Electronic Supplementary Material (ESI) for Nanoscale Advances.

Supplementary Information for

Zero-mode waveguides can be made better: fluorescence enhancement with rectangular aluminum nanoapertures from the visible to the deep ultraviolet

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This document contains the following supporting information:

S1. Influence of the milling depth

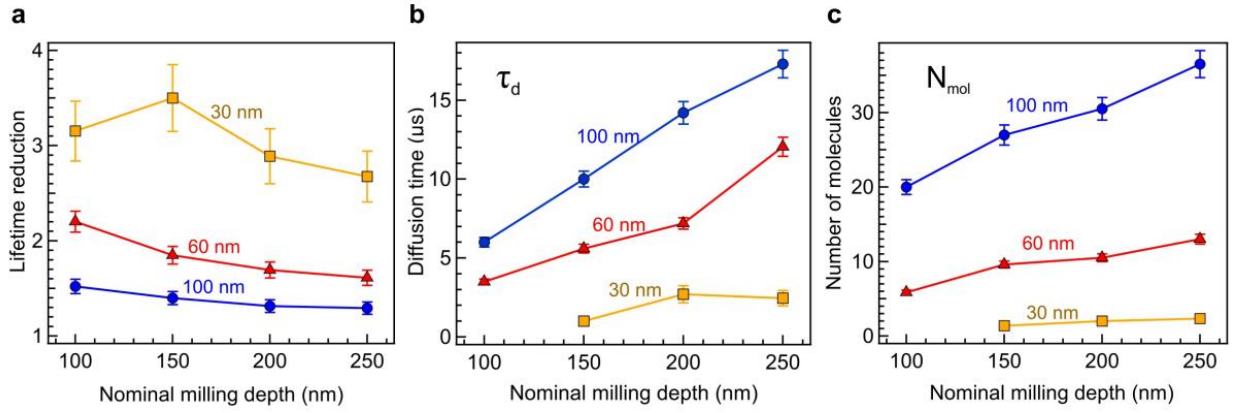

Figure S1. Variation of the milling depth of circular nanoapertures. Experiments are performed in the UV on p-terphenyl molecules dissolved at 10 µM concentration in cyclohexane. (a) Lifetime reduction in 30 nm, 60 nm, and 100 nm apertures milled at various depths. (b) Diffusion time and (c) number of molecules determined by FCS. The nominal milling depth is the input value for the FIB software. We estimate that the real undercut in the dielectric substrate below the aluminum is about 50 nm for a nominal milling depth of 150 nm, which has been chosen as the optimal case for this study.

S2. Single molecule fluorescence time traces in circular ZMWs

In this section, we investigate the fluorescence enhancement of single Alexa Fluor 647 molecules immobilized at the bottom of circular aluminum ZMWs of 110nm diameter. The single molecule fluorescence immobilization is performed by silane-PEG-amine functionalization of the aperture followed by incubation with NHS ester functionalized Alexa Fluor 647 and thorough rinsing to remove unbound molecules. The 25% labeling density of the ZMWs and the single-step photobleaching events indicate true single molecule regime in this work.

While only circular ZMWs have been studied here, the conclusion is that similar fluorescence enhancement and lifetime reduction are observed for both fixed single molecule time trace and diffusing molecule FCS. Both approaches yield similar results, which validates our conclusions for all nanoaperture shapes (circular and rectangular).

