On the fluorescence properties of chromophores near metallic nanostructures

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Chapter 6

Confocal imaging studies on fluorescent dyes placed within an array of Au dots

Abstract

A pattern of Au dots on a glass coverslip has been prepared via gold evaporation in a thermal evaporator under vacuum conditions through a nanostencil consisting of a silicon nitride microsieve with a hexagonal pattern of pores with a diameter of 0.45 μm. The pattern is analysed with light microscopy and SEM. The coverslips are then functionalised with a dye and samples were investigated with confocal microscopy imaging. From the images it is found that quenching of the fluorescence by the small Au dots occurs. However, during the evaporation the gold diffuses on the surface, and a halo of a thin transparent continuous Au layer is formed on the glass surface, which suppresses the fluorescence of the chromophore.
**Introduction**

In Chapters 2 and 3 the effect of gold nanoparticles on the fluorescence of nearby chromophores has been investigated in solution. In these studies, we looked at changes in the average fluorescence intensity of the ensemble. In this Chapter, we wish to investigate the fluorescence of a chromophore placed between an array of gold particles using confocal microscopy.

A regular hexagonal pattern of gold is deposited on a quartz coverslip, after which it is functionalised with a fluorescent dye. We have used the perylene monoimide derivative **P011** (Figure 1; See Chapter 2) as chromophore. Our collaborators have predicted that when a perylene diimide chromophore is sandwiched between two nanospheres, its radiative rate is strongly increased and consequently the brightness of the molecule is enhanced.\(^1\) Further investigations have been carried out on systems of metal nanoparticles places in a 2-dimensional array in the XY plane (each particle with a diameter of 25 nm).\(^2\) These studies showed that strong fluorescence enhancement of chromophores can take place and the factor of enhancement of the relative brightness can be up to two orders of magnitude at interparticle distances ranging from 5 to 35 nm. In our experiments, the pattern of Au dots provides such locations and it may be possible to find local ‘hot spots’ in the pattern using the fluorescence of **P011**. At this stage, these experiments are only exploratory, and so, the confocal microscopy studies were done on a continuous thin layer of chromophores deposited from a solution of \(10^{-5}\) M in toluene, not yet on single molecules.

![Figure 1: Chemical structure of P011.](image)

Confocal microscopy is a useful method to find exact positions where fluorescence quenching or enhancement occurs. An advantage of this analysis tool is that is does not involve averaging over the whole ensemble, and local ‘hot spots’ can be found this way. Microscopy experiments are a useful tool for finding local effects of metal nanostructures. For example, on silver fractal-like structures which are coated with a protein labelled with a chromophore revealed local positions where the fluorescence intensity of the chromophore was increased.\(^3\) In addition, studies on Cy5 bound to silver nanoparticles of \(~20\) nm did show enhancement of the fluorescence accompanied with a faster fluorescence rate and improved photostability.\(^4\) Stronger enhancement of the fluorescence by
silver dimer. Likewise, by depositing fluorescently labelled DNA strands on a porous silver layer, increased fluorescence intensity of single molecules has been shown at certain positions. Also stronger photoluminescence is observed in so-called “Bowtie” antennas, consisting of two metallic triangles facing tip to tip that are separated by a small gap because of significant electric field enhancement by coupling between the metal nanostructures.

The technique used here to make regular and well-defined nanostructure patterns of metals or semiconductors on surfaces is shadow mask evaporation. It makes use of a nanostencil (Figure 2) consisting of a silicon nitride membrane with pre-defined patterns (microsieve) as a mask for physical vapour deposition (PVD) of metals, or other materials, under high vacuum conditions. It is called shadow mask evaporation since the nanostencil forms a local ‘shadow’ for the deposited material. The nanostencil is attached to a substrate (a quartz coverslip) during evaporation of a gold wire under ultra high vacuum conditions. It is thus possible to make nanostructures on a surface by evaporation of metal through the holes of the nanostencil, after which it sublimes onto a substrate (Figure 3). Three dimensional structures of metals, semiconductors or even complex oxides have been successfully prepared using this technique. It is a promising tool with increasing interest in the field of nanotechnology. One example where this method is employed is in fabrication of Si nanorods on a surface. On a silicon layer, a thin layer of Au dots is deposited by PVD, which in turn functions as an etching mask for the underlying silica substrates in a reactive ion beam etching process.

