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Electronic Supplementary Information

Table S1. Calculation of agglomeration in Immunoassay test (experiment 5, blank, Au NPs 60 nm on Au film, total area 36 μm^2).

№	Nm	Nd	Nt	Na*	Number of NPs in agglomers	Agglo-meration, %	Total number of NPs	Total number of NPs per 1 μm^2
1	37	13	2	6	27	61	96	2.7
2	39	12	3	8	76	74	148	4.1
3	24	7	3	11	64	78	111	3.1
4	40	6	5	3	18	53	85	2.4
5	22	9	2	4	38	74	84	2.3
Mean	32	9	3	6	45	68	105	2.9
Mean, %	30.4	8.57	2.85	5.71	42.8	64.7	100	

*Na – number of agglomerates.

Table S2. Calculation of agglomeration in Immunoassay test (Experiment 5, MPT64 0.8 nM, Au NPs 60 nm on Au film, total area 36 μm^2).

№	Nm	Nd	Nt	Na	Number of NPs in agglomers	Agglo-meration, %	Total number of NPs	Total number of NPs per 1 μm^2
1	111	23	13	10	61	57	257	7.1
2	146	23	10	10	53	47	275	7.7
3	122	37	12	14	81	61	313	8.7
4	144	24	7	14	86	52	299	8.3
5	101	28	18	13	77	65	288	8.0
Mean	125	27	12	12	71	54	286	7.9

Table S3. Surface roughness of the substrates as determined by AFM

Surface	Au film	Ag film	Si wafer	Al film
Mean value, nm	2.82	3.58	3.87	3.23
RSD, %	17.4	8.02	3.19	12.6

Table S4. Characteristics of the obtained samples (mean calculated values).

Experiment №, analyte	Substrate	NPs, D nm	Ibg/Im/Id/It cps, (norm. to to 1 mW laser power)	Id/Im, Standard error BG corrected	It/Im, Standard error BG corrected	EFm x10^6 mean, max, min	EFd x10^6 mean, max, min	EFt x10^6 mean, max, min	Nm/ Nd/ Nt
1, 2-MOTP	Au	Au, 80	15/1013/ 2848/-	**2.84,** 0.53		**10.9**	**15.5**		15/
						17	**29**		10/-
						2.6	**6.5**		
1, 2-MOTP	Ag	Au, 80	14/1081/ 3670/-	**3.42,** 0.60		**11.7**	**20.0**		10/
						17	**31**		10/-
						7.6	**6.3**		
2, 2-ATP	Au	Ag, 100	9/2128/ 6446/-	**3.04,** 0.48		**5.0**	**7.6**		33/
						16.2	**13.7**		18/-
						1.2	**3.1**		
2, 2-ATP	Al	Ag, 100	8/923/ 2445/ 5997	**2.66,** 0.5	6.54 1.5	**2.2** **4.3** **0.38**	**2.9** **8.0** **1.2**	**4.7** **16.5** **1.3**	18/ 15/ 18
2, 2-ATP	Ag	Ag, 100	12/10376/2 2757/-	**2.20,** 0.27		**24**	**27**		40/
						60	**114**		28/-
						3.7	**15**		
3, 2-ATP	Au	Au, 60	10/120/ 333/564	**2.93,** 0.34	5.05 1.66	**0.7** **3.0** **0.14**	**1.1** **1.5** **0.7**	**1.2** **3.6** **0.41**	40/ 22/ 8
3, 2-ATP	Ag	Au, 60	20/399/ 1283/-	**3.33,** 0.50		**2.5**	**4.1**		22/
						3.9	**8.2**		14/-
						1.2	**1.5**		
3, 2-ATP	Si	Au, 60	8/14.1/ 70.0/97.1	**9.55,** 0.44	13.7 0.437	**0.043** **0.057** **0.033**	**0.20** **0.4** **0.098**	**0.20** **0.24** **0.15**	28/ 23/ 6
4, (532) 4-NBT hIgG*	Au	Au, 60	19/43.9/ 76.5/-	**2.26,** 0.24		**0.011** **0.014** **0.006**	**0.012** **0.023** **0.007**		20/ 19/-
4, 4-NBT hIgG	Au	Au, 60	9/1048/ 2403/-	**2.35,** 0.28		**0.44** **0.76** **0.29**	**0.51** **0.94** **0.22**		20/ 12/-
5, 4-NBT, MPT64	Si	Au, 60	8.5/15.4/ 753/1028	**107,** 12	148 13.8	**0.003** **0.004** **0.002**	**0.16** **0.62** **0.016**	**0.14** **0.29** **0.03**	15/ 25/ 10
5, 4-NBT, MPT64	Au	Au, 60	12/1039/ 2577/-	**2.49,** 0.39		**0.44** **0.94** **0.71**	**0.5** **1.5** **0.25**		33/ 17/-
5, 4-NBT, MPT64	Ag	Au, 60	21/810/ 2491/-	**3.12,** 0.48		**0.34** **0.70** **0.18**	**0.52** **1.0** **0.19**		17/ 15/-
6, 2-MOTP 1nM **	Au	Au, 80	10/95/ 277/ 513	**3.15,** 0.57	5.94 1.09	**6625** **13201** **3101**	**10463** **27107** **3953**	**13139** **21001** **8131**	16/ 18/ 5
7, 4-NBT	Au	Au, 100	13/1200/ 3411	**2.86** 0.38		**0.25** **0.35** **0.13**	**0.35** **0.53** **0.20**		15 8
Total of 615 nano antennas : monomers 342, dimers 226, trimers 47.									

