**Enhanced fluorescence from metal nanoapertures: physical characterizations and biophotonic applications**

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**ABSTRACT**

Nanoapertures milled in opaque metallic films offer a simple and robust photonic tool to significantly enhance the fluorescence of single molecules. We provide a detailed physical characterization of this phenomenon for apertures milled in gold and aluminum, and discuss its application to biophotonics. For the first time, the most general figures are provided to predict the awaited enhancement factors for almost every kind of fluorescent molecule. This knowledge is essential to discuss the ability to detect low-quantum yield species. We also report the first demonstration of single metal nanoapertures to perform DNA hybridization sensing, and measure similar enhancement factors as for experiments on diffusing molecules.

**Keywords:** Metal-enhanced fluorescence, plasmonics, nanoaperture

**1. INTRODUCTION**

Fluorescence spectroscopy plays the largest role today for optical investigations at the single molecule level. Efficiently detecting the optical signal from a single molecule has therefore become a major goal with applications in chemical and biophysical analysis. The general strategy to detect the fluorescence signal from a single molecule uses a confocal microscope with a high-end immersion objective, providing high resolution and large numerical aperture to collect the fluorescence light. Despite its extreme sensitivity down to the single molecule, this approach remains restricted by the phenomenon of optical diffraction, which limits the size of the analysis volume and the detected fluorescence rate per molecule.

Metallic nanostructures offer promising ways to enhance the fluorescence emission of single molecules.\(^1,2\) An elegant way of circumventing the limits set by diffraction uses a single nanometric aperture milled in an opaque metallic film. As illustrated on Fig. 1, the nanoaperture acts as a spatial filter located directly into the object plane, restricting the number of molecules that are excited by the laser beam. When the aperture diameter is sufficiently reduced below the cut-off diameter of the fundamental excitation mode that may propagate through the aperture, the light inside the aperture is confined to an evanescent mode, with a decay length of a few tens of nanometers.\(^3\) A second major effect brought by the nanoaperture is significant enhancement of the detected fluorescence rate per emitter. This effect was first reported using rhodamine 6G molecules in isolated 150 nm diameter apertures milled in an aluminum film, showing a 6.5 fold enhancement of the fluorescence rate per molecule.\(^4\) Recently, we have achieved further enhancement up to 25-fold by tuning the plasmon properties of the nanoapertures.\(^5\)

In this contribution, we first characterize the phenomenon of nanoaperture-enhanced fluorescence and give a detailed physical analysis for apertures milled in gold and aluminum (Section 2). While gold offers a stronger...
Figure 1. Use of a metal nanoaperture to restrict the analysis volume and enhance the fluorescence rate per molecule in a fluorescence confocal microscope.

plasmonic response, aluminum provides an extended spectral range to implement nanoapertures from the UV to the near IR. Applications using metal-enhanced fluorescence in the ultraviolet region are more likely to take advantage of aluminum properties.

In any experiment of single molecule fluorescence enhancement, the values reported for the fluorescence gain are tightly bound to the initial quantum yield of the molecule. Depending on the choice of the fluorophore’s quantum yield, what are the fluorescence enhancement factors that can be typically awaited in nanoapertures? Based on our detailed analysis of the fluorescence phenomenon, we are able to answer this question in Section 3.1.

The ability to detect molecular species with low quantum yields gives a first example of the use of nanoaperture-enhanced fluorescence for biophotonics. In Section 3, we also discuss the application to perform fast fluorescence analysis and to detect single molecule DNA hybridization.

2. EXCITATION AND EMISSION CHARACTERIZATION IN METAL-ENHANCED FLUORESCENCE

2.1 Fluorescence characterization procedure

To investigate any enhancement of the fluorescence signal in a nanoaperture, it is of crucial importance to quantify the fluorescence count rate detected per molecule $CRM$. This requires the knowledge of the actual number of emitters $N$ contributing to the global fluorescence signal for each experimental run. This issue is addressed via Fluorescence Correlation Spectroscopy (FCS).

