
Biophotonics applications of nanometric apertures

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Abstract: Nanometric apertures in a metallic film are easy to produce, robust and highly reproducible nanophotonic devices that possess a number of desirable properties for biophotonics. In this review, we will describe the exciting applications of sub-wavelength apertures towards the sensitive and specific characterisation of molecules.

Keywords: nanometric apertures; fluorescence microscopy; surface plasmons; spectroscopy.

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1 Introduction

The ability to reliably produce nanometric structures with a resolution down to a few nanometers opens the way for promising photonics applications (Barnes et al., 2003; Ozbay, 2006). Such devices allow to locally confine the light, inducing large absorption and diffusion cross sections and enhancing the local electromagnetic fields. These effects have also a strong relevance for biophotonics, where molecular detection methods based on photonic emission (fluorescence and Raman scattering) have gained a wide popularity in various fields such as biology, medicine, chemistry and materials sciences. The sensitivity of most applications relies quite directly on the intrinsic molecular brightness, thus increasing the optical absorption and emission of individual molecules by metallic nanostructured substrates has become a key issue (Lakowicz, 2005; Aslan et al., 2005; Lakowicz, 2006). Up to now, a large part of the international scientific attention was devoted to the study of metallic nanoparticles fixed on a substrate (either single or in colloids) (Lakowicz, 2005; Lakowicz et al., 2003). However, among the different kind of nanostructures, nanometric apertures milled in a metallic film (single or dispatched in an array) bear surprising optical properties, such as for instance a large transmission at specific wavelengths much greater than the aperture diameter (Genet and Ebbesen, 2007; Ebbesen et al., 1998; Lezec et al., 2002; Degiron et al., 2004).

Milling nanoholes in a metallic film is an intuitive way to manufacture new nanophotonics devices that are robust and highly reproducible. Although this concept appears very simple, such apertures bear attractive physical properties. First, they allow to localise and confine the light in spots much smaller than the volume set by the diffraction theory, with applications in topics such as near-field probes and optical data

storage (Genet and Ebbesen, 2007; Lezec et al., 2002). Second, since an aperture milled in a real metal with finite thickness and conductivity is far different from the case of a hole in an infinitely thin perfect conductor, so-called extraordinary transmission occurs, which leads to a wealth of applications such as surface plasmons excitation or wavelength filtering (Degiron et al., 2004; Popov et al., 2005). Altogether, these electromagnetic properties and the possibility to combine the structures with microfluidics on a lab-on-a-chip format make nanoapertures a relevant technology for interrogating individual molecules (Craighead, 2006), which opens new prospects in biotechnology. Applications range from molecular diagnostics to DNA sequencing and screening for rare molecular types Eigen and Rigler (1994).

A recent comprehensive review has extensively described the electromagnetics phenomena occurring in a sub-wavelength metallic aperture, together with its nanophotonics applications (Genet and Ebbesen, 2007). In this contribution, we will focus on the biophotonics applications of nanometric apertures. After a general introduction on fluorescence studies in restricted observation volumes (Section 2), we will first detail the use of isolated subwavelength apertures to confine the observation volume below the far-field diffraction limit, in the case of single molecule analysis in solution (Section 3) or in a lipid membrane (Section 4). A second major phenomenon that occurs inside these structures is the local enhancement of the optical emission (Section 5). Finally, the implementation of nanoapertures for biosensing will be discussed (Section 6).

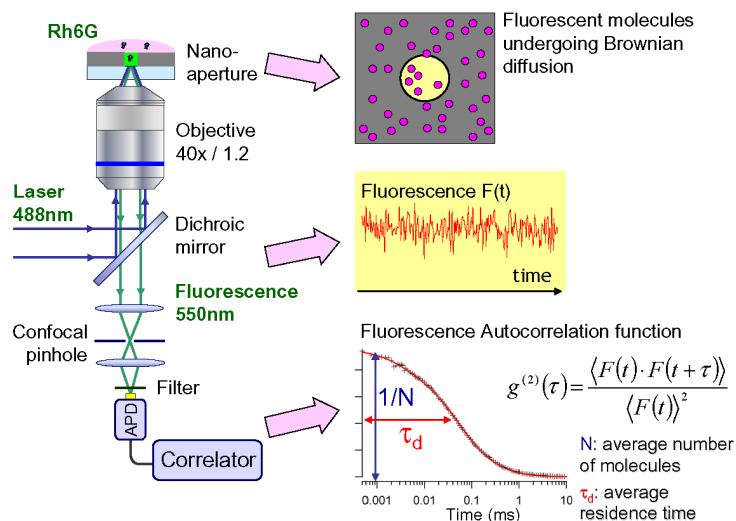
2 Performing fluorescence correlation spectroscopy at physiological concentrations: a need for observation volume reduction