* Laser wavelength 532 nm ** assuming that each AuNPs covered with 14 molecules of 2-MOTP as calculated

Table S5. Mean values for distance between NPs (Gap) calculated from TEM data.

NPs	Gap, TEM, nm (±Standard error, nm)	N gaps
AuNPs, 60 nm in ERL, 4-NBT, dimers	3.1±0.6	15
AuNPs, 60 nm in ERL, 4-NBT, all agglomerates	3.5±0.5	38
AuNPs 60 nm, 4-ATP, dimers	1.1±0.3	16
AuNPs, 60 nm, 4-ATP, all agglomerates	1.0±0.2	42
AuNPs, 80 nm, 2-MOTP, dimers	0.8±0.2	15
AuNPs, 80 nm 2-MOTP, all agglomerates	0.8±0.2	30

Table S6. The values of SD and SEM (standard error of mean) for SERS intensities.

Experiment N, analyte	Sub-strate	NPs, nm	SD, for monomers, %	SEM for monomers, %	SD for dimers, %	SEM for dimers, %
1) 2MOTP	Au	Au, 80	41.8	7.52	42.7	9.33
1) 2MOTP	Ag	Au, 80	43.4	12.6	54.9	14.2
2) 2-ATP	Au	Ag, 100	50.6	10.3	41.1	9.70
2) 2-ATP	Al	Ag, 100	44.9	10.6	60.5	15.6
2) 2-ATP	Ag	Ag, 100	53.0	9.33	42.6	8.05
3) 2-ATP	Au	Au, 60	36.8	7.41	20.9	4.47
3) 2-ATP	Ag	Au, 60	29.8	6.35	47.4	12.6
3) 2-ATP	Si	Au, 60	13.2	2.49	40.7	8.49
4) 4-NBT*(532) hIgG	Au	Au, 60	28.6	6.41	36.9	8.26
4) 4-NBT hIgG	Au	Au, 60	30.6	7.96	35.8	10.3
5) 4-NBT, MPT64	Si	Au, 60	15.2	3.92	111	22.3
5) 4-NBT, MPT64	Au	Au, 60	42.1	7.33	57.4	13.9
5) 4-NBT, MPT64	Ag	Au, 60	40.1	9.72	47.6	12.3
6) 2MOTP 1nM**	Au	Au 80	46.7	11.6	65.9	15.5

Enhancement factor calculations

$EF = (I_{SERS}/I_{Raman})(N_{SERS}/N_{Raman}) = (I_{SERS}/I_{Raman})(N\nu_{BULK}\ V_{Laser}/C_S)$

In Experiment 1 (AuNPs 80 nm/ Au film)

EF (monomer) = (795/171.4)(100500/1.88E+11) = 9.44E+06

N_{Raman} = (density (g/ml)*Avogadro's number/Molar mass 2-MOTP)* V_{Laser} (μm^3)/10^{12} (μm^3/ml) = 1.88E+11, Here density(g/ml) =1.152, FM= 140.2 g/mole

N_{Raman} = (1.152 * 6.02E+23 / 140.2) * 38 / 1E+12 = 1.88E+11

N_{SERS} = NPs Surface area (nm^2)/ MOTP Surface cross section (nm^2) = 2.01E+4/0.2 = 100.5x 10^3 molecules/NP., where NPs Surface area (nm^2) = 4*pi*(Radius NPs nm)^2 4*3.14*40^2 = 2.01E+4 (nm^2), 0.2 nm^2 is surface cross section of 2-MOTP.

In Experiment 1 for dimer: EF (dimer) = (2258/171.4)(201000/1.88E+11) = 1.23E+06. where N_{SERS} = 2*100500.

Experiments 2 to 5 follow the same procedure using very conservative assumption for EF calculation (monolayer coverage with thiol molecules) .

