Multifocal Two-Photon Microscopy for Bio-Sensing Using Gold Nanorods

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Abstract

Gold nanorods (GNRs) are plasmonic nanoparticles of which the plasmon resonance wavelength is influenced by the near-field surroundings. Even binding of single proteins with GNRs can cause a shift in their resonance wavelength. Previous studies have used this resonance shift to detect single binding events of proteins with GNRs. However, those results are limited in their throughput and sensitivity. Here we use a multifocal two-photon microscopy technique to measure hundreds of single GNRs simultaneously with high spectral sensitivity and signal-to-noise ratio. Using numerical simulations we determined how we can optimise the homogeneity of the illumination pattern over an area of $200 \times 200 \, \mu m^2$ and minimise the melting of GNRs during measurements. Finally, we measured GNRs in various concentrations of fibronectin proteins and found an increase in power spectral density of the two-photon luminescence signal for fibronectin concentrations of $1.25 \, \mu g/mL$ to $2.5 \, \mu g/mL$. Through better understanding of the setup we can now perform reliable spectral measurements of hundreds of individual GNRs. This should make two-photon microscopy techniques more competitive with bio-sensing experiments based on scattering of GNRs.
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Introduction

During the past decades, gold nanorods (GNRs) have been an important research topic because of their unique properties as plasmonic resonators. Those properties include their high brightness and stability, as well as the dependence of their absorption spectrum on various adjustable parameters like the refractive index of the medium. This makes it possible to use GNRs for various applications in biology, physics and medicine [1, 2]. Two important applications of GNRs in (bio)physics are that they can enhance the emission of other fluorescent molecules by several orders of magnitude [3] and that they can be used for the sensing of single molecules through induced plasmon resonance shifts [4, 5].

The aim of this thesis is to track and characterise hundreds of single GNRs simultaneously and consistently in a multifocal two-photon microscope over a timescale of multiple minutes. We then want to use this to measure the plasmon resonance shift due to single-molecule interactions with GNRs.

1.1 Gold nanorods

Gold nanorods are plasmonic nanoparticles in which free conduction electrons can couple with incident electromagnetic waves to create oscillations of coupled electrons. This phenomenon is known as surface plasmon resonance (SPR). The magnitude of this SPR depends on the frequency, and therefore the wavelength, of the incident signal. The resulting spectrum has peak intensities at two resonance frequencies, resulting from electrons oscillating along the longitudinal and the transverse axes of the nanorods:
Introduction

Electric field

Electron cloud

GNR

a) Longitudinal SPR

b) Transverse SPR

Figure 1.1: Schematic illustrations of the electron oscillations which cause the longitudinal (a) and transverse (b) resonance modes in GNRs. Figure adapted from [6].

If the plasmon frequency is in resonance, absorption of the photons by the quantized electrons in the nanorods is increased for that wavelength. Relaxation of those electrons happens under the emission of a photon. The exact wavelengths at which this resonance happens depend on the aspect ratio of the GNRs and the refractive index of their near-field surrounding medium. When tracking a single GNR, its dimensions (and therefore its aspect ratio) do not change as long as it is not subject to thermal reshaping or melting. The surrounding medium can, however, change due to a change of the solution in which the GNRs are placed or due to a (single) molecule attaching to the GNR. The latter property lies at the heart of single-molecule bio-sensing using GNRs, which has been shown experimentally before [4, 5, 7–9]. When GNRs are illuminated at a certain wavelength and the resonance spectrum of the GNR changes due to the binding of a molecule, the magnitude of the SPR at that wavelength changes. This leads to a sudden change in the signal emitted by the GNR, making it possible to measure single binding events.

1.2 Two-photon luminescence

In photoluminescence experiments, a single photon excites an electron to a higher energetic level. The electron can then fall back to its original level under emission of a photon. If two separate photons are absorbed nearly instantaneously (within $\sim 0.5 \text{ fs}$ [10]), a phenomenon known as two-photon luminescence (TPL) occurs. The absorption of the two photons leads to the emission of a single photon with a higher energy and thus a lower wavelength. This means that the difference in wavelength between the excitation light and the emitted photons by the GNRs is larger.
than for one-photon luminescence, making it easier to spectrally distinguish them. This decreases the background signal and therefore increases the resolution. In addition, infrared light can be used for the excitation of the GNRs in TPL. This is known to have a much higher penetration depth through tissue and cells than visible light [11], making it well-suited for biological and medical purposes. Finally, the probability of TPL scales quadratically with the excitation intensity. This makes that the resonance peaks of the nanorods are quadratically narrower, which results in a higher sensitivity to changes in the resonance spectrum.

1.3 Bio-sensing fibronectin

Bio-sensing using the SPR shift of single gold nanorods has been shown before by various authors. This can be done using receptors on the GNRs to bind to other proteins [4, 7, 8] or without additional receptors [5, 9]. Ament et al [5] in 2012 performed bio-sensing with the blood plasma protein fibronectin (molecular weight 450 kDa) for single GNRs using single-photon scattering microscopy: see figure 1.2. Here, we will study the bio-sensing of different concentrations of fibronectin using gold nanorods in multifocal two-photon microscopy. This makes it possible to measure hundreds of GNRs simultaneously [12] due to the wide-field scanning and gives a higher sensitivity due to the two-photon luminescence.

![Figure 1.2: The resonance wavelength shift for a single gold nanorod in a solution with fibronectin. Arrows indicate single protein attachment events. Figure from [5].](image-url)
Chapter 2

Theory

2.1 Surface plasmon resonance

The surface conduction electrons in plasmonic nanoparticles, like GNRs, can couple with electromagnetic waves with wavelengths larger than the size of the particles due to their collective oscillation modes. After excitation of those electrons, they tend to return to their initial state due to the Coulomb forces between the displaced electrons and the nuclei of the particle. The oscillations of the electromagnetic waves result in oscillations of the conduction electrons at the same frequency as the light: Surface plasmon resonance (SPR).