For our research purposes, the shadow mask evaporation method is used to deposit a pattern of gold islands on quartz microscopy coverslips. A number of nanostencils (Figure 2) have been received from Aquamarijn Micro Filtration B.V., and used as such. This nanostencil contains a microsieve of silicon nitride. The sieve consists of 14 fields of a porous layer of 140 µm wide. Between
received from Aquamarijn Micro Filtration B.V.,12 and used as such. This nanostencil contains a microsieve of silicon nitride. The sieve consists of 14 fields of a porous layer of 140 μm wide. Between each field is a non-porous beam of 75 μm wide. In addition, each layer also has a set of thin non-porous beams of 3 μm through the porous layer. The porous layer consists of a hexagonal pattern of pores of 0.45 μm diameter. Extrapolation of the predictions of our collaborators1 indicates that if a chromophore is placed between these Au dots, the relative brightness will be strongly enhanced. The thickness of the microsieve itself is 1 μm.

![Figure 3: Schematic representation of the gold evaporation experiments. A: Placement of the nanostencil to the substrate; B: Evaporation of metal through the silicon nitride membrane; C: Removal of nanostencil revealing a pattern on the substrate. Adapted from reference 8.](image)

**Results and discussion**

**Sample preparation**

A pattern of Au dots was prepared on a quartz coverslip via gold evaporation through a nanostencil under vacuum conditions in a Balzers thermal evaporator. The resultant pattern is analysed with light microscopy and Scanning electron microscopy (SEM). The resultant gold pattern can be observed with a normal light microscope, and shows the regular hexagonal pattern, as well as the small gaps of 3 μm (Figure 4). The SEM measurements were performed at the Vrije Universiteit Amsterdam. Based on these images (Figure 5) the hexagonal pattern of the Au dots is apparent; the Au dots have a diameter of 400 - 500 nm and the distance between the Au dots is about 100 - 150 nm.

Not in all the Au patterns the dots were well ordered. In some cases, it is clear that an Au dot is not present on the pattern, as can be seen in Figure 4B, and examples are found where on the sides of the regular pattern the Au dots were somewhat scattered over the surface resulting in an irregular pattern.
A thin layer of a pure chromophore ($P011$) was thereafter spincoated on these glass coverslips. Two kinds of samples were prepared: One with a thin layer of polystyrene polymer as a spacer between the Au pattern and the layer of chromophores (Type I), and one where the pure chromophore was spincoated directly on top of the Au pattern (Type II) without polymer spacer. The thickness of the polymer spacer was estimated at 40 nm. At this stage, these experiments are only exploratory; a continuous layer of chromophores has been made on the coverslips from a solution of...
10^5 M in toluene. A graphical representation of the analysed samples is shown in Figure 6. The samples were analysed with confocal microscopy imaging. The samples can be placed on the objective either with the glass coverslip ('gold down'), or turned around and placed on another coverslip ('gold up'). In the first case the excitation light and the emitted light from the fluorescent dye layer needs to pass through the gold layer, in the second case, the fluorescent dye is directly excited.

**Confocal microscopy imaging**

*Type I: ‘gold down’ approach*

The coverslip with the polystyrene spacer between the Au pattern and the chromophore layer was placed on the objective of the microscope face up, so that the Au pattern is underneath the chromophore ('gold down'). The samples are excited with light of 488 nm and the fluorescence from the sample, after passing the dichroic mirror, passes through a notch filter and an emission filter, to remove reflected and scattered laser light, before it reaches the detector. A 2-dimensional fluorescence image in the XY plane has been made of the sample in this configuration. An example is shown in Figure 7; the light microscope image is shown in Figure 7A. The corresponding fluorescence image is shown in Figure 7B as an inverse contrast image in which dark means high fluorescence intensity. An overlay of both images is shown in Figure 7C. No fluorescence is observed between the Au dots in the Au pattern, but at positions in the sample where no Au dots are present, the fluorescence from the chromophore is observed. Outside the Au patterns a uniform layer of fluorescence is detected, indicating that the chromophores are distributed fairly homogeneously. The fluorescence image itself is exactly complementary to the image of the light microscope. On the border of the Au patterns a thin line corresponding to the gap of 3 μm between the regular patterns of fluorescence can be observed (Figure 7B). The cross-section perpendicular to this line is shown in Figure 7D. It has a constant width (FWHM = 2 μm) along the line that is somewhat thinner than the 3 μm gap between the Au dots, and the intensity does not change either. Moving towards the end of the pattern, the Au pattern becomes thinner and the fluorescence intensity increases gradually. This is shown in the three parallel cross-sections shown in Figure 7E. The fluorescence lifetime of the sample (~ 3.7 ns) does not vary, which suggests that all detected fluorescence stems only from non-quenched chromophore.