Among the large number of optical methods that have been developed for biological and chemical investigations, fluorescence microscopy plays the largest role today, especially in the field of single-molecule analysis. Fluorescence bears not only a high intrinsic optical efficiency, but also provides information about the molecular environment and structure in many different ways: brightness, lifetime, anisotropy or spectrum. Among these observables, monitoring the temporal fluctuations of the fluorescence intensity from a few emitters has proven to be a powerful and versatile tool. This technique, named Fluorescence Correlation Spectroscopy (FCS) or Fluorescence Fluctuation Spectroscopy (FFS), can in principle provide information about any molecular dynamic process on the nanosecond time range and longer that induces a change in fluorescence intensity (Rigler and Elson, 2001; Zander, 2002). For instance, fluctuations occur when molecules diffuse in and out of an observation volume, or when reaction kinetics or conformational changes induce a change in the fluorescence brightness. Statistical analysis of the fluctuations is generally achieved by computing the fluorescence intensity correlation function. FCS is commonly implemented to access a wide range of specific molecular parameters, including translational and rotational diffusion, molecular concentrations, chemical kinetics, and binding reactions.

The prerequisite of FCS is to monitor on average only a few fluorescent molecules in order to induce significant fluctuations. This explains why FCS is tightly bound to confocal microscopy to reduce the analysis volume down to the diffraction limit (Rigler and Elson, 2001), as illustrated on Figure 1. State-of-the-art confocal microscopy can provide observation volumes down to 0.2 fl (= 0.2 μm^3). This amounts to a useful

concentration in the nanomolar range in order to isolate only a few tens of molecules. However, the molecular concentrations found in living cells or cell membranes is often quite large, typically in the micro to the millimolar range. Many enzymatic reactions are also naturally effective at ligand concentrations of micro to millimolars. Arbitrarily reducing the ligand concentration may lead to chemical pathways alteration and artifacts. Performing FCS at biologically more relevant concentrations requires the development of analysis volumes with nanometric dimensions, yielding a volume reduction by at least three orders of magnitude as compared to standard confocal microscopy.

Figure 1 Principle of fluorescence correlation spectroscopy: the fluorescence intensity temporal fluctuations originating from a well-defined volume are recorded and correlated to yield estimates for the average number of molecules observed and the characteristic fluctuation time (see online version for colours)



Recent advancements implemented to decrease the FCS detection volume below that of normal confocal fluorescence microscope are reviewed in reference (Blom et al., 2006). Among the different classical approaches, Total Internal Reflection Illumination (TIRF) reduces the longitudinal dimension of the observation volume by a factor of ten, but with no restriction in the lateral dimensions (Thompson et al., 1981; Hassler et al., 2005). Structured illumination has also been implemented with FCS, for instance with interference fringes produced on a mirror (Lenne et al., 2002). This strategy opens a new time window with the fringes-to-fringes diffusion effect, and enables probing the diffusion properties inside cellular compartments smaller than the transverse resolution of a diffraction-limited confocal microscope. Finally, the technique of Stimulated Emission Depletion (STED) was implemented with FCS (Kastrup et al., 2005), yielding a 25-fold reduction of the axial diffusion time, equivalent to a 5-fold reduction of the focal volume.

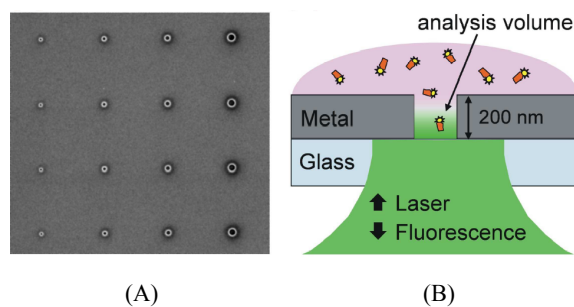
To further decrease the FCS observation volume, nanometric structures have to be combined with fluorescence microscopy techniques (Blom et al., 2006). Subwavelength apertures milled in a metal film are particularly relevant and will be discussed hereafter. An alternative takes advantage of micro- and nanofluidics channels with dimensions

smaller or equal to the width and depth of a diffraction-limited detection volume in confocal microscopy (Brinkmeier et al., 1997; Foquet et al., 2002, 2004; Lenne et al., 2002). The best results achieved an effective detection volume of a few tens of attoliters (Foquet et al., 2002, 2004). A third strategy uses the apertured tips developed for Near-Field Scanning Optical Microscopy (NSOM) to achieve observation volumes with lateral dimensions on the order of 50 nm (Synge, 1928; Betzig and Chichester, 1993; Lewis et al., 2003). This technique reaches presumably observation volumes down to the zeptoliter range, but it suffers from complex probe manufacturing and cannot be straightforwardly implemented in parallel configuration. The apertureless NSOM should attain even lower excitation volumes (Blom et al., 2006), but this approach suffers from a significant background emerging from the whole detection volume.