This phenomenon was first described mathematically by Mie in 1908 [13] for spherical metal nanoparticles. In 1912, Gans [14] elaborated on this theory by formulating a description for nanorods by approximating them as prolate spheroids with a long axis of length $a$ and shorter axes of lengths $b = c$. If such a prolate spheroid is irradiated parallel to one of its axes $i$, this theory states that the resulting polarizability is given by [12]:

$$ \alpha_i = \varepsilon_0 V \frac{\varepsilon' - 1}{1 + L_i (\varepsilon' - 1)} $$  \hspace{1cm} (2.1)

where $\varepsilon_0 = 1/(\mu_0 c^2)$ is the permittivity of vacuum and $V \approx \frac{4\pi}{3} \frac{a^2 b^2}{4} = \frac{2}{3} \pi ab^2$ is the volume of the nanoparticle. $\varepsilon' \equiv \varepsilon/\varepsilon_m$ is the ratio between the complex permittivity of the material of the particle ($\varepsilon$) and the permittivity of the medium surrounding the particle ($\varepsilon_m$), which depends on the...
refractive index of the medium \((n_m)\) through [15]

\[
\epsilon_m = \frac{n_m}{\mu_m c^2}
\]  

(2.2)

with \(\mu_m\) the permeability of the medium. \(L_i\) is the depolarisation factor for each axis (with \(\sum_i L_i = 1\)), defined as [12, 14]:

\[
L_a = \frac{1}{R^2 - 1} \left( \frac{R}{2\sqrt{R^2 - 1}} \ln \left( \frac{R + \sqrt{R^2 - 1}}{R - \sqrt{R^2 - 1}} \right) - 1 \right)
\]  

(2.3)

\[
L_{b,c} = \frac{1 - L_a}{2}
\]  

(2.4)

where \(R\) is the aspect ratio \((R \equiv a/b > 1)\) of the nanoparticle. Since the polarizability in equation 2.1 is at a maximum for \(1 + L_i(\epsilon' - 1) = 0\), the plasmon resonances are found for \(\epsilon' = 1 - 1/L_i\), which has a solution for \(L_a\) and a solution for \(L_b = L_c\). These solutions lead to two plasmon resonance peaks in the spectrum of the particle: the longitudinal mode for \(L_a\) and the transverse mode for \(L_b\) and \(L_c\) (see figure 2.1).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig2.1.png}
\caption{The experimental bulk absorbance spectrum of plasmonic gold nanorods with \(R = 3.3\) shows a transverse and a longitudinal plasmon resonance peak. Figure from [16].}
\end{figure}

Since \(\epsilon' = \epsilon'(n_m)\) and \(L_i = L_i(R)\), equation 2.1 states that the resonance wavelengths for plasmonic nanoparticles depend on the refractive index of the surrounding medium and the aspect ratio of the nanoparticle. In general, both an increase in the refractive index of the medium and an increase in the aspect ratio result in an increase in the longitudinal resonance peak (redshift).
Using the polarizability, it is possible to determine the absorption cross-section of a nanorod with a length which is much smaller than the wavelength of the light using [17]:

\[
\langle \sigma_{\text{abs}} \rangle = \frac{k}{3} \text{Im} (\alpha_a + \alpha_b + \alpha_c) \tag{2.5}
\]

where \( k = \frac{2\pi}{\lambda} \) is the wavenumber and \( \lambda \) the wavelength.

In this thesis, all measurements and numerical simulations are done with gold nanorods with a median length of 40 nm and width of 10 nm (see section 3.3), such that \( R = 4 \) and \( r_{\text{eff}} = \left( \frac{3}{4\pi} V \right)^{1/3} \approx 9.0 \text{ nm} \). From calculations by Jain in 2006 [18] we can find that the extinction cross-section for the GNRs used in our experiments is \( \langle \sigma_{\text{ext}} \rangle \approx 3.5 \times 10^{-15} \text{ m}^2 \) and \( \frac{\langle \sigma_{\text{scat}} \rangle}{\langle \sigma_{\text{abs}} \rangle} \approx 0.04 \) such that

\[
\langle \sigma_{\text{abs}} \rangle = \left( 1 + \frac{\langle \sigma_{\text{scat}} \rangle}{\langle \sigma_{\text{abs}} \rangle} \right)^{-1} \langle \sigma_{\text{ext}} \rangle \approx 3.4 \times 10^{-15} \text{ m}^2 
\approx (60 \text{ nm})^2 \tag{2.6}
\]

### 2.2 Two-photon microscopy

Standard microscopy methods function based on the absorption of a single photon which excites an electron to an energetically higher state. Due to thermal relaxation, this electron then falls back to its initial state under the emission of a photon. However, if two photons are absorbed within \( \sim 0.5 \text{ fs} \) [10], two-photon luminescence (TPL) can occur. This happens through a two-step process in which both photons cause an absorption transition of electrons near the Fermi-surface [19]; see figure 2.2. One electron, from the \( sp \) band, makes an intraband transition to above the Fermi energy level, after which the second electron, from the \( d \) band, fills the hole which the first electron left behind. The first electron can then decay radiatively to the resulting hole in the \( d \) band under emission of a photon with a higher energy than the individual excitation photons.

Two-photon luminescence results in a quadratic dependence on the excitation intensity, resulting in narrower spectra [20]. For GNRs, the theoretical absorption peak follows a Lorentzian-squared curve.
Theory

Figure 2.2: (a): The two-photon absorption process for two degenerate symmetry points (\(X, L\)) in the band structure. Absorption happens in two sequential transitions, each induced by a single photon. The first photon (1) excites an electron to above the Fermi energy level through an \(sp \rightarrow sp\) intraband transition. The second photon (2) excites an electron to recombine with the resulting hole in the \(sp\) band, leaving a hole in the \(d\) band. Figure from [21]. (b): The TPL spectrum (red dots) follows the square of the Lorentzian absorption curve. The blue line is the single-photon absorption curve and the dashed green line is the blue line squared, which overlaps with the green dots. Figure from [20].

2.3 GNR melting

2.3.1 Melting energy

The absorption and scattering properties of GNRs strongly depend on their shape, especially the aspect ratio. Therefore, if the shape of a GNR changes due to thermal processes, this can obstruct sensing experiments. Previous research [22] studied the melting temperature for GNRs of shape (\(R = 4.1\)) and size (length = 44 nm) comparable to the ones in our experiments. Experimentally, this energy was found to be around 16 \(fJ\), whereas the value calculated using thermodynamics based on bulk properties of gold was found to be around 60 \(fJ\). However, GNRs are known to already deform below this temperature through local melting and surface diffusion [23–25].