In Experiment 6:

EF (mean for monomers) = (94.5/171.4)(14.01/1.88E+11) = 6.63E+9

N_{SERS} = 14.01 molecules per one NP, N_{SERS} = number of analyte molecules per one NPs = N molecules analite per nm^2 (on NPs and on Substrate) / One NPs Surface area (nm^2) = 6.94E-4 / 2.01E+4 = 14.01

One NPs Surface area (nm^2) = 4*pi*(Radius NPs nm)^2 4*3.14*40^2 = 2.01E+4 (nm^2)

N molecules of analyte per nm^2 (NPs+ Substrate) = N molecules analyte in drop / (NPs Surf in drop and Substrate Surface nm^2) = 1.51E+10 / 2.16+13 = 6.97E+4

NPs Surf in drop and Substrate Surface (nm^2) = All NPs Surface area in drop (nm^2) + Substrate Surface area (nm^2) = 1.96E+12 + 1.96E+13 = 2.16+13

Substrate Surface area (nm2) = 1.96E+13 = pi R^2 = 3.14*2500000^2

All NPs Surface area in drop (nm2) = 1.96E+12 = One NPs Surface area (nm2) * Number NPs in drop = 2.01E+4*9.75E+7 = 1.96E+12

N NPs in the drop = 9.75E+7 = V drop NPs per L * concentration, particles/L = 2.5E-5 * 3.9E+12

Example and order of combining Raman signal map and AFM map for Ag NPs 100 nm on Al substrate in experiment 2. Original files (Origin 2015 and Aist-NT SPM) can be downloaded from: https://www.dropbox.com/sh/7zpavdfitarpvbp/AAC7yWdfoUxZ2FkcbqUu0wUsa?dl=0

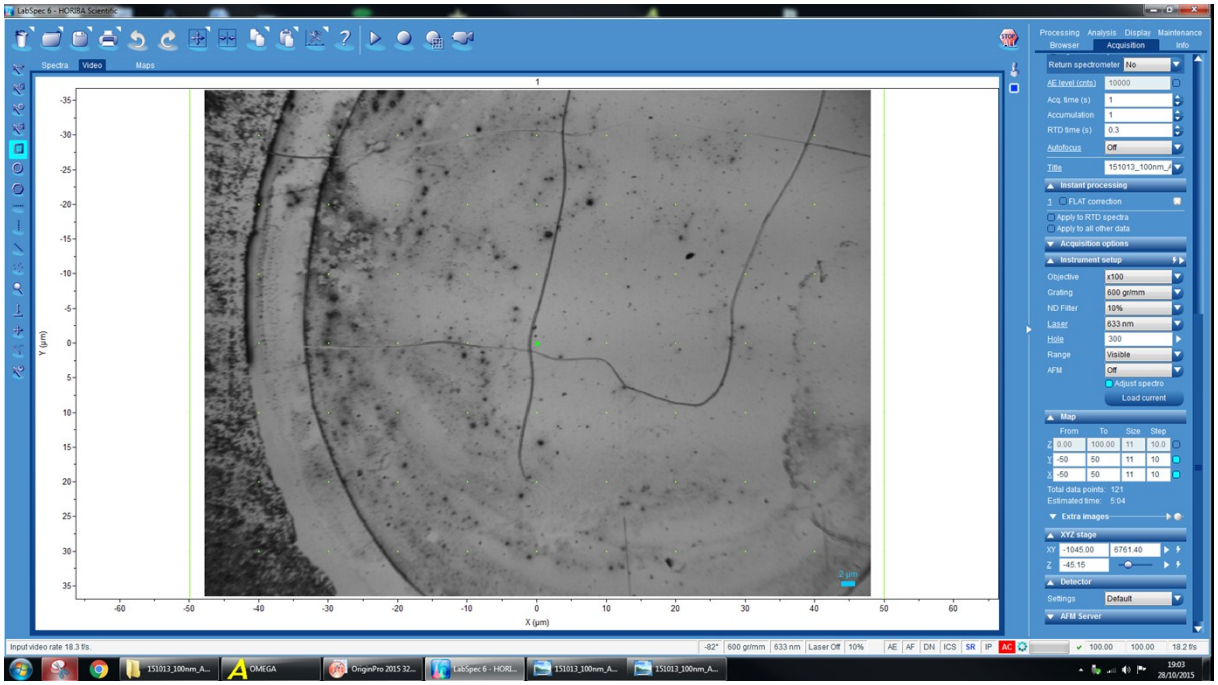


Fig. S1 Screen photo (objective x10) before recording Raman map, in the center of this photo smaller maps (using objective x100) were recorded.