3 Single molecule fluorescence analysis in a single aperture

A simple and elegant way of generating a reduced analysis volume in FCS relies on nanometric apertures milled in an opaque metallic film. As illustrated on Figure 2, the nanostructure itself acts as a pinhole filter directly placed into the object plane of a standard epi-illumination confocal microscope. When the aperture diameter is sufficiently reduced below the cut-off diameter of the fundamental excitation mode that may propagate through the (waveguiding) hole, the light inside the aperture is confined to a rapidly decaying evanescent mode, with a decay length of a few tens of nanometers (such devices have thus been named zero-mode waveguides (Levene et al., 2003)).

Figure 2 (A) MEB picture of 150, 220, 360 and 450 nm diameter apertures milled by focused ion beam on a 200 nm thick aluminum film deposited over a standard glass coverslip and (B) Experimental arrangement of the single aperture in epi-illumination configuration (see online version for colours)



To our knowledge, the first implementation of this concept dates back to 1986 (Fischer, 1986), where apertures of diameters between 180 nm and 480 nm in thin films of silver or gold supported by a glass slide were used as probes to detect the properties of an adjacent liquid containing fluorescent molecules. Even in the absence of efficient detectors and laser sources, sensitive measurements of refractive-index differences of the order of 10^{-4} were demonstrated, and a global enhancement of the fluorescence brightness was reported.

A significant step forward was performed by the groups of Harold Craighead and Watt Webb at Cornell University (Ithaca) in an outstanding contribution using modern nanofabrication and characterisation methods (Levene et al., 2003). With

aperture diameters down to 30 nm, detection volumes of a few tens of zeptoliters ($1 \text{ zL} = 10^{-21} \text{ L}$) have been generated, which are about four orders of magnitude smaller than diffraction-limited confocal volumes. As an example of the effectiveness of nanoapertures for performing single-molecule experiments at high concentrations, DNA polymerase activity has been monitored at $10 \mu\text{M}$ dye concentration with an average of 0.1 molecule inside a 43-nm-diameter aperture. However, for experiments conducted on ultrasmall structures, the signal to noise ratio comes close to one, as a consequence of different effects. First, the fluorescence emission is strongly affected by the metal in the close vicinity of the structure (see discussion in Section 5). Second, the background noise becomes then significant, due to the metal auto-fluorescence and the large pool of dyes above the aperture that induce some fluorescence leakage back to the detector. Even if it tends to decrease the amplitude of the FCS correlation curve, it should be noted that the non-fluctuating nature of the background still enables a discrimination against the signal. Therefore, detecting the presence of a millisecond-scale enzymatic reaction event or a microsecond-scale diffusion event is always possible even for a low signal-to-noise ratio, which is a general property of FCS.

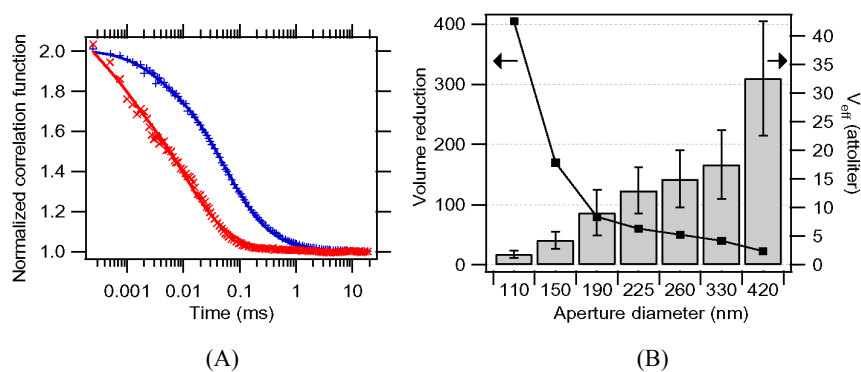
This pioneering work has led to a number of studies combining nanometric apertures with FCS. In a second contribution (Samiee et al., 2005), the group of Harold Craighead derived an empirical model for the FCS autocorrelation function inside a nanoaperture. This model holds in the limit of a small diameter to height ratio, where the diffusion can be assumed to be mainly one-dimensional. This method is used to measure the oligomerisation of the bacteriophage λ repressor protein (CI) at micromolar concentrations. Thanks to 50 nm diameter apertures, a binding constant of $4.6 \mu\text{M}$ was determined for the formation of CI tetramers.

The evolution of the observation volume vs. the aperture diameter was extended to the range of diameters between 100 nm and 400 nm, both in epi- (Rigneault et al., 2005) and trans- (forward) (Leutenegger et al., 2006) illumination configuration. Knowing the initial molecular concentration, the observation volume is readily deduced from the mean number of molecules N that is derived by the amplitude of the FCS correlation function. A clear reduction of the observation volume is always observed, which comes close to the geometrical nanohole volume. As illustrated on Figure 3, a 110 nm diameter aperture was demonstrated to probe a volume of 1.8 attoliters, which corresponds to a 400x volume reduction as compared to confocal microscopy. We point out that if the trans-illumination performs nearly as well as the epi-configuration (Leutenegger et al., 2006), the lower amount of background noise and the ‘double’ filtering induced by the aperture (on both the illumination and emission pathways) always play in favour of the epi-illumination configuration.