The maximum energy absorbed by a single immobilised GNR can be estimated by approximating the point-spread function (PSF) of the incident laser beam as a 2D Gaussian profile with [26]

\[
\sigma_x = \sigma_y = \sigma_{PSF} \geq 0.25\lambda/NA
\]

with NA the numerical aperture of the microscope. The peak fluence of
2.3 GNR melting

this pulse with total energy $E_{\text{pulse}}$ is then given by [27]

$$F_{\text{max, pulse}} = \frac{E_{\text{pulse}}}{2\pi \sigma_{\text{PSF}}^2} \leq \frac{8(NA)^2}{\pi \lambda^2} E_{\text{pulse}}$$  \hspace{1cm} (2.8)

such that the energy $E_{\text{max, GNR}}$ absorbed from a single pulse by a single GNR with absorption cross-section $\langle \sigma_{\text{abs}} \rangle \ll \sigma_{\text{PSF}}^2$ is approximately

$$E_{\text{max, GNR}} = \langle \sigma_{\text{abs}} \rangle F_{\text{max, pulse}} = \frac{\langle \sigma_{\text{abs}} \rangle}{2\pi \sigma_{\text{PSF}}^2} E_{\text{pulse}}$$ \hspace{1cm} (2.9)

2.3.2 Timescales

The timescales on which melting and reshaping happen in metal nano-structures has been worked out theoretically and numerically by Ekici et al. [28], who describe three fundamental processes involved in the processing of energy after an ultrafast laser pulse to a particle. The energy is first absorbed by the electrons in a non equilibrium distribution, which relaxes through electron-electron scattering within $10 - 50 \, fs$. Thermal equilibrium between the lattice and the electrons is then reached in $10 - 50 \, ps$ through electron-phonon coupling, increasing the particle temperature. The particle then decreases in temperature through transfer of thermal energy to the surrounding medium. This happens by phonon-phonon coupling in $100 \, ps - 1 \, ns$, depending on the intensity of the laser pulse and the size of the particle.

For a laser with the same repetition rate ($80 \, MHz$) and a comparable pulse duration ($250 \, fs$) to our set-up, they reported a mean temperature increase of $3^\circ \, C$ during the first few pulses, after which it did not substantially increase for the following pulses. Both this change in temperature and the timescales at which the different heat transfers happen can be seen in figure 2.3.
**Figure 2.3:** Simulations of the temporal temperature profile for electrons and the gold atoms in an $48 \times 14 \text{ nm}^2$ GNR and the water in its surroundings. Values are for laser pulses with a fluence $F_{\text{pulse}} = 4.70 \text{ J/m}^2$. Figures from [28]. (a): The changes for a single pulse on the picosecond scale. Inset: The full electron temperature evolution. (b): The changes for a series of pulses on the nanosecond scale.
Materials and methods

3.1 Experimental setup

The two-photon multifocal scanning microscope shown schematically in figure 3.1 was used for all experiments in this thesis. The excitation laser used is a tunable Chameleon Ultra Ti:Sapphire laser with a pulse width of 140 fs and a repetition rate of 80 MHz [29]. The wavelength can be tuned between 690 and 1020 nm. A Faraday isolator (Broadband Faraday Optical Isolator, Newport, USA) is used to prevent unwanted feedback into the laser cavity. The beam is expanded using two expansion lenses, after which a 750 nm-longpass filter blocks the low-wavelength tail of the laser and prevents it from being mixed with the luminescence signal from the GNRs at a later stage. A diffractive optical element (DOE), custom-made by HOLOEYE Photonics Germany, splits the laser beam into a triangular lattice of 25x25 beamlets with a lattice spacing of approximately $d = 8 \mu m$ in the image plane. A zero-order block consisting of a piece of soldering tin on a cover slip blocks the zero-order of the diffraction pattern. A scanning mirror (FSM-300, Newport, USA) then moves the focused beamlets in an Archimedean spiral pattern (see section 3.2) to create a more homogeneous illumination pattern in the field of view. An ND-filter (Thorlabs, USA) attenuates the beamlets to reduce the illumination intensity. A $\lambda/4$ waveplate (Thorlabs, USA) converts the linearly polarised light of the laser to circularly polarised light. The beam then passes the aperture of the water-immersion objective ($NA = 1.10$, Nikon CFI75 Apo 25XC W). The sample (see section 3.3 for the preparation process) is placed on an XY-stage (PKTM50, Owis, Germany) which can be moved with two degrees of freedom to scan the sample. The luminescent signal emitted by the nanorods is collected by the objective after which a dichroic mir-
Materials and methods

Figure 3.1: Scheme of our two-photon multifocal scanning microscope. Figure adapted from [12].

ror (700dcr, Chroma, USA) deflects it through a 720 nm-shortpass filter (720SP, Semrock, USA) which blocks the residual laser and scattering light. The signal is finally focused on an CMOS (Complementary Metal Oxide Semiconductor) camera (Hamamatsu, Orca Flash4.0 v2, 82 % peak QE), which collects the signal. The camera has a field of view of 2048 × 2048 pixels resulting, for our set-up, in 0.26 × 0.26 µm per pixel. Typically measurements were performed at only 512 × 512 pixels, leading to a total field of view of 133 × 133 µm.

3.2 Archimedean spiral scanning

To increase the scanned surface area, the laser beam is split into an array of 25x25 separate focal points. During a measurement, every one of those focal points moves over the surface in the shape of an Archimedean spiral to produce a more homogeneous excitation profile [30]. The time-dependent position of the focal points with respect to their initial position is given by:

\[
x(t) = A \tau \sin (2\pi n \tau) \quad y(t) = A \tau \cos (2\pi n \tau)
\]

with \( \tau(t) = \sqrt{t/T} \exp \left( \frac{(t/T)^2 - 1}{2\sigma_G^2} \right) \)
3.2 Archimedean spiral scanning

where $A$ is the amplitude of the spiral (in our set-up, a scanning voltage of 1 V corresponds to an amplitude of 15.45 $\mu m$ in the image plane), $n$ is the number of spiral arms, $T$ is the total exposure time and $\sigma_G$ is a measure for the width of the resulting profile. Integrated over a full spiral scan, every single spiral ideally produces a 2D super-Gaussian intensity distribution:

$$I(r) \propto \exp \left( \frac{r^2}{2\sigma^2} \right)$$

with $r$ the distance from the centre of the spiral pattern and $\sigma$ the width (HWHM) of the Gaussian. Multiple super-Gaussian profiles can, if they are close enough to each other, form a 2D flat-topped profile. As the ratio between the distance between the Gaussians and their widths decreases, the flat-topped profile approaches the equation [31] (see figure 3.2)

$$I(r) \propto \exp \left( -\left(\frac{r}{w}\right)^n \right)$$

where $w$ is the width of the resulting intensity profile, $r$ is the distance from the centre of the profile and $n \geq 2$ is the number of Gaussian profiles (in our case, the number of Archimedean spirals per axis) of which the super-Gaussian constitutes. The higher the value of $n$, the steeper the edges of the the illumination profile become.

