

Supporting Information

Enhanced Single Molecule Fluorescence and Reduced Observation Volumes on Nanoporous Gold (NPG) Films

Yi Fu*, Jian Zhang, Kazimierz Nowaczyk, and Joseph R. Lakowicz

Center for Fluorescence Spectroscopy, Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, 725 West Lombard Street, Baltimore, MD 21201.

S1. NPG film fabrication.

The free-standing NPG films were fabricated with slightly modification to the method developed by Ding¹⁵ and Ciesielski⁷. The 12 carat gold leaves (Ag₆₅Au₃₅) were manufactured by Eytzinger (Crocodile Brand). Pre-thiolated coverslips were prepared by immersing the cleaned slides in a methanol solution containing 20mM mercaptopropyltrimethoxysilane for 40 mins. The gold leaf was cut into a desired sample size and was placed gently on a wet coverslip surface. The slide was slowly dipped into a beaker of 70% nitric acid. The coverslip was removed as the leaf floated freely on the surface of the acid. After dealloying for 6 hrs, the slide was dipped into the acid at an angle positioning beneath the floating leaf. The slide was slowly withdrawn after the top surface contacting the edge of the leaf. As the slide was removed, the leaf adhered smoothly on the surface. The dealloyed leaf was then transferred to a second beaker of deionized water for rinsing following the similar procedures. After final rinse step, the thiolated glass coverslip was used to remove the leaf from the water. The sample was left to dry under vacuum for 24 hrs. The thiol groups on the surface bonded to the NPG surface and formed the firmly attached NPG film on coverslips. An EDX spectrum of the nanoporous confirms that the complete removal of silver in the etching process (Figure S1).

S2. Immobilization of Cy5 on NPG film.

1 mg of the NHS-ester Cy5 (GE, Lifesciences) was dissolved in 50 μ l absolute, amine-free DMF to make a final concentration of approx. 25 μ M stock solution. The NPG film was thiolated by wetting the surface with 2-aminoethanethiol ethanol solution (50mM), after gently rinsed with ethanol, the film was floating on the surface of a bicarbonate buffer (pH 9.0, 50 mM) containing 5nM NHS-ester dye (100nM concentration was used to prepare the samples reflectance imaging experiments). The distance between the fluorophore and the NPG surface is estimated to be 3-4 nm upon conjugation. The leaf was kept in buffer for reaction overnight at room temperature. Similar procedures from above were used to collect the film from the buffer. The film was rinsed and kept in vacuum overnight before the test.