FCS combined with nanometric apertures has been recently extended to dual-colour Fluorescence Cross-Correlation Spectroscopy (FCCS) case (Wenger et al., 2006). The principle of FCCS is presented on Figure 4: each reaction partner is labelled with spectrally separable fluorophores and the distinct fluorescence intensities are then cross-correlated to get a measure of the colocalisation efficiency, accessing a wide range of molecular parameters such as association/dissociation kinetics and enzymatic activities (Bacia et al., 2006). Although FCCS achieves a high signal specificity, the experimental realisation of a dual-colour FCCS setup is demanding, as it requires exact and stable superposition of the two confocal observation volumes. Compared to a conventional FCCS confocal microscope setup, FCCS within a single nanoaperture brings some useful improvements (Wenger et al., 2006):

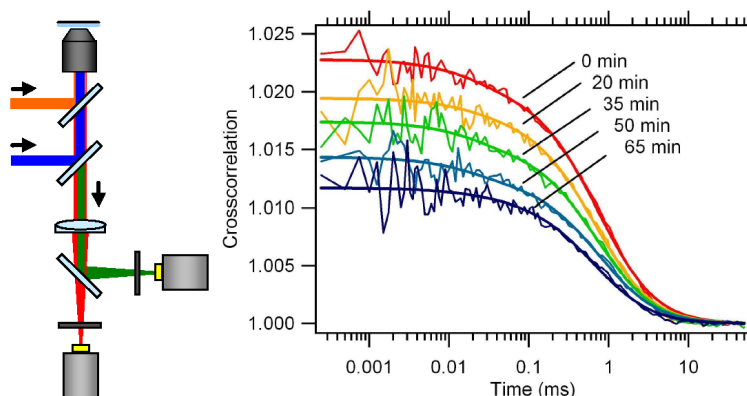
- the overlap of the two excitation beams and the optical alignment are greatly simplified by the nanoaperture which defines the analysis volume
- no confocal pinhole is needed, relaxing the requirement for accurate correction of chromatic aberrations and accurate alignment
- compared to two-photon excitation, the use of nanoapertures keeps the intrinsic advantages of dual-colour FCCS to use standard fluorophore constructions with high absorption cross-section and well-defined absorption/emission spectra with large Stokes shifts
- experiments can be conducted at micromolar concentration, allowing for an increase up to three orders of magnitude compared to standard FCS setups.

Figure 3 (A) FCS correlation curves for rhodamine 6G excited at 488 nm inside a 150 nm diameter aperture (x) and in open solution (+) and (B) observation volume measured by FCS in nanoapertures and volume reduction as compared to the confocal microscope setup (see online version for colours)



Source: Derived from Rigneault et al. (2005)

Figure 4 Principle of dual-colour FCCS and cross-correlation curves during a cleavage reaction carried on a solution with 20 μ L DNA substrate at 2 μ M, 2 μ L EcoRI of activity 10 u/ μ L and 2 μ L Tris-HCl/MgCl₂/NaCl buffer (10x) in a 340 nm diameter nanoaperture (see online version for colours)



Source: Adapted from Wenger et al. (2006)

4 Sub-diffraction diffusion analysis within lipidic membranes

Revealing the dynamic organisation of the cell plasma membrane at the submicron level is a challenging task (Marguet et al., 2006). On one hand, electron microscopy resolves nanometric features, and yet it cannot be easily applied to live cells in physiological conditions. On the other hand, standard optical microscopy unravels details of live cell membranes, but is limited by the diffraction phenomenon to a resolution of about 200 nm. Therefore, new methods providing dynamic information on lipids and proteins in live cell membranes with nanometric resolution are required. To go beyond the diffraction barrier, a promising strategy relies on nanometric apertures milled in a metallic film to confine the probed area.

The first proof-of-principle towards the use of nanometric apertures to probe the plasma membrane of a live cell on a sub-diffraction area was reported in 2005 (Edel et al., 2005). Rat basophilic leukemia cells were grown and labelled with DiI-C18 fluorescent lipids prior to incubation on an aluminum film containing 100 nm diameter apertures. The single apertures act as pinholes directly located under the cell membrane and restrict the observation area below the diffraction limit. While monitoring lipid diffusion along the cell membrane, the authors showed that confinement with a nanohole largely reduced fluorescent contributions from the cytosolic pool that is present when using standard confocal microscopy.