![Figure 3.2: The flat-topped profile (blue line, $w = 14.46 \mu m$ in equation 3.4 yields $R^2 = 0.9995$) resulting of a summation of 14 perfect Gaussians (grey areas, $\sigma = 1.4 \mu m$, $d = 2 \mu m$).](image)
3.3 Sample preparation

For all experiments GNRs with an SPR peak of \( \sim 800 \text{ nm} \), a length of 40 \( \text{nm} \) and a diameter of 10 \( \text{nm} \) (giving an aspect ratio of 4.0) were used. Those GNRs were produced commercially by Nanopartz [32] and are coated with the surfactant cetrimonium bromide (CTAB). The stock solution with the GNRs was first diluted four times with HPLC-grade water and sonicated for 20 minutes to prevent aggregation of the nanorods. A small volume of this mixture (usually about 100 – 200 \( \mu \text{L} \)) was then deposited on an ethanol-cleaned glass microscope slide onto which the GNRs could sediment. After approximately five minutes the remainder of the GNR-mixture was removed from the microscope slide. The cover slip was placed in a sample holder and immersed in HPLC-grade water.

3.4 GNR detection

For the detection of GNR traces and the extraction of single-GNR intensity time traces, a Python-program was used which determined the peak locations and intensity profiles in a wide-field measurement. First, global peak locations were determined by finding the pixels in the time-averaged data (see appendix A.1) where the intensity was greater than the median intensity multiplied by a certain threshold value (typically 1.15). Local coordinates were then determined by fitting a 2D-Gaussian to the time-averaged image in an area around the global coordinates to find the sub-pixel peak locations. The temporal intensity profile for every peak was determined by spatially averaging the intensity in the 11 \( \times \) 11 pixel-area around the peak location for each frame, and subtracting the median value of the intensity of the whole field of view from the values.

3.5 Simulations of the illumination pattern

To understand the illumination pattern of our set-up, we used numerical simulations. In those simulations, every spiral scan was discretized into approximately 5000 time-steps. The field of view was split up into a discrete number of pixels. In every time step for every pixel, the distance to each beamlet was determined, resulting in the absorbed energy based on a Gaussian approximation for the PSFs [26]. The energy per GNR could then be determined by scaling the values based on the difference between the area of a pixel and the absorption-cross section of a GNR.
Chapter 4

Results

To perform wide-field bio-sensing, we need to have a homogeneous illumination pattern in the xy-plane to prevent changes in the emitted signal caused by fluctuations in the illumination intensity. Moreover, for spectroscopy it is essential that this homogeneous illumination pattern persists at different excitation wavelengths. In addition, it is important to have GNRs which do not reshape or melt during measurements, as the aspect ratio strongly influences the optical properties. The first two sections of this chapter study the relevant parameters for producing a consistently homogeneous illumination pattern based on numerical simulations. The third and the fourth section characterise the melting of GNRs. Finally, in section 4.5 the results of GNR-based bio-sensing measurements in a solution with unlabeled proteins are presented.

4.1 Homogeneous illumination

A homogeneous multifocal illumination pattern is important to be able to perform consistent, reproducible microscopy measurements. It has been shown before that this is possible by scanning a periodical lattice of individual beamlets in an Archimedean spiral pattern [30], following equations 3.1 and 3.2. In the set-up shown in section 3.1, the lattice spacing in the DOE pattern is three times larger than in this previous work. We first tested if the same scanning pattern can be used to produce a comparably homogeneous illumination pattern for the current set-up. We used numerical simulations to optimise the scanning parameters and lattice spacing to generate a flat-topped illumination pattern and to describe what happens if such criteria are not fulfilled. Figure 4.1 presents various spiral patterns,
Figure 4.1: Numerical simulations of the spiral scanning illumination for forming a homogeneous intensity distribution. For all figures, a PSF-width of 0.6 µm is used. (a): The normalised super-Gaussian formed by spiral-scanning a single laser beam for a spiral with a small amplitude ($A = 3.5 \mu m$, $\sigma_G = 0.85$, $n = 12$). The blue line is the illumination pattern over a single axis resulting from integration of the PSF of the laser over time (grey curves). The red line is a Gaussian fit yielding $\sigma = 1.43 \mu m$. The inset is the pattern made by the beamlet. (b): The normalised illumination pattern for 14 spirals with lattice spacing $d = 2.3 \mu m$. The red line is a flat-topped fit giving $w = 16.5 \mu m$. (c): The resulting two-dimensional illumination pattern. (d-f): The same results as (a-c) for a larger lattice spacing: $d = 4 \mu m$. The flat-topped profile was fitted resulting in $w = 28.3 \mu m$. (g-i): The
same results as (a–c) with 2 instead of 12 spiral arms. The Gaussian was fitted resulting in $\sigma = 1.33 \, \mu m$ and the flat-topped profile was fitted giving $w = 16.1 \, \mu m$. (j–l): The results for spirals with a larger amplitude ($A = 15 \, \mu m$, $\sigma_G = 0.85$, $n = 24$) and with a typical lattice spacing for the set-up described in section 3.1 (8 $\mu m$). The Gaussian has been fitted giving $\sigma = 5.35 \, \mu m$. The inset is a diagram of a fraction the spiral pattern (to scale). (m): The normalised velocity for the laser beam at the positions where it crosses the x-axis for small spirals (circles) as in figures (a) and (d) and for the large spiral (stars) as in figure (j). (n): The dependence of the coefficient of variation of the central part of a flat-topped profile on the ratio between the width of the spirals and the distance between their centres.