In FCS, the temporal fluctuations $F(t)$ of the fluorescence signal are recorded, and the temporal correlation of this signal is computed $g^{(2)}(\tau) = \langle F(t)F(t+\tau) \rangle / \langle F(t) \rangle^2$, where $\tau$ is the delay (lag) time, and $\langle \rangle$ stands for time averaging. Analysis of the correlation function provides a measure for the number of molecules $N$ needed to compute the count rate per molecule $CRM = \langle F \rangle / N$. Let us point out that as a consequence of the stochastic nature of the FCS technique, all the presented fluorescence data are spatially averaged over all the possible molecule orientations and positions inside the detection volume.

We have developed a specific experimental procedure to unravel the origins of the fluorescence enhancement near a photonic structure, and distinguish between the gains in excitation intensity and the gains in emission rate. This procedure can be briefly summarized as follows: the fluorescence rates per molecule $CRM$ are measured for increasing excitation powers. The resulting data points are fitted according to the model $CRM = AI_e / I_s^2$, where $I_e$ is the excitation intensity, $I_s$ the saturation intensity, and $A$ is a constant proportional to the molecular absorption cross-section, quantum yield and setup collection efficiency. We deduce from the fits the fluorescence enhancements at the two extreme cases below saturation $I_e \ll I_s$ and at saturation $I_e \gg I_s$. In the saturation regime, the fluorescence rate enhancement is determined only by the gain in emission $\eta_{em}$. In the low excitation regime $I_e \ll I_s$, the fluorescence enhancement $\eta_F$ is proportional to the gains in emission $\eta_{em}$ and local excitation intensity $\eta_{exc}$, and inversely proportional to the gain in total fluorescence decay rate $\eta_{tot}$: 
\[ \eta_F = \eta_{em}\eta_{exc}/\eta_{tot}. \]

Using supplementary fluorescence lifetime measurements to determine the alteration in the total fluorescence decay rate \( \eta_{tot} \), it is therefore possible to extract the gain in local excitation intensity from the fluorescence enhancement in the low excitation regime. This unambiguously separates the excitation and emission contributions to the total fluorescence enhancement.

### 2.2 Fluorescence enhancement in gold and aluminum apertures: a detailed comparison

We now apply this procedure to characterize the fluorescence enhancement found in gold and aluminum apertures. While the case of gold apertures has already been discussed in, \(^7\) the detailed comparison with aluminum apertures is completely new.

For all experiments reported here, we use an aqueous solution of Alexa-Fluor 647 fluorescent molecules (quantum yield in water is 30\%) deposited on top of the sample with micromolar concentration. These molecules are constantly diffusing in and out of the aperture, thereby limiting photobleaching. The excitation source is a CW He-Ne laser operating at 633 nm focused by water-immersion objective with 1.2 NA. Single photon detection is performed by avalanche photodiodes with 670 ± 20 nm fluorescence bandpass filters.

Measurements of the fluorescence rates per molecule \( CRM \) for increasing excitation powers can be reliably done by FCS. Figure 2 illustrates the evolution of the \( CRM \) for 120 and 190 nm diameter apertures milled in gold or aluminum. While it is apparent that gold provides a stronger enhancement, Figure 2 also shows that the behavior found below and at saturation are quite different for both metals.

For each aperture diameter, we numerically fit the \( CRM \) data points to infer the fluorescence enhancements at the two extreme cases below saturation and at saturation. The fluorescence gains in the regime below saturation are detailed in Fig. 3(a), they are an intricate combination of both gains in excitation intensity, quantum yield and collection efficiency. In the saturation regime, the fluorescence enhancement is equivalent to the gain in emission \( \eta_{em} \), which is displayed in Fig. 3(b). Next, we use fluorescence lifetime measurements to quantify the alteration in the total fluorescence decay rate \( \eta_{tot} \). This value is used to extract the gain in local excitation intensity \( \eta_{exc} = \eta_F\eta_{tot}/\eta_{em} \), and the alteration in the effective quantum yield of the emitter \( \eta_{em}/\eta_{tot} \). These results are displayed in Fig. 3(c) and (d) respectively. Let us point out that the gains in emission and effective quantum yield are to be understood as the gains for the number of photons as detected by our setup. Therefore, they take into account the gain in collection efficiency which adds up to the gain in the photophysical properties of the molecule.