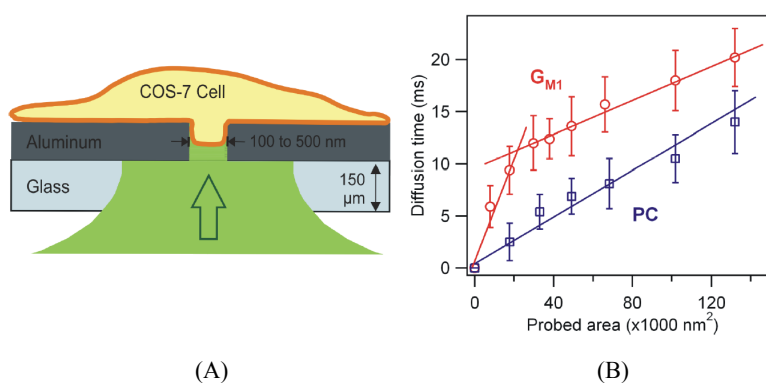
Studies on supported lipid bilayers can also benefit from the confinement brought by the nanoapertures. This topic is specially relevant to study systems involving ligand-receptor interactions. A crucial issue is to determine whether the lipid bilayer will invaginate or not into the structure. A first report suggests that invagination is dependent on membrane rigidity (Samiee et al., 2006): FCS could be performed on POPC lipids in liquid-disordered phase, while gel phase DSPC membranes did not appear to enter the apertures. The nanoapertures were also used to determine the equilibrium constant of tetanus toxin C fragment bound to a supported POPC lipid bilayer populated by gangliosides at a concentration of 500 nM (Samiee et al., 2006).

Instead of performing measurements on a single aperture size, much information can be gained by observations with increasing probed areas. To demonstrate that the diffusion of single fluorophores in a lipid membrane can be probed below the diffraction limit, circular nanoapertures milled in an aluminum film were used to study the diffusion dynamics of Bodipy-PC fluorophores in aDOPCmultilayer (Wenger et al., 2006). A linear dependence between the mean diffusion time and the aperture surface was found, indicating that the observed diffusion was mainly 2-dimensional and restricted by the aperture size for diameters ranging from 100 nm to 400 nm.

Since the diffusion of molecules in the cell plasma membrane is highly sensitive to lateral heterogeneities, monitoring the lateral diffusion of lipids and membrane proteins is highly relevant in order to reveal the structure and the role of membrane heterogeneities (Marguet et al., 2006). Living cells with fluorescently labelled membrane components (lipid analogs and GFP-tagged proteins) were incubated over isolated nanoapertures milled in an opaque aluminum film with radii between 75 nm and 250 nm (Wenger et al., 2007). Performing FCS experiments with increasing aperture sizes, the diffusion time was plotted vs. the transverse area of the observation volume. Though the exact geometry of the cell membrane invaginated inside the nanoaperture remained unknown, different transient diffusion regimes were observed at different characteristic lengths for several proteins and lipids. For instance, Phosphatidylcholine (PC) lipid

incorporated in the COS-7 cell membranes exhibited free-like diffusion at all length-scales ranging from 100 nm to 450 nm, as an almost linear dependence between the mean diffusion time and the aperture surface was reported (Figure 5). This indicates that the observed diffusion could be considered mainly two-dimensional and limited by the aperture size, and that the aperture did not alter the diffusion process. Under the same experimental conditions, the ganglioside lipid G_{M1} (a putative raft marker) had a drastically different diffusion behaviour, as it experienced a transition to normal Brownian diffusion at an aperture radius of 100 nm. Based on numerical simulations and previous theoretical work (Wawrezynieck et al., 2005; Lenne et al., 2006), this transition was interpreted as the cross-over from confined to normal diffusion, the confinement originating from isolated nanodomains of about 30 nm radius in the case of G_{M1} (Wenger et al., 2007). Two major mechanisms that contribute to the organisation of the plasma membrane could be distinguished, namely a lipid-dependent ‘microdomain’ model and a cytoskeleton-dependent ‘meshwork’ model.

Figure 5 (A) Nanoaperture to probe a sub-diffraction area on a live cell membrane and (B) FCS diffusion time vs. the aperture area for two types of lipidic membrane components (see online version for colours)



Source: Adapted from Wenger et al. (2007)

Nanoapertures are thus demonstrated to be valuable tools to monitor the diffusion of molecular components in the plasma membrane at high spatial and temporal resolutions, with the cells being kept under physiological conditions and with ultra-low amounts of incident laser light. This yields a technique having both high spatial and temporal resolution together with a direct statistical analysis.

5 Fluorescence emission enhancement

Apart from the observation volume limitation, a second major effect brought by the subwavelength aperture is its ability to significantly enhance the fluorescence emission, which is likely to play a key role in fluorescence-based assays. A first hint for the presence of fluorescence brightness enhancement within nanoapertures was reported in Fischer (1986), and yet, the lack of efficient detectors and laser excitation together with a rather involved experimental configuration complicated the analysis of the measured data.