as well as the resulting illumination patterns. The best results are obtained when the spiral approximates a perfect Gaussian profile. Figures 4.1e and 4.1f show that the illumination pattern becomes less homogeneous if the distance between focal points becomes large compared to the width of single-spiral super-Gaussians (figure 4.1d). This ratio determines the coefficient of variation (the standard deviation of the intensity divided by the intensity) for the flat part of the flat-topped profile. Figure 4.1g shows that deviations from the single-spiral super-Gaussian increase when the number of spiral arms per spiral decreases, which results in less homogeneous illumination profiles in figures 4.1h and 4.1i. For a single spiral in figure 4.1j, a dip appears at the top of the super-Gaussian. It shows that for a larger lattice spacing, the homogeneity of the illumination pattern decreases due to a change in the shape of the single-spiral pattern. This results in inhomogeneities in figures 4.1k and 4.1l. The change in amplitude of the PSF is caused by a change in velocity of the laser beamlets, as can be seen in figure 4.1m. Numerically calculated values for the dependence of the coefficient of variation on this ratio for perfect Gaussians can be found in figure 4.1n. Based on this figure, we can conclude that the homogeneity of the intensity profile depends on how well the single-spiral illumination pattern compares to a Gaussian profile, which is better for smaller spirals. Furthermore, the smaller the ratio between the width of the spirals and the distance between the beamlets, the more homogeneous the profile becomes.
4.2 Wavelength-dependence of illumination pattern

The previous section discussed the creation of a homogeneous illumination pattern based on a fixed width of the point-spread function (PSF) of each laser beamlet and a constant lattice spacing. However, in our actual measurements those parameters change when the wavelength of the laser changes. This can, for example, become important when the spectra of GNRs are determined by sweeping the laser wavelength.

Figure 4.2b shows the measured peak-to-peak distance in the DOE pattern as well as the peak width, which is defined as the half width at half maximum as in [26], for each beamlet as the wavelength changes. Compared to the theoretical diffraction limit, the PSF width was consistently approximately three times higher. Measured widths were used to numerically calculate the illumination patterns at various wavelengths for spirals with different numbers of spiral arms. The resulting patterns are shown in figure 4.2c for the lowest wavelength of 744 nm and in figure 4.2d for the highest wavelength of 850 nm.

We next computed the absorbed energy per duty cycle per GNR at different locations for all wavelengths, using an absorption cross-section of \((60 \, nm)^2\) as discussed in section 2.1. The absorbed energy is shown in figure 4.2e for six different GNR locations. Especially for the lower wavelengths, strong variations in the absorbed energy are visible. A change in absorbed energy for different wavelengths results in fluctuations in the emitted 2-photon luminescence. It is therefore important that the illumination pattern is not only homogeneous across the field of view, but also across different wavelengths. In figures 4.2f and 4.2g the illumination pattern is shown for spirals with a higher number of spiral arms. Also for lower wavelengths, a homogeneous pattern appears in which the single spiral arms can no longer be distinguished. The resulting energy per duty cycle per GNR is shown in figure 4.2h. This makes that figure 4.2 demonstrates that the fluctuations in the energy per GNR as a function of the number of arms per spiral are lower for spirals with a higher number of arms.
4.2 Wavelength-dependence of illumination pattern

**Figure 4.2:** Simulations of the wavelength-dependence of the generated illumination pattern for three beams. (a): Measures of the peak-peak distance and the peak width (HWHM) without spiralling. (b): Experimental wavelength-dependence of the peak-peak distance (blue) and the peak-width (red) as measured using the two-photon fluorescence of a Rhodamine 6G-solution. Larger wavelength values could not be determined due to low Rhodamine absorption [33]. The solid red line indicates the diffraction limit for our microscope (equation 2.7). (c-e): The change in illumination pattern for spirals with 24 spiral arms per spiral at the lowest (c, 744 nm) and the highest (d, 850 nm) wavelengths. Coloured dots are locations of which the wavelength-dependent total absorbed energy per duty cycle for a typical GNR is shown in the corresponding colour in (e). Values are based on $P_{\text{laser}} = 280 \, \text{mW}$, $T = 0.1 \, \text{s}$. Other simulation parameters are $\sigma_G = 1.15$ and $A = 15 \, \mu \text{m}$. The inset in (e) shows a section of the shape of a single spiral. (f-h): The same simulations as in c-d, but with 42 spiral arms per spiral.
4.3 Melting of GNRs

When a GNR melts, the longitudinal resonance band blueshifts towards the transverse band, causing the spectral response to move outside the range of the excitation laser. It can then no longer be used for bio-sensing using 2-photon luminescence in the infrared part of the spectrum. Therefore it is important to understand how and when they melt.

When a sample of GNRs on a glass surface is continuously imaged at a fixed wavelength, the 2-photon luminescence of individual GNRs can be followed in time. We observed a variety of behaviours. Examples of the different types, selected from 1442 measured traces, are shown in figure 4.3. The majority, 63% of the traces, stay luminescent during the entire experiment of 100 s (figure 4.3a). However, their intensity is not always constant. Some of those traces contain step-wise changes, indicating that those might be clusters of GNRs. 6% of the traces have an intensity that nearly instantly decreases to zero such as the one shown in figure 4.3b. 23% of the traces rapidly decrease to zero intensity after having had a consistent non-zero signal for some time (figure 4.3c). 7% first show a short increase in intensity before disappearing (figure 4.3e). A possible explanation for these two types of traces is shown in figures 4.3d and 4.3f. When the resonance peak of the GNR is at a wavelength which is higher than the laser wavelength, the blue-shifting of the spectral response as the rod melts first induces an increase in intensity before it vanishes. If the resonance peak is below the laser wavelength, the blueshift drops the intensity without an initial increase.