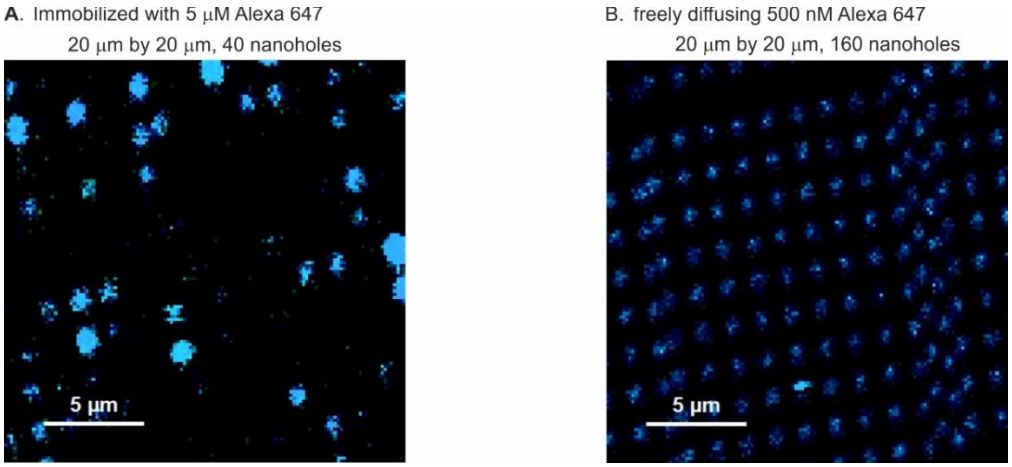

Figure S2. Comparison of the images of the nanoholes obtained in (A) immobilized and (B) freely diffusing conditions respectively. Under immobilized condition only 40 nanoholes out of 160 are grafted by a fluorophore. The grafting efficiency is 25%. From this value and assuming the distribution of molecules follow a Poisson distribution, the probability of finding one molecule per ZMW is 22%, the probability of finding more than one molecule is 3.5%.

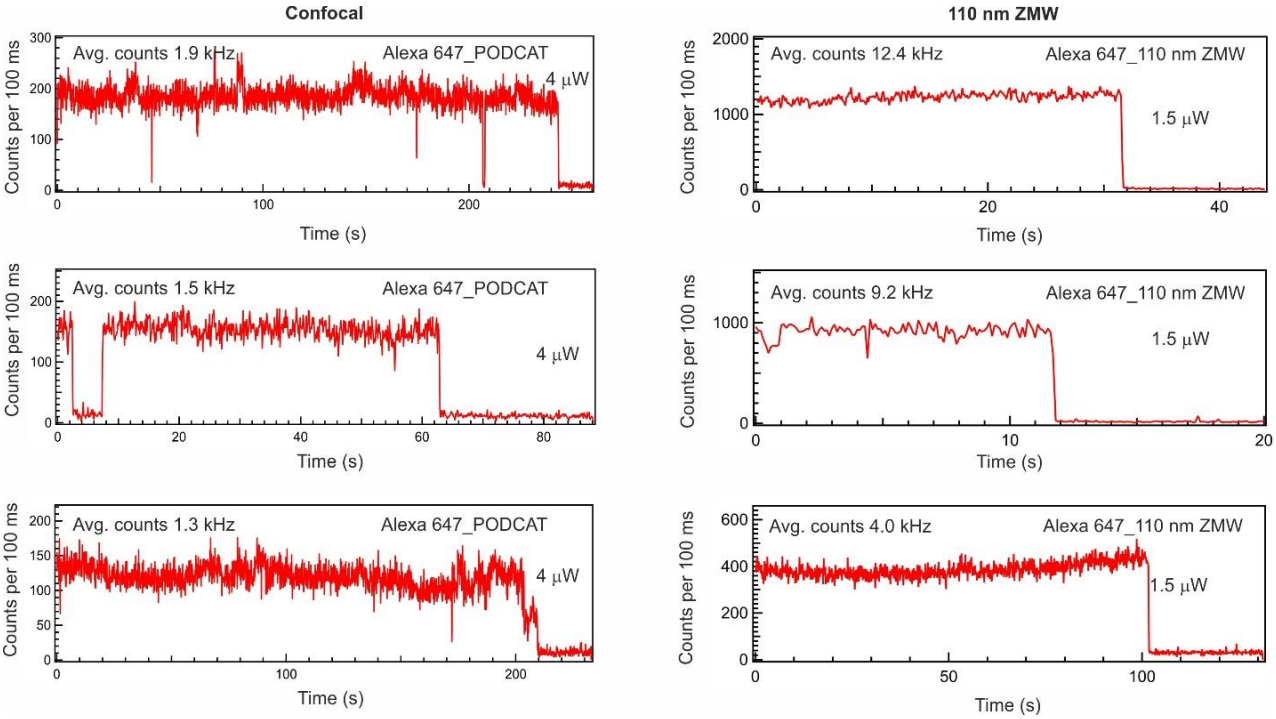

Figure S3: Comparison between the fluorescence time traces obtained for the immobilized single molecule in confocal (left panel) and inside a 110 nm ZMW (right panel). Please note that the measurement in confocal and in the presence of ZMW are carried out at 4 μW and 1.5 μW respectively.

Table S1: Comparison of count rate per molecule (CRM) between the diffusion and immobilized measurements. Both CRMs are computed back to the same 20 μW excitation power.