A part of the sample with defects on the Au pattern is shown in Figure 8. The fluorescence intensity image in Figure 8B shows that the emission can be observed through large, but also through small defects, on the Au pattern. Moreover, the overlay image (Figure 8C) indicates that the fluorescence is observed through all defects in the patterns, indicating that the polymer film and the...
chromophore layer are uniformly present on the Au pattern and that the polymer solution has not floated off the rough surface of the pattern during spincoating (lotus effect). 

**Figure 7**: Results of the confocal microscopy imaging: type I sample, ‘gold down’. A: Light microscopy image (70 × 80 μm); B: Fluorescence image (inverse contrast, dark means high intensity; 80 × 80 μm); C: Overlay of both. Panels D and E: cross-sections of the fluorescence intensity along the lines shown in panel B.

**Figure 8**: Results of the confocal microscopy imaging: type I sample, ‘gold down’. A: Light microscopy image (70 × 80 μm); B: Fluorescence image (inverse contrast; 80 × 80 μm); C: Overlay of both (45 × 45 μm).
Chapter 6

Type I samples: ‘gold up’ approach

To verify whether the Au pattern is transparent for the wavelengths of excitation and emission, the sample is turned so that the Au pattern is above the chromophore layer with regard to the excitation beam (‘gold up’ approach). In this setting, the incident laser light and the emission light do not have to go through Au the pattern. The fluorescence image of such a sample consists of a homogeneous layer independent of the presence of the pattern (Figure 9A) and in this case, no influence of the Au pattern is observed.

![Figure 9: A: Fluorescence image (inverse contrast; 80 × 80 μm) of the type I sample, ‘gold up’. B: Reflection image (inverse contrast; 80 × 80 μm) of the Au pattern without loading of polystyrene spacer or chromophore, using an increased excitation laser power.](image)

When the laser power of the excitation light is increased by nearly three orders of magnitude, the reflected laser light is not completely suppressed by the emission filters, so that it is detected by the detectors. The reflection of the gold allows the observation of the Au pattern on the coverslip, which is not functionalised with the chromophore (Figure 9B). The gap of 3 μm is also visible in such a fluorescence image. By this means, in the type I ‘gold up’ samples, both fluorescence of the chromophore and reflection of the gold pattern can be detected in one image (Figure 10). The black line in the cross-section across the gap (Figure 10C) is averaged over multiple cross-sections which themselves are indicated in gray. The FWHM is the same as in the previous samples (‘gold down’ approach). As there appears no evidence of quenching, it is believed that the absence of fluorescence from the gold pattern in the ‘gold down’ samples is solely caused by a shielding effect of a continuous layer of gold on the Au pattern. In addition, the fluorescence lifetime of this sample does not differ from that of the normal chromophore.
Figure 10: Results of the confocal microscopy imaging: type I sample, ‘gold up’. A: Light microscopy image (70 × 70 μm); B: Fluorescence image with increased laser intensity (inverse contrast; 80 × 65 μm). Panel C: cross-section of the fluorescence intensity along the line shown in panel B.

*Type II samples: ‘gold down’ approach*

The second set of samples does not contain a polymer spacer, but instead the chromophore is directly spincoated on top of the Au pattern (type II samples). The fluorescence images (Figures 11 and 12) of these samples when viewed through the Au pattern reveal that, like in the type I samples, no fluorescence is observed between the Au dots in the regular Au pattern. The fluorescence images on the defects of the Au pattern show fluorescence of the chromophores except in close proximity of the Au dots. For example in Figure 11B, the shape of the fluorescence matches the shape of the Au pattern in the same area. The distance in which the fluorescence is suppressed, however, is much larger than is expected. A close-up on one area (Figure 11C) clearly indicates that the fluorescence intensity is diminished at distances > 1 μm from an Au dot. On the border of the Au patterns a thin line of fluorescence can be observed, corresponding to the gap of 3 μm between the regular patterns. The width of this line (FWHM in Figure 11E: a: 1.1 μm, b: 1.3 μm, c: 1.5 μm) and the fluorescence intensity (Figure 11D) vary when going along the layer. In addition, the width is clearly less than that of the type I sample.

Some clusters of aggregates of chromophores can be seen in the light microscopy images (Figure 12A); these aggregates are also clearly visible in the fluorescence images (Figure 12B). Using them as anchors, the two images can be overlaid. It is in this case found that at positions on the irregularities of the Au pattern, the fluorescence intensity is lower than at positions of the sample where no gold is present (outermost left in Figure 12). It seems likely that, on the substrate, there is a layer of Au that is not visible with the light microscope, which quenches the fluorescence of the chromophore.
Figure 11: Results of the confocal microscopy imaging: type II sample, ‘gold down’. A: Light microscopy image (70 × 70 μm); B: Fluorescence image (inverse contrast; 80 × 80 μm). Panel C: Overlay of the light microscopy image and the fluorescence image (30 × 30 μm). Panel D: Cross-section of the fluorescence intensity (logarithmic scale) along the 3 μm gap. Panel E: Cross-section of the fluorescence intensity along the lines shown in panel B.