Figure 4.3g shows a histogram of the measured resonance wavelengths of 67 GNRs on the same sample. Based on the ratio between traces which do and which do not show a peak in the intensity before going to zero, one would expect that the ratio between traces which melt with a peak and those which melt without a peak to be comparable to the ratio between the number of GNRs with a resonance wavelength above and below the wavelength used in figures 4.3a-f. This ratio was approximately 1:3.3 (7% and 23%) for the time traces. In the spectra, the ratio was 1:0.7 (37 traces with $\lambda_{\text{res}} > 785 \text{ nm}$ and 26 traces with $\lambda_{\text{res}} < 775 \text{ nm}$). This implies a different nature of the various observed types of melting. Note however that a dip occurred in the spectra at 780 nm, suggesting that several GNRs were melted before a spectrum could be taken. This does not explain the different ratios though, as such effect would decrease GNRs with resonance wavelengths higher than than and lower the excitation wavelength alike. Therefore we cannot say with certainty what causes the different types of melting behaviour.
4.3 Melting of GNRs

Figure 4.3: GNR stability during spiral-scanning 2-photon microscopy. Different types of GNR-melting or reshaping occur. Percentages indicate the number of traces which follow the described behaviour out of a total of 1442 traces in five measurements. (a): An example of an uncategorised trace, i.e. a trace which does not show melting or which is unstable. (b): An example of a trace which vanishes nearly instantly. (c): An example of a trace abruptly vanishing without an initial increase in intensity. (d): An illustration of a possible explanation to the type of trace shown in figure (c). As the aspect ratio of a GNR decreases due to
Results

If a GNR with a resonance wavelength below the laser wavelength abruptly blueshifts, this results in a decrease in signal intensity. (e): An example of a trace of which the intensity increases for a short time before vanishing. (f): A possible explanation to the type of trace shown in figure (e). If a GNR with a resonance wavelength above the laser wavelength melts, the intensity can increase before it vanishes. (g): The distribution of the spectrum resonance peaks measured on the same sample as figures (a), (b), (c) and (e). Before measuring the spectra, the laser was put into focus at 780 nm. Only traces where the Lorentzian-squared spectrum was fitted with $R^2 \geq 0.7$ are included. Spectra were measured with $A = 11.5 \mu m$, $\sigma = 1.2$ and $n = 12$ spiral arms. Examples of individual traces can be found in Appendix A.2.

4.4 Timescales in GNR melting and reshaping

In order to decrease the amount of melting of our GNRs, we need to properly understand the timescales and energies involved in our measurements. Based on the earlier work [28] discussed in section 2.3.2, we know that the timescales involved in the absorption of energy by the plasmonic electrons and the recovery of thermal equilibrium happen in the order of magnitude of a few nanoseconds. They also showed that the temperature does not substantially increase after the first few pulses, after which the average temperature has increased by approximately $3^\circ C$. Our laser has a repetition time of $12.5 ns$ but there are several additional timescales involved. This means that we need to study the exposure of single GNRs over many pulses to quantify the relevant energies around the melting-threshold to understand GNR reshaping in our set-up.

Figure 4.4a shows the time-scales of the various processes involved in the melting of GNRs and the time-scales involved in our measurements. It shows that the absorption of energy by the plasmonic electrons through electron-electron scattering is short enough to happen within the duration of a single pulse. It also visualises that the heating of the GNRs through electron-phonon scattering and the recovery of thermal equilibrium with the surroundings through phonon-phonon coupling both happen at time scales which are several orders of magnitude smaller than the repetition time of the laser.

Figure 4.4b shows the power distribution of the absorbed energy within a single pulse, which follows a sech$^2$-curve [29]. Integrating this curve yields the energy per pulse. Figures 4.4c, 4.4d and 4.4e show the distribu-
4.4 Timescales in GNR melting and reshaping

Figure 4.4: The timescales involved in the melting or thermal reshaping of gold nanorods. (a): The relevant timescales for the energy processing by GNRs (black arrows) and the relevant timescales for the set-up used in our experiments (dashed blue lines). Adapted from Ekici et al [28]. (b-e): Visualisations of numerical simulations of the energy per pulse for a single GNR at the femtosecond single-pulse timescale for $d = 5.5 \mu m$, $A = 7 \mu m$, $\sigma_G = 1.15$, $\sigma_{PSF} = 0.48 \mu m$, $P_{laser} = 280 mW$ and $T = 0.1 s$, with 20 arms per spiral. (b): A single laser pulse on the femtosecond timescale. (c): The laser has a repetition rate of 80MHz, such that pulses arrive at the nanosecond timescale. (d): As a beamlet...
crosses a GNR at the millisecond timescale, the absorbed energy per pulse depends on the distance of the centre of the pulse to the GNR, causing a time-dependent change in energy per pulse. (e): A full duty cycle of spiral scanning with various peaks. (f): The pattern the three beamlets follow during a single spiral scan which was simulated in figures (b-e) and (g). (g): The total energy distribution of the three beamlets per scan. Values are determined for single GNRs based on an absorption cross-section of $(60 \text{ nm})^2$. The red dot is the GNR location corresponding to figures (c-e).

In conclusion, figure 4.4 shows that the absorbed energy per pulse for a single GNR strongly changes during a spiral scan. This makes that there is a chance of melting during only a fraction of the total duration of such a scan. When the energy is at a peak, the important parameter which influences the chance of melting is the amount absorbed energy per pulse.

4.5 Bio-sensing of fibronectin using GNRs

It has been shown before that gold nanorods can be used for the detection of single unlabeled proteins. Here, we want to follow the experiment by Ament et al., who measured fibronectin using 1-photon scattering of gold nanorods. We aim to benefit from the good signal-to-noise ratio and spectral sensitivity of multifocal two-photon microscopy.

Figures 4.5a and 4.5b show two intensity traces for GNRs in a HEPES
4.5 Bio-sensing of fibronectin using GNRs

Figure 4.5: Measurements of GNRs in different concentrations of fibronectin in HEPES. (a): A single GNR-trace in 1.25 µg/mL fibronectin. The right part of the figure shows a histogram with the distribution of the measured intensity. Red circles indicate detected peaks in the intensity histogram. (b): A single GNR-trace without fibronectin in the sample. (c): The mean standard deviation in the measured intensity per sample for various concentrations of fibronectin. Data points are based on 45 traces on average. (d): The mean power spectral density of all traces per fibronectin concentration on a log-log plot. Values in the legends refer to fibronectin concentrations in µg/mL. Data are based on 207 traces for 5 µg/mL fibronectin, 149 for 2.5 µg/mL, 128 for 1.25 µg/mL, 146 for 0.625 µg/mL and 91 for no fibronectin. (e): The spectral density of all traces for fibronectin concentrations of 0 µg/mL, 0.625 µg/mL and 5 µg/mL, for the lowest frequencies on a lin-log plot. (f): The same as figure (e), for concentrations of 0 µg/mL, 1.25 µg/mL and 2.5 µg/mL. (g): The average Fourier transforms of traces without fibronectin in HPLC-grade water (23 traces) and in HEPES (24 traces), measured on another sample than the data shown in figures (a-g).
buffer with and without 1.25 µg/mL fibronectin. More examples of traces at various concentrations can be found in appendix A.3. Individual traces are complemented with histograms of the intensity distribution per trace. One would expect to see a shot noise limited constant intensity for samples without fibronectin, and clear steps when fibronectin binds or unbinds. Those steps can then be distinguished as peaks in the histograms, as shown by Ament. In figure 4.5a we see the fluctuations in the intensity when fibronectin was added to the sample. The changes however were hardly discrete, and the intensity histogram shows a continuous distribution. The control experiment, with HEPES buffer but without fibronectin, on the contrary shows a two-level distribution (figure 4.5b). Such instabilities were common, as can be seen in Appendix A.3, and were also observed in our melting experiments. Nonetheless, the intensity fluctuations in the presence of fibronectin were striking and therefore two other approaches were used to quantify the fluctuations, based on the standard deviation of the traces and on their Fourier transforms.