![Figure 2. Fluorescence rates per molecule as measured by FCS for apertures milled in gold (red) and aluminum (blue). Grey crosses indicate the reference signal for molecules in water solution.](image-url)
Figure 3. Influence of the nanoaperture on the fluorescence signal versus the aperture diameter. Red circles are for gold, blue squares for aluminum. (a) Total fluorescence enhancement at low excitation powers. (b) Total fluorescence enhancement at the fluorescence saturation limit; this factor is equivalent to the gain in detected emission rate $\eta_{em}$. (c) Gain in local excitation intensity $\eta_{exc}$. (d) Gain in the effective quantum yield of the emitter $\eta_{em}/\eta_{tot}$, which corresponds to the gain in emission divided by the fluorescence lifetime reduction. Note that the gains in emission and effective quantum yield take into account the increase in the fluorescence collection efficiency.

It is apparent from Fig. 3 that the nanoaperture affects both emission and excitation process. Both effects contribute to the enhanced fluorescence rates. The shift in the optimal diameter leading to maximum fluorescence enhancement can be viewed as a direct consequence of the difference in the complex permittivity for the metals. These curves show that the higher value of fluorescence gain for gold is related to a larger enhancement of the emission rate, which tend to indicate a stronger coupling of the single emitter to plasmonic modes in the structure in the case of gold. Finally, we point out that our experimental results stand in good agreement with the numerical predictions based on the finite elements method and differential theory.

3. BIOPHOTONIC APPLICATIONS OF NANOAPERTURE-ENHANCED FLUORESCENCE

3.1 Detection of low quantum yield species
For Alexa Fluor 647 which has a quantum yield of about 30%, the maximum fluorescence enhancement found in aluminum apertures is 8-fold, while for Rhodamine 6G the enhancement is 6-fold in water (quantum yield about 90%). There is obviously a strong dependence on the fluorescence enhancement upon the fluorophore initial quantum yield: for a perfect emitter (quantum yield close to one), all enhancement origin is to be found on excitation intensity or collection efficiency, as no gain can be found on the quantum yield. Therefore, the
question is: what are the fluorescence enhancement factors that can be typically awaited in nanoapertures depending on the fluorophore’s initial quantum yield? Our detailed analysis of the fluorescence phenomenon in section 2 enables us to answer this question.

Without the nanostructure, the fluorescent molecule has a radiative rate \( k_r \), a non-radiative rate \( k_{nr} \), and a quantum yield \( \phi_0 = k_r / (k_r + k_{nr0}) \). The setup collection efficiency is \( \kappa_0 \) and the excitation rate is \( k_e \). With the introduction of the nanostructure, the radiative rate is modified to \( k_r \), the collection efficiency is \( \kappa \), and the excitation rate \( k_e \). The quantum yield now reads \( \phi = k_r / (k_r + k_{nr0} + k_{abs}) \), where the internal non-radiative rate is kept constant, and a new non-radiative decay route \( k_{abs} \) is introduced, for instance to take into account the ohmic losses into the metal and non-radiative energy transfers to the free electrons in the metal.\(^{11}\)

At moderate excitation powers (below fluorescence saturation), the fluorescence enhancement \( \eta_F \) is proportional to the gains in local excitation intensity \( \eta_{exc} \), emission \( \eta_{em} \), and inversely proportional to the gain in total fluorescence decay rate \( \eta_{tot} \). This enhancement can be explicitly written as:

\[
\eta_F = \eta_{exc} \eta_{em} \eta_{tot} = \frac{k_e}{k_{e0}} \frac{\kappa}{\kappa_0} \frac{k_r}{k_{r0}} \frac{k_{r0} + k_{nr0}}{k_r + k_{nr0} + k_{abs}} \tag{1}
\]