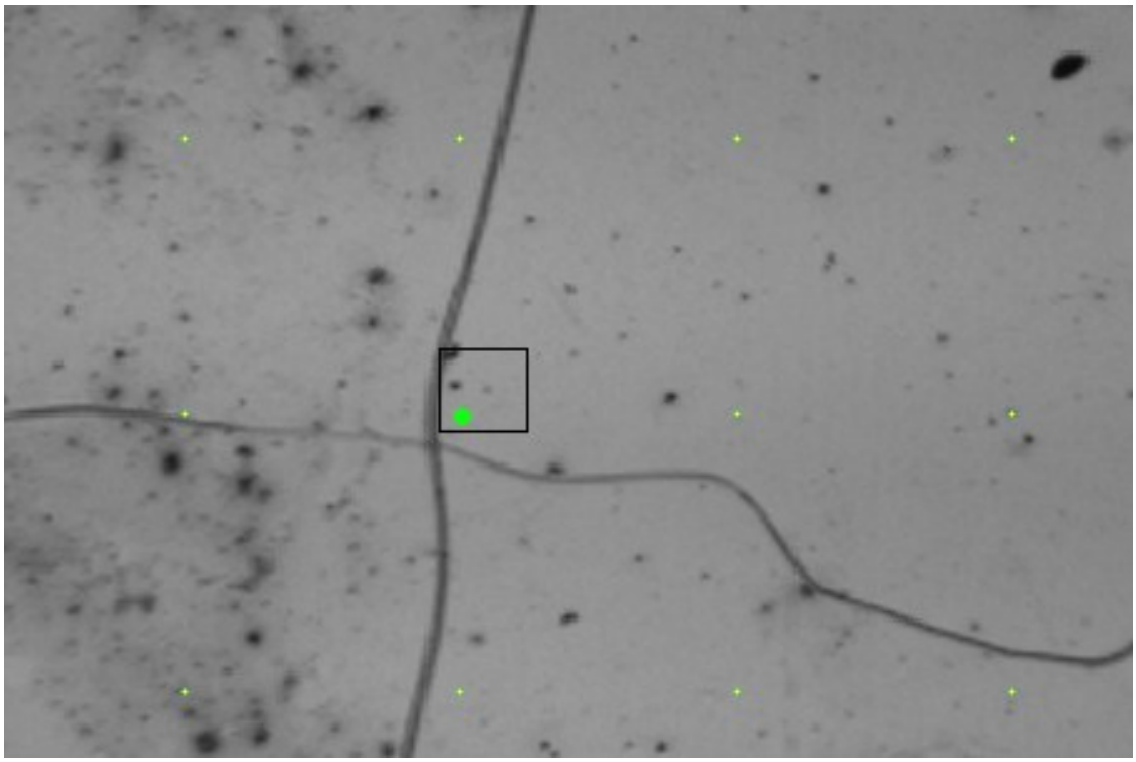


Fig. S2 Screen photo (objective x10) before recording Raman map (100x100 μm,), in the same place as AFM map.