Nominal **Milling depth**	**CRM in diffusion (FCS)**	**CRM in immobilized case (single molecule)**
0.3 μm	97.2 ± 1.8 kHz	58.5 kHz
0.35 μm	67.0 ± 4.3 kHz	68.0 kHz
0.4 μm	78.9 ± 5.6 kHz	40.0 kHz
0.45 μm	59.2 ± 6.7 kHz	48.0 kHz
0.5 μm	76.8 ± 1.4 kHz	57.3 KHz

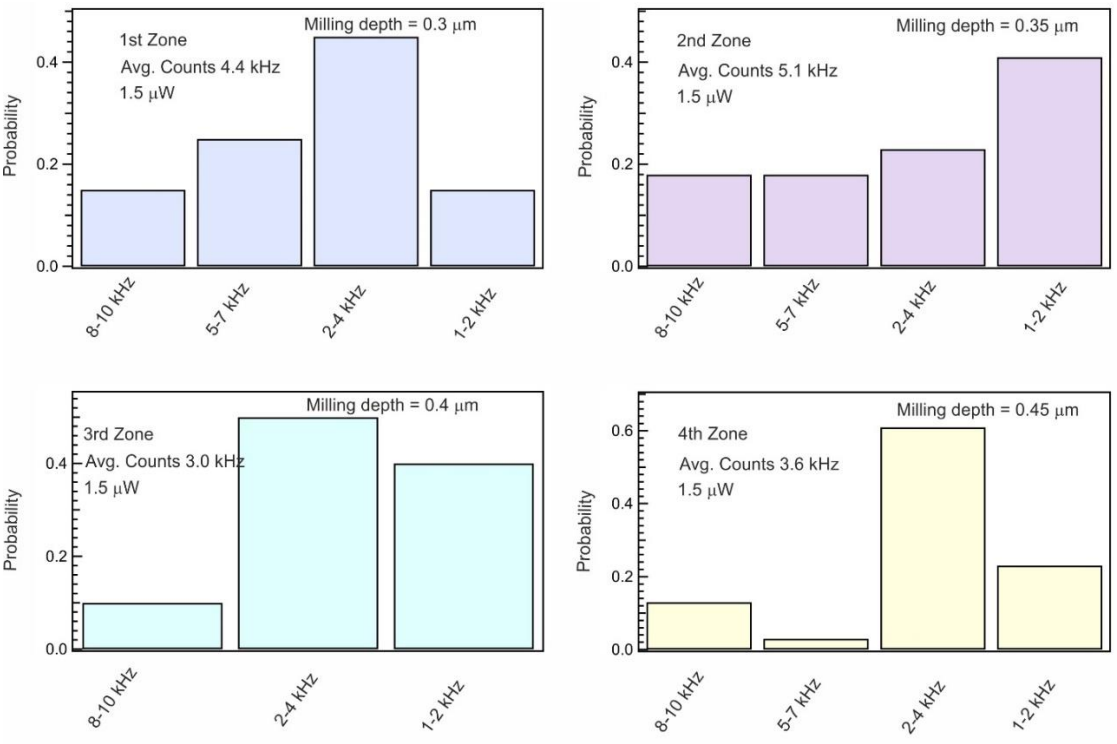

Figure S4. Intensity distributions for different ZMWs featuring different nominal milling depths. 20, 17, 10, and 31 molecules were used to construct the distribution for 1st, 2nd, 3rd, and 4th zone respectively. The excitation power is 1.5 μW.

The fluorescence lifetime is always shorter in presence of ZMW. The lifetime ranges from 0.26 ns-1.0. The intensity time trace which display good fluorescence counts, has a fluorescence lifetime ranging from 0.7 ns – 0.9 ns. The traces which display a lifetime around ~ 0.3 ns also has weak fluorescence counts indicating metal induced quenching. More than 2-fold decrease in the fluorescence lifetime is observed inside ZMW than the confocal reference (Fig. S5).

In conclusion, considering the case of circular 110nm diameter aluminum ZMW, we find that similar results are obtained for the fluorescence brightness enhancement and the fluorescence lifetime reduction independently of the method chosen of (i) FCS on freely diffusing molecules or (ii) fluorescence time traces acquired for immobilized molecules. This fully confirms our claims and conclusions in the main manuscript.