Independently of this pioneering study, emission enhancement for fluorescent molecules dispatched over a square array of nanoapertures was reported in Liu and Blair (2003, 2004). The peak fluorescence occurred under conditions of maximum transmission of the excitation light and excitation of surface plasmons on the metal–air surface, when the nanohole array was rotated to achieve the Bragg resonance condition. Under resonant transmission conditions, the net gain in fluorescence output normalised to the aperture fill fraction was estimated to nearly 40. This study was extended to randomly placed nanoapertures in gold films, showing that random hole structures could also enhance the fluorescence output by about seven times (Liu et al., 2004). A second feature shown that fluorophores placed in close vicinity to the metal surface of the periodic array can couple to surface plasmons and emit from the back side at specific angles (Liu et al., 2004). Complete isolation from this fluorescence signal was obtained by passivating the top surface.

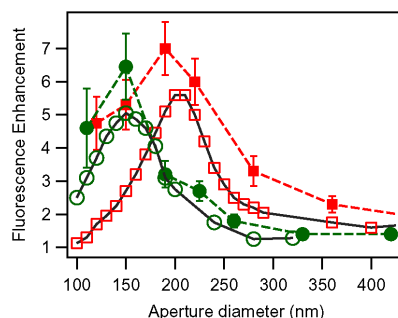
Instead of rotating the aperture array to achieve the resonant surface plasmon excitation, a different approach is to tune the resonance by changing the spacing between the holes, in order to maximise the fluorescence from a specific dye. To this end, nanoholes arrays with distinct periodicities were fabricated and spin-coated with polystyrene films containing the fluorescent dye oxazine 720 (Brolo et al., 2005). Enhanced fluorescence was observed when the geometric characteristics of the arrays allowed for an enhancement in the transmitted excitation. As a consequence of the larger excitation intensity under resonant conditions, the fluorescence measurement sensitivity (signal changes vs. concentration changes) appeared significantly larger than that obtained from equivalent films on glass substrates.

In a second contribution, the surface plasmons resonances of the nanohole arrays were tuned around the photoluminescence peak of cadmium sulfide quantum dots in contact with the incident side of the arrays (Brolo et al., 2006). This realised the first example of direct coupling of the quantum dots photoluminescence to surface plasmons modes. The maximum luminescence enhancement occurred when the resonance from the nanohole array matched the Qdot luminescence spectrum, showing enhancement in the spontaneous emission by two orders of magnitude even after excluding the enhanced transmission of the nanohole array. Under that conditions, the PL transmitted spectrum followed the distribution of the plasmonic resonance of the array.

In all the experiments reported above in this section, the configuration was set in transillumination: the dyes were excited via resonant transmission of the laser through the nanoholes arrays. This makes the interpretation of the measurements rather involved, as the amount of collected fluorescence depends on the transmission efficiency of the excitation line, together with the collection efficiency of the emitted light, the alteration of the photokinetics rates of the dyes and their relation with surface plasmons modes. Moreover, the relative influence of the local (single aperture) and collective (nanohole array) effects remains difficult to quantify for nanoapertures arrays. It is thus worth looking at the physical phenomena occurring at an isolated nanohole, preferably in epi-illumination configuration with a high numerical aperture, in order to simplify the data analysis, minimise the background and improve the observation volume confinement. Besides, the fluorescence signal should be normalised to the emission per molecule, to avoid any artefact linked to the correction for different filling fraction of the apertures. For that purpose, FCS turns out to be a valuable technique since it enables a direct quantification of the number of molecules monitored during an experimental run.

Our team has recently devoted much attention to this point, showing that a single subwavelength nanowell could significantly enhance the fluorescence rate emitted by a single fluorescent molecule. Using single rhodamine 6G molecules in isolated 150 nm diameter apertures milled in an aluminum film, a 6.5 fold enhancement of the fluorescence rate per molecule was reported as compared to free solution (Rigneault et al., 2005). The fluorescence lifetime appeared also to be dramatically reduced inside the aperture, indicating that the molecular energy levels' branching ratios were strongly affected. Instead of quenching the dyes, the local density of states alteration brought by the aperture allowed for a higher radiative rate without decreasing the fluorophore quantum efficiency. The combination of this effect together with an increase in the local excitation intensity led to an overall fluorescence enhancement, as displayed on Figure 6. This was confirmed using a rigorous electromagnetic theory of light diffraction in cylindrical geometry. At low excitation powers (well below saturation), the fluorescence enhancement was linked to the appearance of the fundamental mode propagating inside the aperture (Popov et al., 2006). The cutoff condition led to modes with a low group velocity, and to an increased local density of states allowing a higher excitation intensity as compared to a diffraction-limited beam.