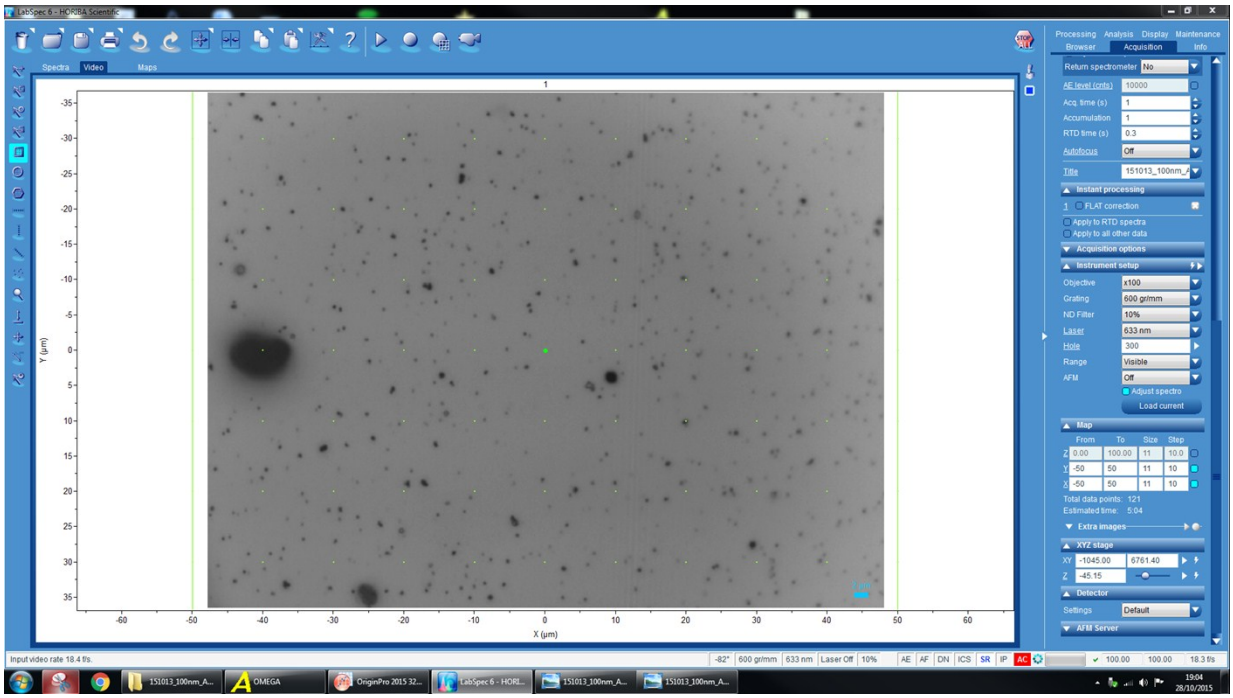


Fig. S3 Screen photo (objective x100) before recording Raman map (100x100 μm), in the same place as AFM map.

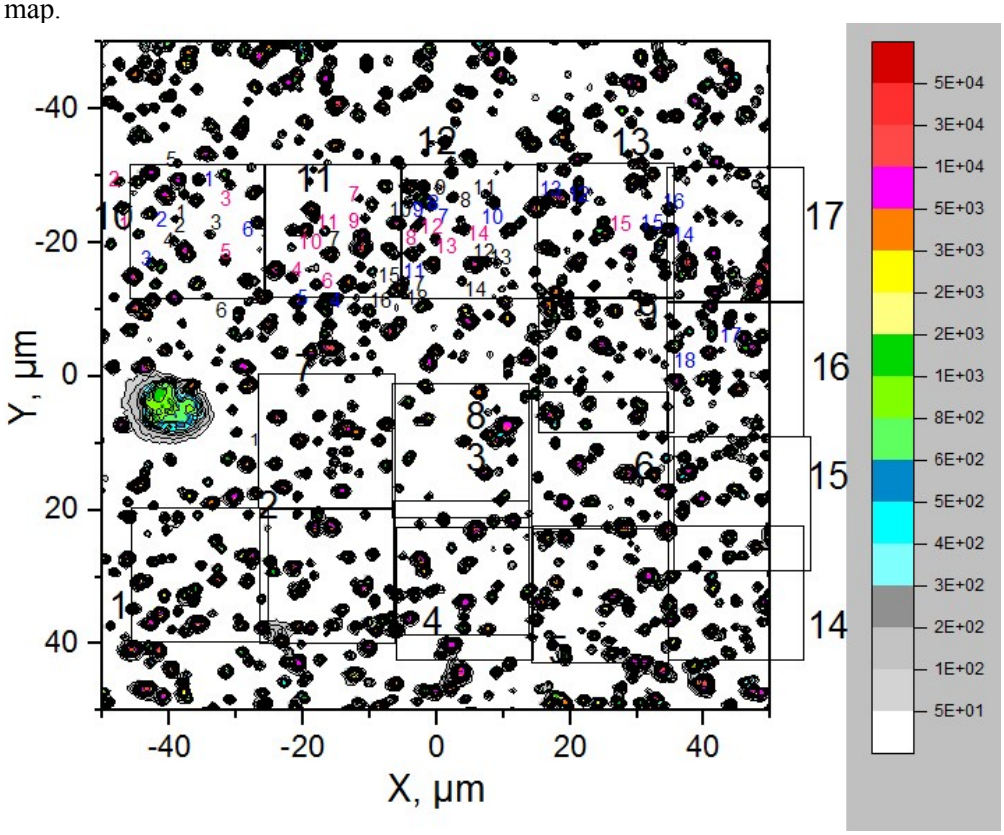


Fig. S4 SERS signal determination from nanoparticles, (Raman intensity map 100x100 μm, step 0.5 μm), places marked by squares where AFM maps 20x20 μm have been recorded.