After some basic algebra, this equation can be expressed to introduce the initial quantum yield \( \phi_0 \):

\[
\eta_F = \frac{k_e}{k_{e0}} \frac{\kappa}{\kappa_0} \frac{k_r}{k_{r0}} \frac{1}{(1 - \phi_0) + \phi_0 \zeta} \tag{2}
\]

with \( \zeta = (\frac{k_r}{k_{r0}} + \frac{k_{abs}}{k_{r0}}) = \frac{\eta_{tot} + \phi_0 - 1}{\phi_0} \). A very nice point is that the quantity \( \zeta \) can be calibrated for a given experimental characterization of the structure (such as the one described in Section 2), and then used as a fixed parameter. For each nanostructure investigated, equation (2) can then be plotted as a function of the initial quantum yield \( \phi_0 \).

Figure 4 displays the fluorescence enhancement factors \( \eta_F \) for increasing initial quantum yield \( \phi_0 \). These curves provide the most general information to predict typical fluorescence enhancement factors. They are intended to serve as reference basis when designing nanoaperture-enhanced fluorescence experiments for a wide range of different fluorophores and spectral ranges. Lastly, we point out that the enhancement factors found for gold in Fig. 3 and 4 can be further increased by optimizing the adhesion layer to the glass slide,\(^5\) which was not the case here.

Figure 4. Fluorescence enhancement factors versus the fluorophore’s initial quantum yield as given by Eq. (2) for apertures milled in gold (a) and aluminum (b). The aperture diameters are expressed as units of the excitation wavelength \( \lambda \) for an aperture on glass substrate and molecules dissolved in water. Choice of the excitation wavelength is also important: gold apertures will perform well for \( \lambda > 570 \) nm, while aluminum will work over the entire visible spectral range \( \lambda > 200 \) nm.
3.2 Performing fast FCS at high concentrations

A critical point to keep in mind is that the signal to noise ratio (SNR) in FCS does not depend on the total detected fluorescence, but on the fluorescence count rate per molecule $CRM$ times the square root of the total experiment acquisition time $T_{tot}$ and the correlator channel minimum width $\Delta \tau$: $SNR \propto CRM \sqrt{T_{tot} \Delta \tau}$. This emphasizes the single molecule nature of FCS, and the need to enhance the detected fluorescence signal per molecule.

The data displayed in Fig. 2 shows that $CRM$s greater than a few hundreds of thousands photons per second can be readily obtained in a nanoaperture. Such high $CRM$s remain out of reach for the case of open solution, where fluorescence saturation prevents the count rate from exceeding a few tens of thousands of counts per second. A noticeable point is that even for non-optimized structures, it is still possible to get $CRM$s significantly above those in the case of open solution.

Typically, $CRM$ enhancements over five-fold can be readily obtained with a large range of nanoaperture designs, with either gold or aluminum, covering an extended range of fluorophores with different spectral properties or initial quantum yield. Since the SNR in FCS scales by the product $CRM \sqrt{T_{tot}}$, a five-fold increase in $CRM$ already amounts to a 25-fold reduction of the experiment duration, enabling fast FCS analysis with improved accuracy. To illustrate this, gold nanoapertures have been used to monitor enzymatic cleavage reactions with FCS using sampling times of one second. We believe that this opens new opportunities for probing of specific biochemical reactions that require fast sampling rates.

3.3 DNA hybridization sensing

All fluorescence enhancement reported so far using single nanoapertures have been performed on molecules diffusing in a solution that covers the sample. To what extent does the fluorescence enhancement factors translate to the case of molecules bound to the aperture bottom surface? A first hint can be found in, where an array of randomly placed nanoapertures in gold could also enhance the fluorescence output by about 7 times. Hereafter, we will provide experimental evidence that the enhancement phenomenon found for molecules diffusing is still present for bound molecules. Actually, it turns out that the enhancement factors expressed per molecule are quite comparable in both cases.