Figure 6 Experimental fluorescence enhancement (filled markers) and theoretical computations of the excitation intensity enhancement (empty markers) vs. aperture diameter in an aluminum film. Circles correspond to rhodamine 6G excited at 488 nm, while squares refer to cyanine 5 excited at 633 nm (see online version for colours)



Source: Adapted from Popov et al. (2006)

Experiments have also been conducted on rectangular subwavelength apertures milled in aluminum films (Wenger et al., 2005). This shape allows the activation of two distinct modes by changing the angle between the linearly-polarised incoming electric field and the longitudinal axis of the aperture. Since the waveguide's cut-off is different for each axis, the penetration depth of the excitation light within the aperture can thus be significantly tuned, offering new possibilities to dynamically tailor the observation volume. Whereas for both polarisation directions the molecular environment remained almost the same (as shown by the same fluorescence lifetime), the fluorescence rate enhancement varied strongly with the polarisation direction: a high transmission of the excitation field did not yield any fluorescence enhancement whereas an evanescent coupling of the pump field induced a significant increase in the detected count rate per molecule. These results confirmed that the fluorescence enhancement found for rhodamine 6G was mainly related to the excitation near field intensity within the subwavelength aperture.

The emission properties of a fluorophore in a single nanoaperture have been theoretically studied independently of the excitation mechanism (Liu et al., 2005; Mahdavi and Blair, 2006). Round, square, and triangular nanoapertures were investigated, with an improvement in radiative efficiency of about three times for optimal nanohole dimensions. These results do not depend on the excitation mechanism, and are therefore valid for chemiluminescence, electroluminescence, and photoluminescence, making the conclusions relevant to a number of biotechniques.

The significant fluorescence increase obtained for small-radius apertures appears especially interesting because it allows for the possibility to significantly reduce the observation volume while still detecting a sufficient signal. This yields an efficient signal-to-background discrimination, even with attoliter volumes and single molecule resolution (Wenger et al., 2006). Moreover, for a properly tailored subwavelength aperture, count rates greater than a few hundred thousands photons per second and per molecule were readily obtained, whereas for a single molecule in open solution the fluorescence saturation prevented the count rate to exceed a few tens of kilocounts per second. Other types of structured apertures could provide even higher fluorescence rate enhancement, while still enabling efficient signal-to-background discrimination, such as for instance bull's eye structures (Lezec et al., 2002; Popov et al., 2005; Nahata et al., 2003; Ishi et al., 2005), nanopockets (Liu et al., 2006) or combined slit and aperture (Liu et al., 2007).

6 Biosensing applications of sub-wavelength apertures

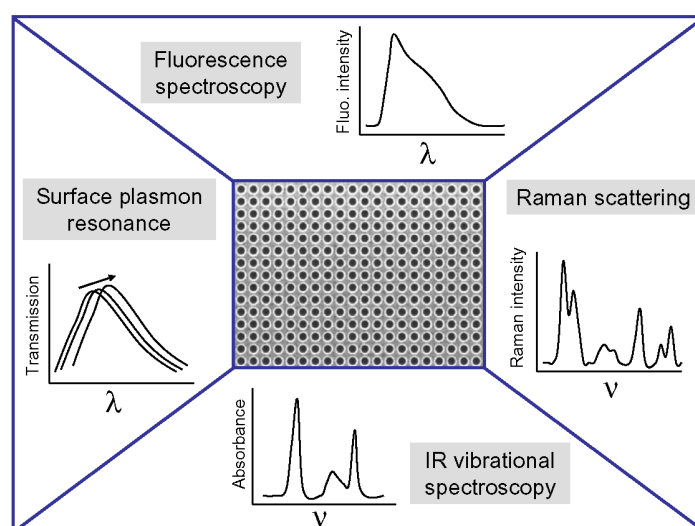
Detecting molecules in real-time with high sensitivity and molecular specificity is of great interest in many fields of biosciences. Intensive world-wide research on new biosensing techniques is motivated by numerous applications in clinical diagnostics, genetic screenings, proteomics, and single-molecule detection. In this context, the combination of molecules and subwavelength apertures is a promising area of application. The electromagnetic fields enhancement, the sensitivity to the dielectric medium in contact with the surface and the simplicity of integrating the arrays have motivated efforts to use them to detect molecules and enhance spectroscopic contrasts (Figure 7).

A first application of the fluorescence enhancement reported in the previous section was devoted to DNA binding events detection (Liu et al., 2004). To perform this affinity sensing, nanoapertures were spotted with probe molecules specific to a target of interest. Bound target oligonucleotides within the nanoholes produced enhanced fluorescence in transmission, while (non-enhanced) fluorescence emitted from unbound molecules lying outside did not couple efficiently through the structure, yielding an high signal-to-background rejection. As a proof-of-principle, real-time detection of 20-base oligonucleotides in solution was performed (Liu et al., 2004). Nanoapertures arrays are particularly relevant for integration with Highthroughout Screening (HTS) methodologies, for applications in drug screening or evolutionary biotechnologies (Koltermann et al., 1998). Combined with FCS or coincidence analysis (Winkler et al., 1999), nanostructures appear very well suited to design miniaturised HTS fluorescence sensors (Wenger et al., 2006).