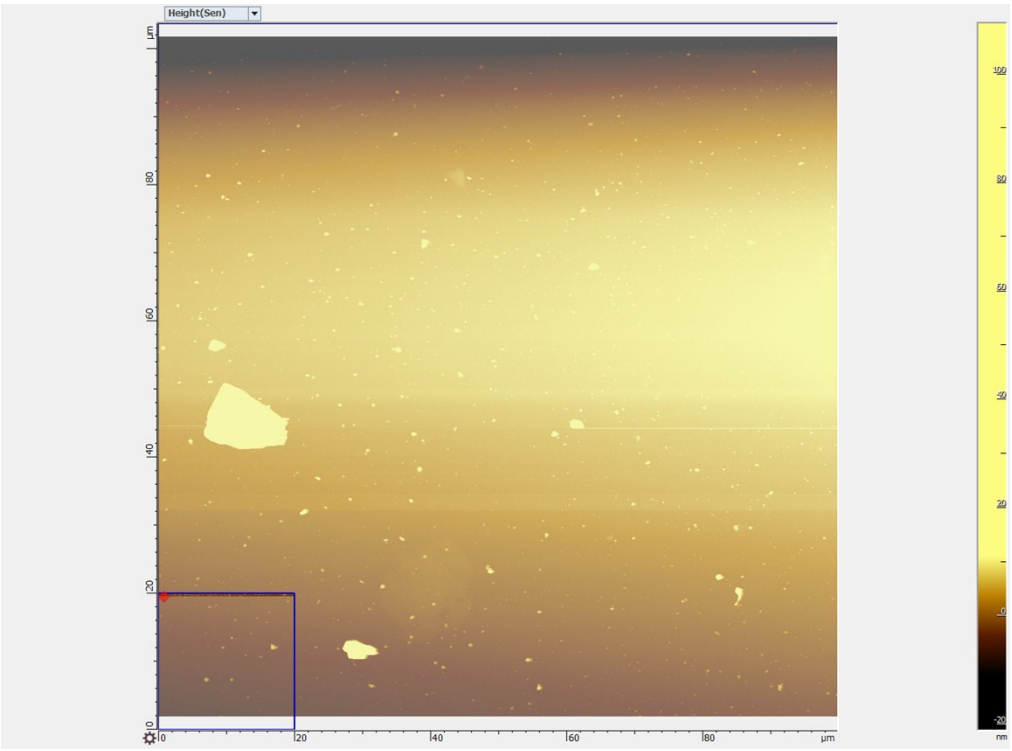


Fig. S5 Screen photo of AFM map, 100x100 µm in same place where Raman intensity map was recorded.

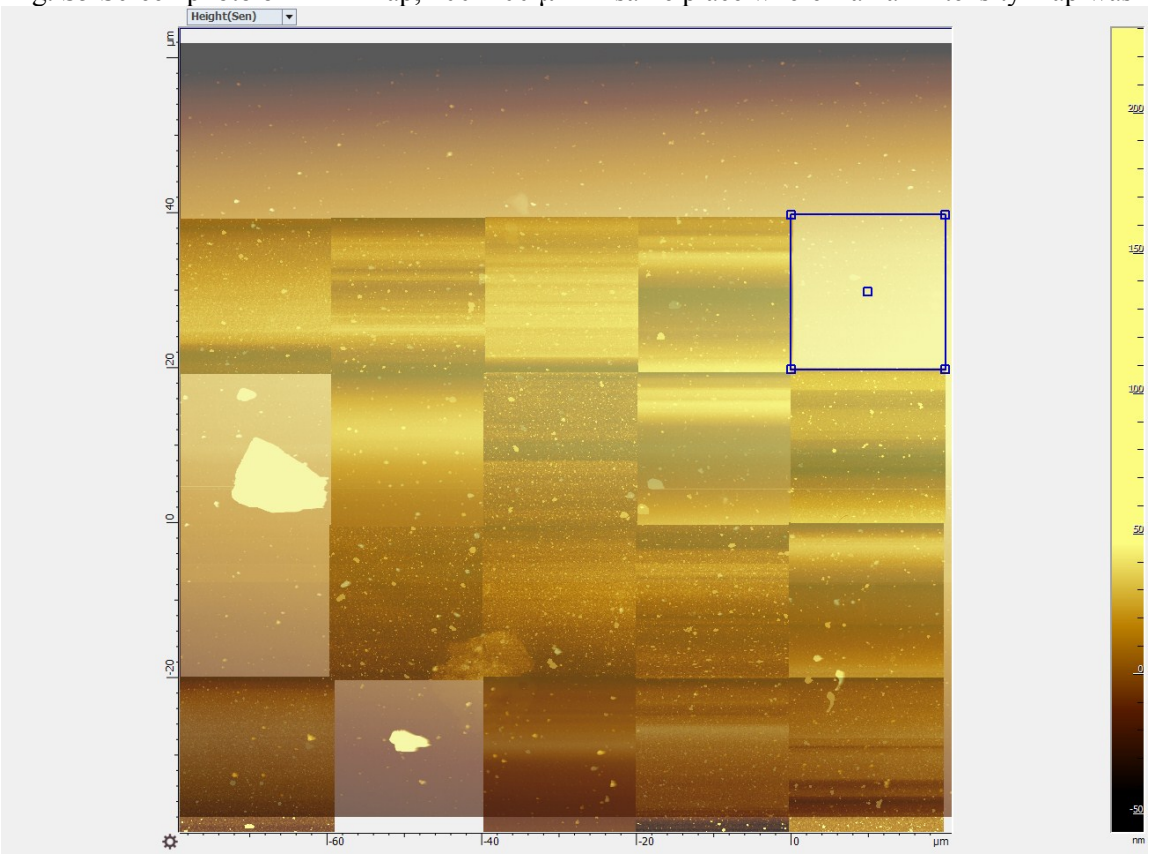


Fig. S6 Screen photo of AFM map 100x100 µm, that was used as mask for recording AFM maps 20x20 µm.