The average standard deviation of each trace is shown in figure 4.5c for increasing concentrations of fibronectin. The standard deviation does not appear to change between the various concentrations of fibronectin. In the Fourier space, as shown in figure 4.5d, it is also hard to distinguish the traces when looking at the full spectrum. But when looking at the lower frequencies, an increased signal was observed (figures 4.5e and 4.5f) between traces with low (0 or 0.625 µg/mL) or high (5 µg/mL) concentrations of fibronectin and intermediate concentrations (1.25 µg/mL, 2.5 µg/mL). In 0.625 µg/mL the binding and unbinding of the fibronectin may be too infrequent for it to be visible in the frequency-domain in figure 4.5e, causing no increase in the spectral density to be observed in this regime. In 5 µg/mL, the steps might be of such high frequency that single steps can no longer be discriminated. The other two concentrations might be in the midway regime, resulting in the increase in signal for low frequencies in figure 4.5f. Finally, figure 4.5g shows a comparison between the same range in the Fourier space for GNR traces in HPLC water and in HEPES buffer, which shows that the spectral density for low frequencies is lower in high-purity water than in the buffer. This last experiment indicates that the step-wise fluctuations which are present in the control experiments without fibronectin may be caused by impurities in the buffer.

Although we cannot discriminate single steps in the intensity traces in figure 4.5, the spectral response of the different samples shows an increase in intensity at low frequencies for certain concentrations of fibronectin.
In this thesis, various aspects which are crucial for multifocal two-photon bio-sensing experiments were studied. First, the formation of a homogeneous illumination pattern across the xy-plane for different wavelengths was examined. Secondly, measurements of the melting frequency and types of melting were presented, as well as a numerical analysis of the timescales and energies involved in the melting process. Finally, measurements of the luminescence intensity of single GNRs in different concentrations of the protein fibronectin were introduced.

All results before the fibronectin-experiment discuss factors which have to be optimised to correctly perform bio-sensing experiments. The two sets of results focus on the illumination pattern. The illumination needs to be homogeneous across different wavelengths to prevent fluctuations in the luminescence signal which are caused by inhomogeneities. Understanding the melting of GNRs in a sensing experiment, to which the third and fourth part of the results are related, is important as it is a limiting factor to the amount of data produced. In addition to that, partial reshaping of GNRs can influence the spectral response of the GNRs, therefore decreasing the consistency and reliability of the obtained data.

Section 4.1 shows that the homogeneity of an illumination pattern depends on how well a single-spiral illumination pattern compares to a Gaussian distribution and on the ratio between the width of the spirals and the lattice spacing. When making spirals with a larger amplitude (as compared to the $A = 2.15 \mu m$ reported previously [30]), a dip in the centre of the intensity profile appears. The velocity distribution indicates that this is caused by an increase in velocity in the beginning of the spiral, leading to
a reduction in intensity. For smaller spirals, the width of the PSF appears to be able to fill this gap. This indicates that for larger amplitudes (or larger \( A : \sigma_{PSF} \) – ratios), it is no longer possible to produce homogeneous illumination patterns using the currently used Archimedean spirals.

For a perfect Gaussian profile, the velocity should scale with the inverse of a Gaussian, i.e.

\[
v_{\text{ideal}}(r) \propto \exp \left( \frac{r^2}{2\sigma_G^2} \right)
\]

(5.1)

In the actual pattern given by equations 3.1 and 3.2, using \( r = \sqrt{x^2 + y^2} = A\tau \) such that \( \tau = \frac{r}{A} \), we find that the velocity of the laser beam can be written as

\[
v_{\text{laser}}(r) = \frac{dr}{dt} + r \frac{d\theta}{dt} = \frac{dr}{dt} + r \frac{d}{dt} \left( 2\pi n \tau \right)
\]

\[
= \left( 1 + \frac{2\pi nr}{A} \right) \frac{dr}{dt}
\]

(5.2)

which does not result in \( v_{\text{laser}} = v_{\text{ideal}} \) with \( r = A\tau \). This makes it impossible to get perfect Gaussians using the current spiralling procedure.

In the case of perfect Gaussians, figure 4.1 indicates that the homogeneity of the flat-topped profiles depends on the ratio \( \sigma/d \). Van den Broek et al. [30] report an intensity distribution with \( \langle I \rangle = 1780 \text{ counts} \pm 0.12\% \), which based on figure 4.1n can be reached if \( \sigma/d \gtrsim 0.6 \).