Together with fluorescence-based devices, Surface Plasmon Resonance (SPR) sensors are widely used for the characterisation and quantification of molecular binding

events in biological systems. Traditionally, an SPR measurement consists of the excitation of extended Surface Plasmons (SP) modes through prism coupling in the Kretschmann configuration, which can be sensitive to surface processes at the submonolayer level. A periodic square array of sub-wavelength holes on gold films was recently used as a SPR sensor to monitor the binding of organic molecules to the metallic surface (Brolo et al., 2004a). This technique is particularly sensitive to surface binding events because it is based upon the resonant SP-enhanced transmission through the array of nanoholes. The sensitivity was found to be comparable to other grating-based SPR devices. The array of nanoholes appears well suited for dense integration in a sensor chip in a collinear optical arrangement providing a simpler setup and a smaller probing area than the typical Kretschmann configuration.

Figure 7 Optical contrasts used for chemical detection and characterisation that benefit from a sensitivity enhancement induced by nanoapertures (see online version for colours)



Single nanometric holes in thin gold films have been shown to also exhibit a (localised) SP resonance in the red to near-infrared region (Prikulis et al., 2004) which results in a peak in the extinction spectrum. The hole plasmon red shifts with increasing refractive index of the surrounding medium, indicating a potential for novel surface-enhanced spectroscopy and biosensing applications. This type of device has been successfully employed to monitor lipid-membrane-mediated biorecognition events (Dahlin et al., 2005), and selective sensing of specific cancer antigens (Gao et al., 2007). Thanks to the simplicity of fabrication, the small probing area, and the simple optical configuration, this technique could be developed into a simple, and inexpensive platform for array-based label-free biosensors.

Although the fluorescence and SPR methods provide a high sensitivity, they lack molecular specificity because their principles of operation rely respectively on large emission spectra and changes in refractive index at the metal-dielectric interface. Molecular specificity is a powerful advantage of SP-enhanced spectroscopic methods such as Surface Enhanced Raman Scattering (SERS) or SP-enhanced infrared vibrational spectroscopy.

SERS is a valuable technique resulting in strongly increased Raman signals from molecules attached to nanometer sized metallic structures, most generally colloidal nanoparticles. SERS has proven to be very sensitive and capable of single-molecule detection (Nie and Emory, 1997). Among the variety of substrates and nanostructures that have been proposed to achieve SERS, periodic arrays of sub-wavelength apertures in ultrathin gold films performed enhanced-Raman spectroscopy of oxazine 720 dyes adsorbed on arrays (Brolo et al., 2004b). The enhancement factor reached a maximum for the array that presented the largest transmission at the excitation wavelength of the laser, showing that the enhancement of the Raman signal was provided by surface plasmon modes. Therefore, by careful engineering of the metallic surface, it is possible to tune the plasmon bands to be resonant with the excitation wavelength found in common Raman spectrometers. Enhancement higher than 10^6 and reproducibility better than 10% were demonstrated using benzenethiol and aminothiophenol molecules as monolayer chemical markers (Perney et al., 2006).

Infrared vibrational spectroscopy is another highly specific technique that benefits from SP electromagnetic enhancement occurring in a nanoaperture arrays. In references (Williams et al., 2004; Rodriguez et al., 2004; Teeters-Kennedy et al., 2006), arrays of square holes in a Ni film coated with Cu oxide were prepared and tuned to the IR region, before a specific molecular monolayer was adsorbed on the top surface of the nanohole array. When the vibrational absorption spectrum of the monolayer was probed, the extracted absorbances were two orders of magnitude stronger than the absorbance of a monolayer on a dielectric surface. The large absorption enhancement was related to the increased interaction between molecules and light trapped momentarily on the surface in surface plasmons. Absorption enhancement of electronic transitions was also reported in the visible range of the spectrum (Dintinger et al., 2006a), although the enhancement factor was limited to about one order of magnitude due to the shorter SP lifetime at wavelengths in the visible range. These studies open opportunities for analysing catalytic events or biological membranes on standard IR absorption spectrometers by simply adding an array in the path of the spectroscopic beam. Furthermore, the ensemble molecule/hole array was shown to act like an all-optical switch, with speeds up to the terahertz range (Dintinger et al., 2006b).

7 Conclusion

Nanoapertures possess a number of desirable properties for biophotonics, such as localisation of excitation light within the nanoholes, strong isolation from emission produced by unbound species, and an apparent increase in absorption and emission yield. The simplicity of the structures and their ease of use should further expand their application towards the real-time detection and identification of a low number of molecules. Methods for the preparation of organised patterns of metallic nanoapertures are readily available and are based on focused ion beam milling, electron beam lithography and photolithography. As an alternative, laser-induced optical breakdown by femtosecond pulses was recently used to mill 50 nm apertures in dielectrics, thanks to a remarkably sharp threshold for laser-induced material damage (Joglekar et al., 2004). Strategies for cost-effective nanoapertures fabrication on a large scale are thus available, and should allow for a wide range of applications in biophotonics.

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