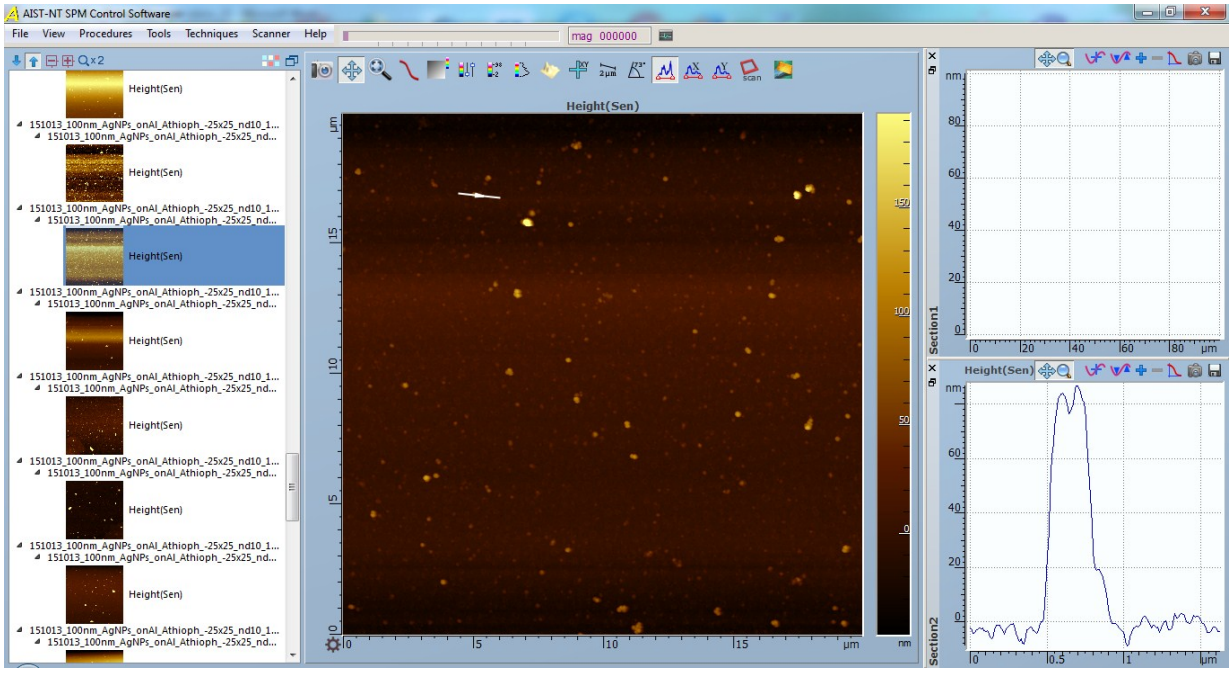


Fig. S7 Screen photo of one of 17 AFM maps 20x20 µm that were recorded using AFM map 100x100 µm as mask.