Figure 12: Results of the confocal microscopy imaging: type II sample, ‘gold down’. A: Light microscopy image (70 × 80 μm); B: Fluorescence image (inverse contrast; 80 × 80 μm); C: Overlay of both. Aggregates of chromophores are encircled in Panels A and B.
Type II samples: ‘gold up’ approach

The sample is turned so that the Au pattern is above the chromophore layer with regard to the excitation beam. These images show that fluorescence of the chromophore on top of the regular Au pattern is completely quenched whereas the fluorescence is observed in defect areas or outside the Au pattern (Figure 13 and 14). Interestingly, as is shown at certain positions in Figure 13B, the fluorescence appears to be also quenched at places where no Au dots are visible in the light microscopy image. This observation is also found in Figure 14. In this figure, the fluorescence image is shown from the 75 μm gaps between two porous fields of the nanostencil. The regular Au pattern is apparent by the absence of fluorescence, but cannot be observed with the light microscope. The distance of the fluorescent layer on this area is much less (Figure 14B; FWHM = 59 μm) than that of the actual beam. This indicates that the fluorescence is still quenched at a distance of 7 μm from the Au pattern at both sides, presumably by a dispersed layer of gold.

Figure 13: Results of the confocal microscopy imaging: type II sample, ‘gold up’. A: Light microscopy image (60 × 70 μm); B: Overlay with the fluorescence image (40 × 40 μm).

Figure 14: Results of the confocal microscopy imaging: type II sample, ‘gold up’. A: Fluorescence image (inverse contrast; 80 × 80 μm); B: cross-section of the fluorescence intensity along the lines shown in panel A.
Chapter 6

Discussion

When the sample with a polymer spacer between the gold and the fluorescent dye is illuminated from below, through the gold, emission is only observed in the areas in which gold is not present, but not in the holes in the gold pattern. When the sample is turned, and the fluorescent layer illuminated directly, strong fluorescence is observed, which is not quenched by the gold layer. The observation that fluorescence is not observed in the first experiment may be explained by the non-transparency of the gold layer, while it is known that metallic films with small submicrometre cylindrical holes transmit light.\(^{15}\) It appears that also in areas close to the deposited gold no light is passed through the sample. This suggests that the deposition process is accompanied by some blurring of the gold layer, as illustrated in Figure 15. The pattern itself is consequently not transparent and blocks the excitation and emission of the chromophore that needs to pass through the Au pattern to reach the detector. This ‘shielding’ effect can explain the complete absence of detected fluorescence from the chromophore on the Au pattern.

![Figure 15](image)

*Figure 15:* A: Schematic representation of the blurring of the deposited nanostructures occurs due to diffusion of the gold.\(^9\) B: Proposed resultant nanopattern after evaporation with a continuous layer of gold formed on the pattern.

Towards the edge of the Au pattern, the thickness of the Au pattern decreases and therefore the pattern is more transparent; a slight increase of fluorescence signal from this area compared to that from the middle of the pattern is observed. When the sample is turned, the fluorescence can be directly monitored. When a spacer (polystyrene film) is present between the chromophore layer and the Au pattern is present (type I samples), no evidence of quenching or enhancement of the fluorescence is found. In this case, the distance between the Au pattern and the chromophore is likely too large to have an effect. When the chromophore is spincoated directly on top (type II samples), the regular Au pattern completely quenches the fluorescence as can be seen when the sample is placed
'gold up'. Interestingly, close to isolated Au dots, the fluorescence is suppressed for a relatively long distance, in the order of micrometres; this cannot be explained by quenching due to distinct Au dots. It is more probable that the diffusion during the preparation of the Au pattern results in the formation of a halo around the Au dots.

**Conclusion and perspectives**

The Au pattern has been successfully prepared on glass coverslips. It has been studied using SEM and can also be observed with light microscopy. Studies using confocal imaging indicated that Au dots indeed quench the fluorescence of the chromophore when it is spincoated directly on top. It is also shown that the Au layer does not consist of satisfactorily separated Au dots but instead a continuous layer of gold has formed during the evaporation, by blurring of the deposited nanostructures due to diffusion of the gold (Figure 15). The layer is even too thick to be transparent for light to pass through, and it shields the excitation light and fluorescence of the chromophore.