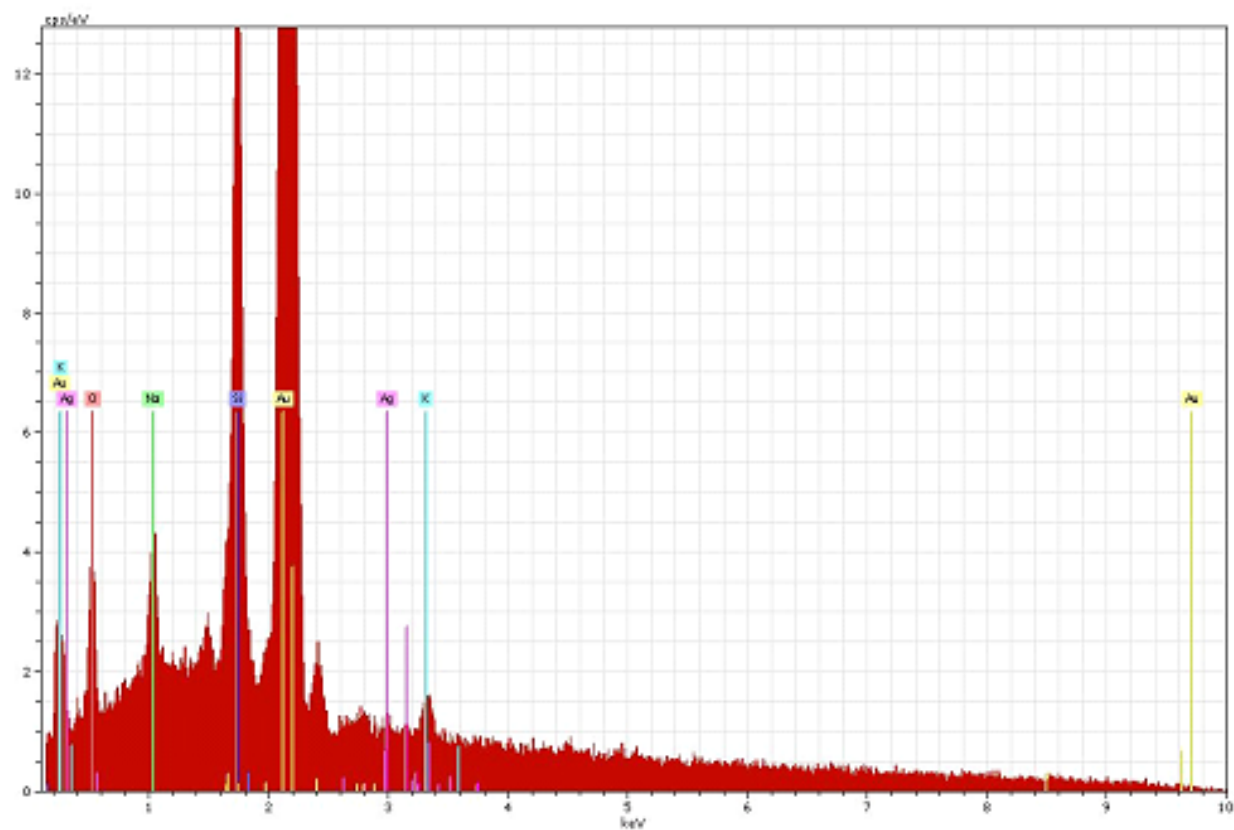


Figure S1. EDX was performed on a glass coverslip covered by NPG film, the analysis showing major element components as follows: Au (30.8%); Ag (1.3%). Si(10.8%), O: (28.5%).

S3. Single molecule measurements.

All single molecule studies were performed using a time-resolved confocal microscopy (MicroTime 200, PicoQuant). A single mode pulsed laser diode (635 nm, 100ps, 40 MHz) (PDL800, PicoQuant) was used as the excitation light. The collimated laser beam was spectrally filtered by an excitation filter (D637/10, Chroma) before directing into an inverted microscope (Olympus, IX 71). An oil immersion objective (Olympus, 100×, 1.3NA) was used both for focusing laser light onto sample and collecting fluorescence emission from the sample. The fluorescence that passed a dichroic mirror (Q655LP, Chroma) was focused onto a 75 μm pinhole for spatial filtering to reject out-of-focus signals and then through a 685/35 nm bandpass filter before reached the single photon avalanche diode (SPAD) (SPCM-AQR-14, Perkin Elmer Inc). Images were recorded by raster scanning (in a bidirectional fashion) the sample over the focused spot of the incident laser with a pixel integration of 0.6 ms. The excitation power into the microscope was maintained close to 100nW. Time-dependent fluorescence data were collected with a dwell time of 50 ms.

TCSPC lifetime analysis. The fluorescence lifetime of single molecules was measured by time-correlated single photon counting (TCSPC) with the TimeHarp 200 PCI-board (PicoQuant). The data was stored in the time-tagged-time-resolved (TTTR) mode, which allows recording every detected photon with its individual timing information. In combination with a pulsed diode laser, total Instrument Response Function (IRF) widths of about 400 ps FWHM can be obtained, which permits the recording of sub-nanosecond fluorescence lifetimes, extendable to less than 100ps with reconvolution. All measurements were performed in a dark compartment at room temperature. Lifetimes were estimated by fitting to a χ^2 value of less than 1.3 and with a residuals trace that was fully symmetrical about the zero axis. Fluorescence intensity decay curves were reconvoluted with the instrument response function and analyzed as a sum of exponential terms:

$$I(t) = \sum_i \alpha_i \exp(-t / \tau_i)$$

Where $I(t)$ is the fluorescence intensity at time t and α_i is a pre-exponential factor representing the fractional contribution to the time-resolved decay of the component with a lifetime τ_i . The contribution of each component to the steady-state intensity is given by