To demonstrate the use of single nanoapertures for affinity biosensing, the gold surface was first passivated using thiophenol, and then a monolayer of unlabelled avidin was formed at the bottom surfaces of the nanoapertures. Isolated nanoapertures were spotted with probe single stranded DNA molecules. After rinsing for unbound molecules, complementary DNA strands (serving as target molecules in this nano-biochip system) labeled with cyanine-5 fluorophores were then introduced over the nanostructure. The fluorescence signal after one hour incubation was monitored using $1 \mu W$ excitation power at 633 nm to measure the number of photons detected within 100 s after laser illumination.

Figure 5 shows the total amount of detected fluorescence versus the aperture diameter (right scale). For this study, we used apertures milled in gold, as they provide the higher fluorescence enhancement. We also compute the fluorescence enhancement per molecule by normalizing the detected intensity $I_{aperture}$ by the reference intensity found for a glass slide $I_{glass}$, corrected by the ratio of the surfaces being probed:

$$\eta_F = \frac{I_{aperture}}{I_{glass}} \frac{S_{glass}}{S_{aperture}}$$

This enhancement factor is also plotted versus the aperture diameter in Fig. 5 (left scale). This enhancement factor follows quite nicely the one obtained with molecules diffusing in gold apertures (see Fig. 3(a)). Metallic nanoapertures are thus demonstrated for use in DNA affinity biosensing with high signal to noise ratio. Here, all nanoapertures were spotted with the same probe DNA strand; in the ultimate limit, each aperture could be derivatized with a different probe.
4. CONCLUSIONS

The fluorescence enhancement found in nanometric apertures is demonstrated to originate from a combination of excitation and emission gains. The very general characterization procedure based on fluorescence correlation spectroscopy and fluorescence lifetime measurements enables a deep understanding of the enhancement phenomenon. The main scientific result is that for the first time general figures are provided to predict the awaited enhancement factor for almost every kind of fluorescent molecule given the essential knowledge of fluorescence quantum yield and absorption/emission spectral range, provided that the fluorescence Stokes shift remains small compared to the excitation wavelength.

Important applications of enhanced fluorescence have been discussed. These include the ability to detect low-quantum yield species and predict the awaited fluorescence enhancement. The gain in count rate per molecule translates into a quadratic reduction of experiment integration time while keeping the same SNR. This illustrates the capability to perform single molecule detection within short time ranges. Lastly, we have reported the first demonstration of single nanoapertures to perform DNA hybridization sensing, and observed similar enhancement factors as for FCS experiments on diffusing molecules.

Last, we would like to emphasize that nanoapertures offer a wide range of applications to biophotonics. These applications are reviewed for instance in. Briefly, the illuminated area well below the diffraction limit has proven of essential interest for FCS studies at high (micromolar) molecular concentrations. It also offers an elegant way to probe an area well below the diffraction limit in lipid bilayers as well as in live cell membranes. The applications can be extended to dual-color cross-correlation FCCS analysis at high concentrations with a simple setup, and to monitor flow mixing. Lastly, a very promising application of nanometric apertures concerns real-time single-molecule DNA sequencing to perform high-throughput high-accuracy DNA sequencing at low costs. We thus believe that nanoapertures offer unique properties to significantly improve the effectiveness of single molecule fluorescence techniques.

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Figure 5. Use of gold nanoapertures to enhance the fluorescence detected for hybridized double stranded DNA molecules labeled with cyanine-5 and bound to the bottom of the aperture. Filled markers (right scale) show the total fluorescence intensity integrated within 100 s after laser illumination. Empty markers (left scale) denote the fluorescence enhancement factor as defined by Eq. (3).
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