Section 4.2 shows that the wavelength of the excitation laser can have a strong influence on the illumination pattern due to the dependence of the lattice spacing and the PSF width on the wavelength. This results in fluctuations in the excitation intensity for individual GNRs when sweeping the wavelength. The strongest fluctuations arise when the smallest width of the PSF along the spectrum is too small compared to the distance between consecutive spiral arms. This is related to the process displayed in figure 4.1n, but for PSFs with varying intensity. If we define \( d_{\text{spiral}} = A/(n - 1) \) as the distance between consecutive spirals, in figures 4.2(c-e) we find that \( \sigma_{PSF,\text{min}}/d_{\text{spiral}} \approx 0.46 < 0.6 \). This results in strong fluctuations in the illumination intensity. In figures 4.2(f-h) we find that \( \sigma_{PSF,\text{min}}/d_{\text{spiral}} \approx 0.82 > 0.6 \), resulting in a much more homogeneous intensity profile for single GNRs across the different wavelengths. Those values confirm that we can use the same ‘rule of thumb’ as the one used
for $\sigma/d$ as a requirement for $n$:

$$n_{\text{spirals}} > 0.6 \frac{A}{\sigma_{\text{PSF, min}}} + 1$$

Section 4.3 shows that traces of melting GNRs sometimes show a peak in the intensity before the intensity drops to zero. Figures 4.3d and 4.3f pose a hypothesis on what the physical principle behind this might be. However, the statistics on the number of traces which do and which do not show the short increase in intensity do not match with the positions of the SPR peaks in figure 4.3g. A higher number of SPR peaks at wavelengths below the laser wavelength would be expected. A possible explanation for this can be found when looking at the selection of the traces used in these data. Only traces which could be fitted to a Lorentzian-squared with $R^2 \geq 0.7$ were included. This excluded traces which were bright but showed unexpected fluctuations in the intensity or had double peaks. This can in some cases mean that clusters of GNRs were being measured, but it can also be related to the illumination pattern. When looking at the scanning parameters ($A = 11.5 \mu m$ and $n_{\text{spirals}} = 12$ arms), we see that the above requirement for $n_{\text{spirals}}$ is not met as long as $\sigma_{\text{PSF}} < 0.63 \mu m$, which is likely to be the case based on the results in figure 4.2b. In figure 4.2(c-h), we see that this mainly causes fluctuations in the intensity for lower wavelengths. This means that if $n_{\text{spirals}}$ was indeed too small, the spectra of GNRs with a lower SPR peak are expected to deviate from the Lorentzian-squared shape more strongly. The result would be that many traces with lower SPR peak wavelengths would be excluded from the histogram since the accuracy of their fit would be decreased. This could explain why there were fewer peaks measured with $\lambda_{\text{SPR}} < \lambda_{\text{excitation}}$ than expected.

The results found by Ekici et al. [28] on the timescales of the thermal processes involved in the heating and cooling down of GNRs indicate that we can look at single pulses to determine the probability of melting. Consecutive pulses do not cause a significant change in temperature, meaning that the chance of melting does not increase for later pulses. Therefore the relevant parameters when discussing the melting probability of a given GNR are the maximum absorbed energy per pulse and the number of time a pulse of such energy reaches the GNR. In figure 4.4 we see that the maximum energy absorbed by a GNR from a single pulse is approximately 14 $fJ$. This is in close agreement with the value which is found when calculated for the same pulse energy and PSF-width using the method which leads to equation 2.9: $E_{\text{max, GNR}} = 14.2 fJ$. This is below the threshold energy to melt a GNR as determined by Link and El-Sayed [22], both when
compared to the experimental value (16 \text{ fJ}) and to the theoretical result (60 \text{ fJ}) they reported. Due to spiral scanning, this value is for a given GNR however only approximated during a small fraction of every duty cycle. This causes the chance of melting to be negligible during the vast majority of the time per duty cycle. If the laser power would be slightly higher or if the width of the PSF would be slightly lower, however, $E_{\text{max,GNR}}$ would quickly exceed 16 \text{ fJ}, significantly increasing the chance of melting per full spiral scan.

The results in section 4.5 show that it is not yet possible to distinguish time traces of GNRs with and without fibronectin based on histograms of the luminescence intensity, which is the method used by Ament for distinguishing them using 1-photon scattering [5]. However, when looking at the power spectral density, an increase in signal for GNRs in fibronectin concentrations of 1.25 \text{ µg/mL} and 2.5 \text{ µg/mL} is visible for lower frequencies. For both lower (0.625 \text{ µg/mL}) and higher (5 \text{ µg/mL}) concentrations, this increase was not visible. This pattern of a change in signal only being visible for certain concentrations is interesting, since those concentrations are in accordance with what Ament called the ‘optimal concentration for the detection of single steps’ [34]. This is an indication that the measured difference in spectral response for the different samples is actually a result of the change in fibronectin concentration.

However, even with this difference being observable it is remarkable that there are steps in the signal visible for control measurements. This can be caused by impurities in the HEPES buffer which was used in all measurements. Figure 4.5g indicates that this might indeed be part of the problem, as measuring a sample of GNRs in HPLC-grade water instead of in the HEPES buffer already strongly decreases the low-frequency signals in the measurements.
Conclusion

We determined the behaviour of the spiral scan-illumination pattern for different lattice spacings and found that it is not possible to create a homogeneous illumination pattern for our current lattice spacing using the spiral scanning system we use now. Nevertheless, for getting an optimal homogeneity given this limitation, we need the spiral width to be large enough with respect to the lattice spacing. When performing spectral scans by sweeping the wavelengths we found that it is crucial to have a high enough number of spiral arms to prevent sudden fluctuations in the luminescence signal, especially for lower wavelengths.

We found that melting of GNRs happens in different patterns, but the hypothesis that this depends on the position of the SPR peak with respect to the excitation wavelength seems to conflict with the SPR peak histogram of the sample. This might be caused by inhomogeneities in the illumination pattern. It would therefore be interesting to replicate this experiment with different parameters. We also determined that we need to look at the peak single-pulse absorption by a single GNR as the main parameter which influences the likelihood of a GNR melting.

Finally, we found that we can, through an increase in the power spectral density for lower wavelengths, see some change in the luminescence signal of GNRs in various concentrations of fibronectin. However, we can not yet measure single steps due to unexplained fluctuations in the signal in control measurements. Contamination of the buffer might play a role in this. It would be interesting to perform this experiment again in the future with a more purified sample in order to make it possible to perform bio-sensing through the detection of single plasmon resonance shifts in the luminescence signal.
Bibliography


A.1 Wide-field luminescence pattern

Figure A.1: The time-averaged \( t = 180 \text{s} \) luminescence signal of a sample of GNRs, used for the determination of single GNR locations. (a): The full 512×512 pixels field of view. (b): The section marked in red in (a) in more detail. The blue square is a residual of the ImageJ software.
A.2 GNR spectrum traces

Figure A.2: Some examples of the single-GNR (λ,I)-spectra which were used for the histogram in figure 4.3g.
A.3 Traces of GNRs in fibronectin

Figure A.3: Intensity traces for individual GNRs in different concentrations of fibronectin, and the corresponding intensity histograms. The traces are part of the results presented in figure 4.5.