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j}.$$

The averaged lifetime is given by

$$\bar{\tau} = \sum f_i \tau_i.$$

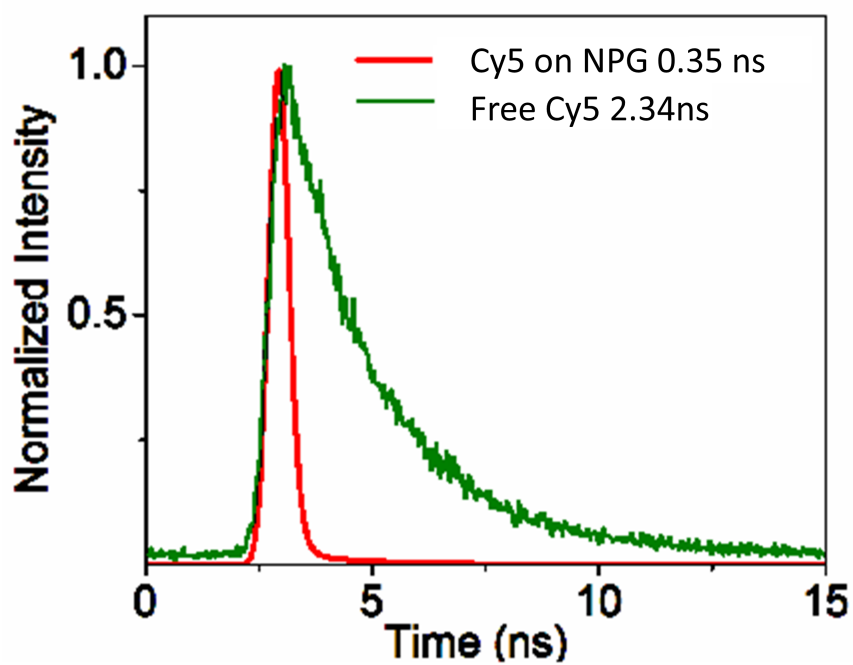


Figure S2. Typical TCSPC intensity decay curves of single Cy5 molecules immobilized on glass (green) and on NPG (red). The mean lifetimes were obtained by exponential fitting with the curves.

	Free Cy5	NPG
Ampl ₁	1942	31.6
Lifet ₁	2.34	1.007
Ampl ₂		683.03
Lifet ₂		0.167
χ^2	1.014	0.895
τ_{av} : (ns)	2.34	0.35

Table S1. Single-molecule lifetime fitting for intensity decays curves shown in Figure S2.

