef + Na/2; Angle = Angle - Na; end
function Img = Mark( Img, Radius, Angle)
    Angle = Angle * (pi/180); % deg -> rad
    x = round( size(Img,2)/2 ); % x-coordinate
    y = round( size(Img,1)/2 ); % y-coordinate
    Img( y-1:y+1, x ) = 255; % vertical line
    Img( y, x-1:x+1 ) = 255; % horizontal line
    x = round( size(Img,2)/2 + Radius * cos(Angle) ); % x-coordinate
    y = round( size(Img,1)/2 + Radius * sin(Angle) ); % y-coordinate
    Img( y-1:y+1, x ) = 255; % vertical line
    Img( y, x-1:x+1 ) = 255; % horizontal line
end

function WriteFile( Img, Path, FileName, ImageNr, Digits )
    FileNr = strcat( '00000000', num2str( ImageNr ) );
    FileNr = FileNr( end-Digits+1 : end );
    imwrite( Img, strcat(Path,FileName,FileNr,'.tif') );
end

function MakeLog( ClkStart, Dataset )
    Path = Dataset{1};
    Folder = strcat( [Path(1:end-1) '/'], ClkStart, ' (' );
    if ~isempty(Folder) Folder = strcat( Folder, 'log.txt' ) end;
    fprintf(fid,['Analysis started: ' ClkStart 'n']);
    fprintf(fid,['Images loaded from: ' Path 'n']);
    Start = Dataset{2};
    Stop = Start;
    StopNr = num2str(Dataset{4}) '.tif';
    Stop = [stop; num2str(Dataset{4}) '.tif'] ;
    Stop = Stop( end-numel(Stop) : end ) ;
    StopNr = [num2str(Dataset{4}) '.tif'] ;
    fprintf(fid,['   ' Start ' ... ' Stop 'n']);
    SizeROI = num2str(Dataset{5});
    fprintf(fid,['SizeROI ' x ' SizeROI ' Region of Interest (ROI)n']);
    CrossSec = num2str(Dataset{6});
    fprintf(fid,['CrossSec ' 'n']);
    fprintf(fid,['   0 for min intensity, 1 for max intensityn']);
    Rmin = num2str(Dataset{7});
    Rmax = num2str(Dataset{8});
    NAngle = num2str(Dataset{9});
    AngleStep = num2str(360/Dataset{9});
    fprintf(fid,['   Angle step: 360deg / ' NAngle ' = ' AngleStep 'degn']);
    fprintf(fid,['dAngle = num2str(Dataset{10})']);
    fprintf(fid,['   Max increment of angle per image: ' dAngle ' degn']);
    fprintf(fid,['Variables saved in "data.mat":n']);
    fclose(fid);
end

function Clk = ClockString
    C = clock;
    year = [0000' num2str(C(1))];
    month = [00' num2str(C(2))];
    day = [00' num2str(C(3))];
    hour = [00' num2str(C(4))];
    minute = [00' num2str(C(5))];
    second = [00' num2str(floord(C(6)))];
    Clk = [ year month day '-' hour minute second ];
end