Advances in preparing a more distinct pattern of Au dots need to be sought. Clearly it is of importance that the nanoparticles are well isolated. A way to remove the diffused gold atoms is by corrective etching,\textsuperscript{9,16} which has been also used to improve nanostructures with smaller dimensions than the Au pattern used in our experiments. The etching time in this cleaning method needs, however, be tuned properly since it depends on various factors (material, thickness). To obtain a pattern of Au dots in which the distance between them is larger, the pattern on the microsieve could be changed. Preferably, also the pore size needs to be smaller than is the case in our experiments. Another way to obtain sharper cone-shaped Au dots is by treating the silicon nitride with a self assembled monolayer of alkyl and perfluoroalkyl chains.\textsuperscript{17} This treatment reduces the diffusion of gold atoms and also prevents clogging of the microsieve. Alternative methods that do not involve the nanostencil can also be considered. For example, annealing of a metal surface yields structured metal nanostructures,\textsuperscript{18} but this method does not provide well ordered patterns. An elegant method is Au deposition through a shadow mask consisting of a highly ordered monolayer of nanospheres.\textsuperscript{19,20} This technique is used to fabricate regular arrays of prismatic shaped Au particles with sufficient distance between them.\textsuperscript{20} By exposing these nanoparticles with intense nanosecond pulsed laser light, the shape of these particles can be changed into colloids while maintaining the regularity of the array.\textsuperscript{21} Another possibility to prepare well-defined Au surfaces is to selectively protect certain regions of a gold layer with a self assembled monolayer of alkanethiols, followed by etching away of the unprotected Au surface.\textsuperscript{22}
Experimental Details

Sample preparation

The nanostencil was fixed to a quartz coverslip which was placed on a holder with the microsieve facing down and put into a Balzers evaporator, approximately 30 cm above the evaporation source. The source consists of a Tungsten spiral which is heated by passing a current through the spiral. Next to the sample a microscope slide was placed as a reference to monitor the progress of Au evaporation. A gold wire of 0.1 g was put inside the Tungsten spiral and the chamber was brought to high vacuum (2 - 2.5 × 10⁻⁶ bar) before the evaporation of gold was started. After evaporation of the gold, the microsieve was carefully taken off the coverslip. The pattern of 14 gold ‘stripes’ on the coverslips with inside a regular hexagonal pattern of small dots can be observed using a light microscope.

Next, the coverslips were thereafter functionalised with a fluorescent compound. In this case, we opted for P011 (see Chapter 2). The Type I samples were prepared by putting a thin layer of polystyrene as spacer on the gold pattern by spincoating a solution of 2% polystyrene in toluene, using standard settings consisting of two successive spincoating programs: 30 s with spinning rate of 6000/min followed by 10 s with spinning rate of 300/min. Hereafter the chromophore is spincoated on top using a solution of approximately 10⁻⁵ M of chromophore in toluene. Additionally, samples were prepared on which the molecule was spincoated on top of the Au dots without the usage of a spacer (Type II).

Confocal microscopy experiments

The confocal microscopy experiments were conducted using a MicroTime 200 confocal microscope from PicoQuant GmbH in our laboratory. Briefly, the laser system is based on a titanium:sapphire laser (Chameleon Ultra-II, Coherent). The output of the laser is tuned at 976 nm, at a repetition rate of 80 MHz. The frequency of the laser light was doubled to generate excitation pulses of 488 nm using a second harmonic generator (SHG). The excitation light was coupled into the adapted confocal unit via a polarisation maintaining monomode fibre. An excitation filter (HQ480/40x, Chroma Tech.) was placed in front of the excitation beam and a dichroic mirror (Z488 RDC, Chroma Tech.) reflected the excitation light to the sample. The emission light passed through the dichroic mirror, a notch filter (488NF, Semrock), a pinhole of 50 μm in diameter and an emission filter (HQ510LP, Chroma Tech.) to a single photon avalanche diode (SPCM-AQR-13, Perkin Elmer).
Typical laser power in these experiments was 100 nW. The fluorescence images were obtained by XY-scanning.

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References:

14 It is known that liquids can slide off rough hydrophobic surfaces due to high surface tension. See: Dettre, R. H.; Johnson, R. E. Contact Angle Hysteresis II Contact Angle Measurements on Rough Surfaces. In Contact Angle, Wettability and Adhesion; Fowkes, F. M. Eds.; American Chemical Society: 1964, 43, 136-144.