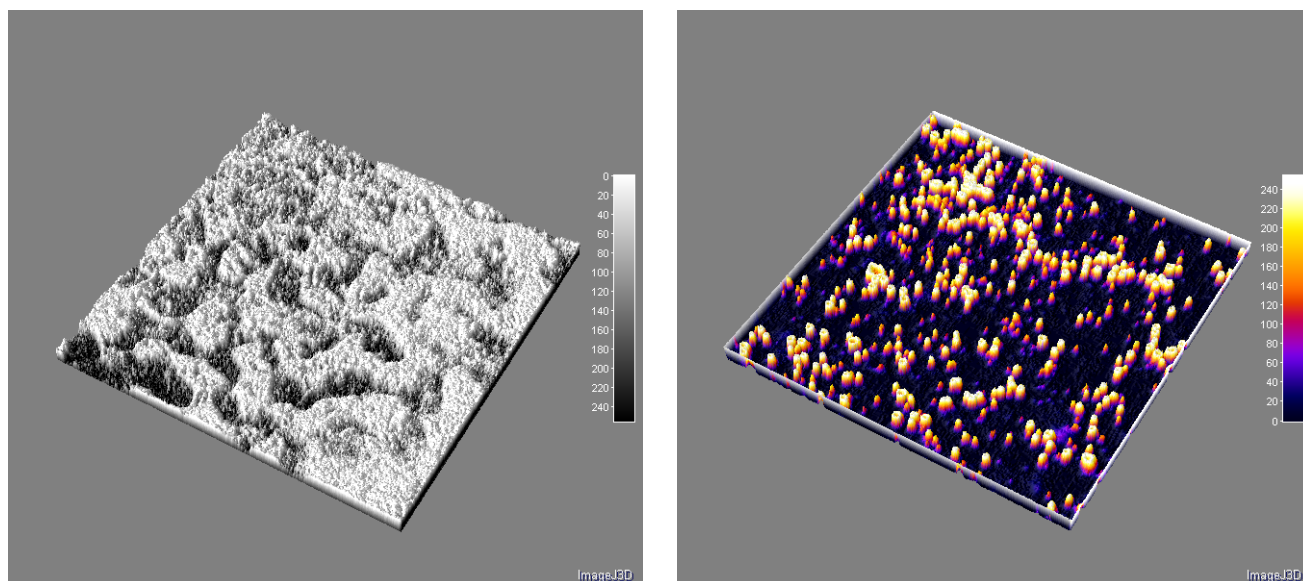


Figure S3. The original reflectance image (left) and fluorescence image (right), both images were scanned from the same sampling area.

S4. Fluorescence Correlation Spectroscopy.

The FCS experiments were performed on a MicroTime 200 confocal microscopy (PicoQuant), equipped with a Olympus NA 1.3, 100X oil immersion objective. The stock solution was further diluted to the desired concentrations with 0.05% Tween 20 added to prevent aggregation and surface adhesion. A 635 nm pulsed diode laser with a repetition rate of 40 MHZ was used as the excitation source. The beam was directed via a dichroic mirror into the microscope. The FCS measurements were performed 10 μm above the glass surface.

Autocorrelation Analysis. The analysis was performed using PicoQuant fitting software. The time traces (Figure S4) recorded were used to calculate autocorrelation function as follows:

$$G(\tau) = \frac{\langle \delta F(t) \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2} \quad (1)$$

where $\delta F(t)$ represents temporal intensity fluctuation, τ is a variable interval, $\langle F \rangle$ is the time average value, averaged over all data points in the time series. The theoretical autocorrelation decay for a single diffusing species is given by equation 2 using the Gaussian detection profile and some complex mathematics:

$$G(\tau) = G(0) \left(1 + \frac{4D\tau}{s^2}\right)^{-1} \left(1 + \frac{4D\tau}{u^2}\right)^{1/2} = G(0) D(\tau) \quad (2)$$

where $G(0)$ is the amplitude at $\tau=0$. D is the translational diffusion coefficient; s and u are the focal volume variants.

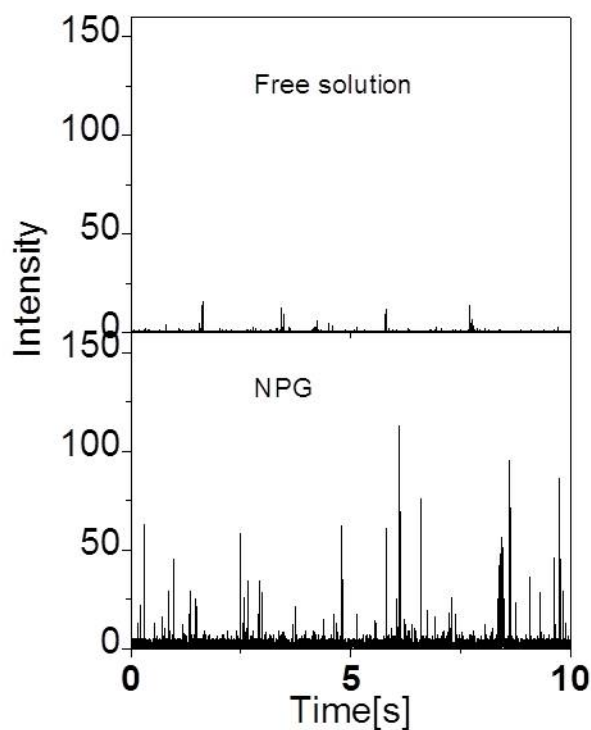


Figure S4. Time transients (a: on glass; b: on NPG) collected were used for further autocorrelation analysis.

References

- (1) Ding, Y.; Kim, Y. J.; Erlebach, J. *Advanced Materials* **2004**, *16*, 1897.
- (2) Ciesielski, P. N.; Scott, A. M.; Faulkner, C. J.; Berron, B. J.; Clifffel, D. E.; Jennings, G. K. *Acs Nano* **2008**, *2*, 2465.