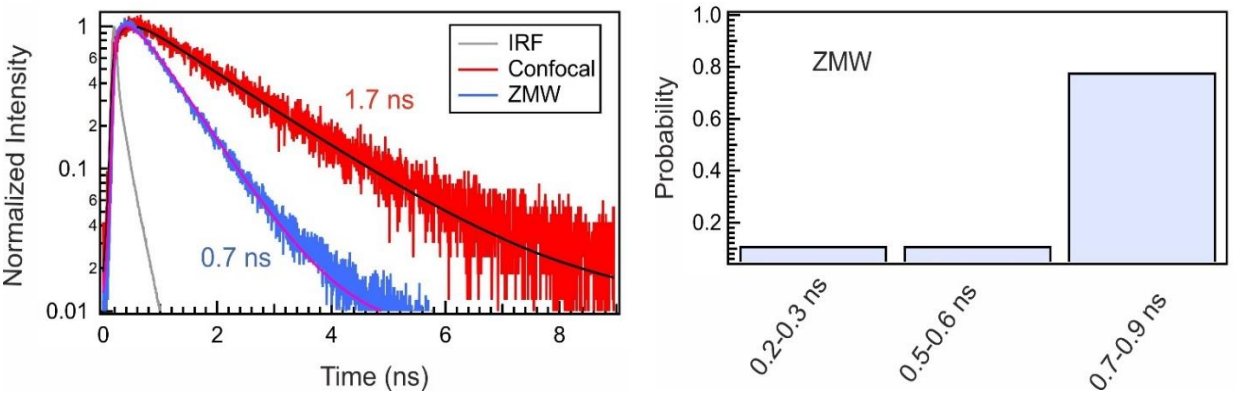

Figure S5. Comparison of fluorescence intensity decay between confocal and ZMW measurements, acquired in immobilized single molecule condition.The lifetime distribution on the right plot is obtained from 18 different Alexa 647 molecules immobilized inside a 110 nm diameter ZMW.

S3. Influence of the excitation power

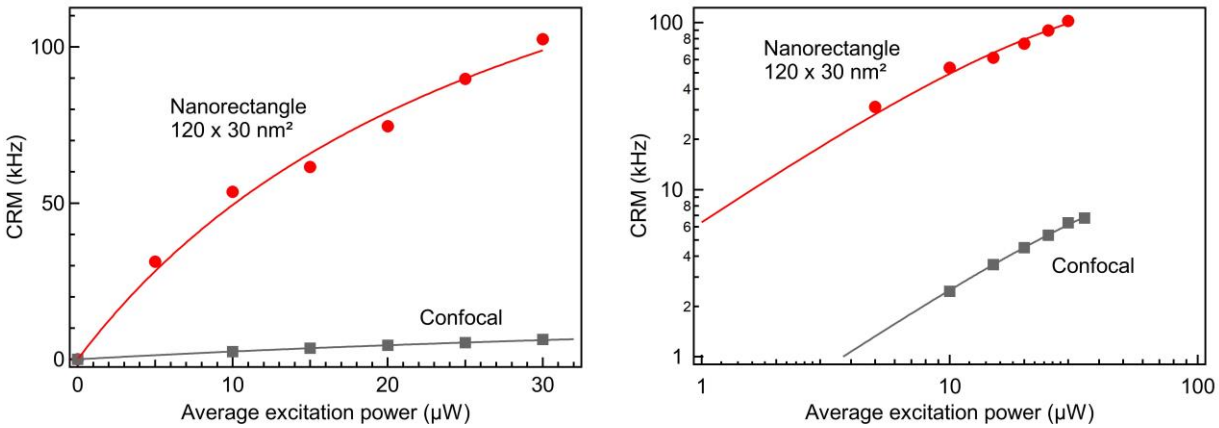

Figure S6. Influence of the excitation power on the fluorescence brightness per molecule (CRM) for diffusing Alexa Fluor 647 molecules in the confocal setup and with a 120 x 30 nm² NR. The left graph is with linear scale, the right graph displays the same data in logarithmic scale.

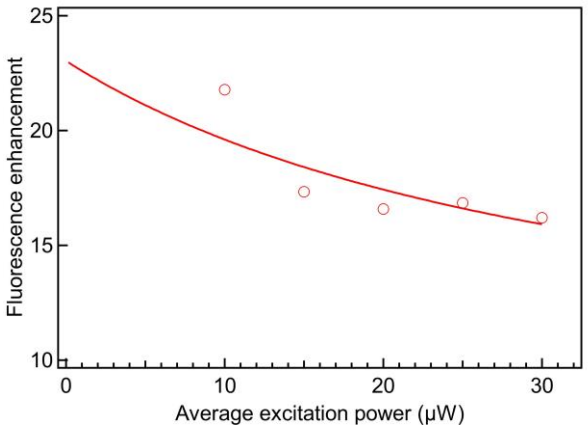

Figure S7. Influence of the excitation power on the fluorescence enhancement factor for diffusing Alexa Fluor 647 molecules in a 120 x 30 nm² NR as compared to the confocal reference.