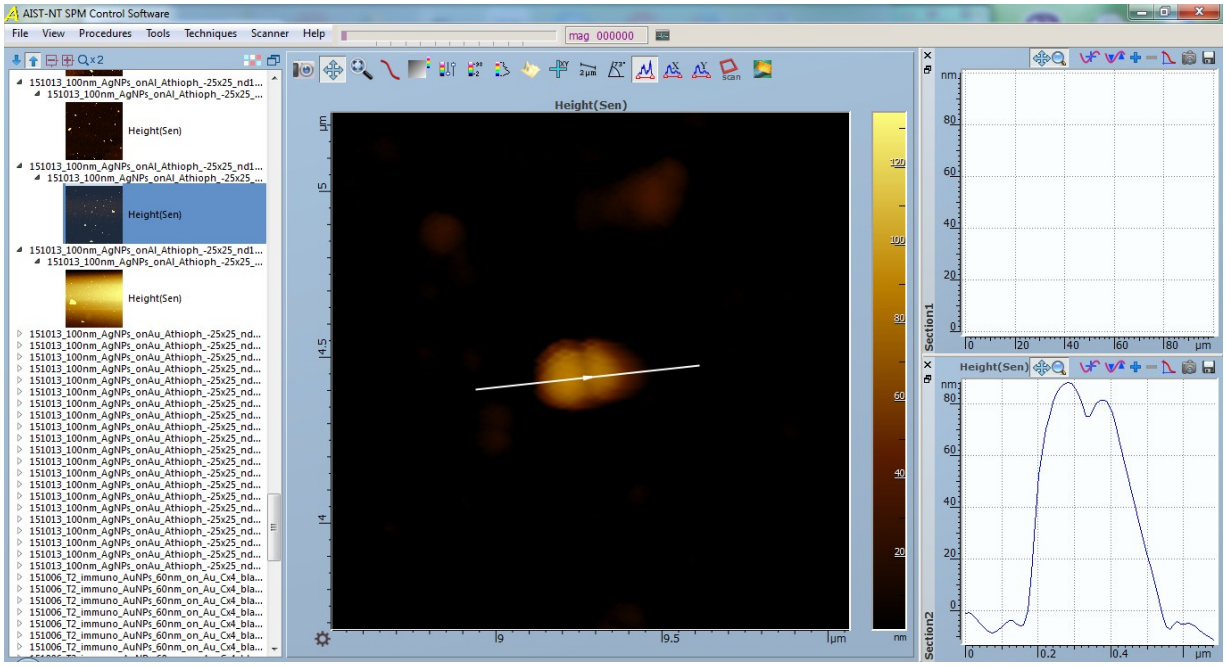


Fig. S8 Screen photo of one dimer (Ag NPs 100nm) on AFM map (20x20µm)

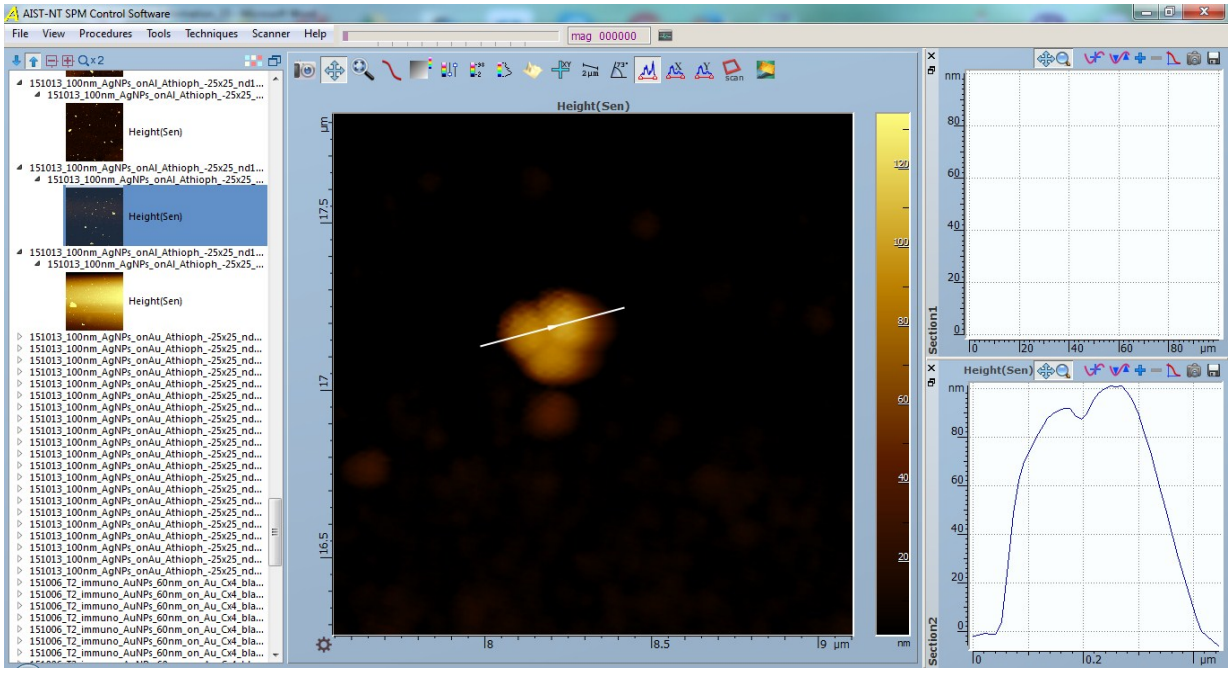


Fig. S9 Screen photo of one trimer (Ag NPs 100nm) on AFM map (20x20µm)

Example and order of combining Raman signal maps and AFM maps for Au NPs 60 nm on Si substrate in experiment 5. Original files (Origin 2015 and Aist-NT SPM) can be downloaded from: https://www.dropbox.com/sh/7zpavdfitarpvbp/AAC7yWdfoUxZ2FkcbqUu0wUsa?dl=0

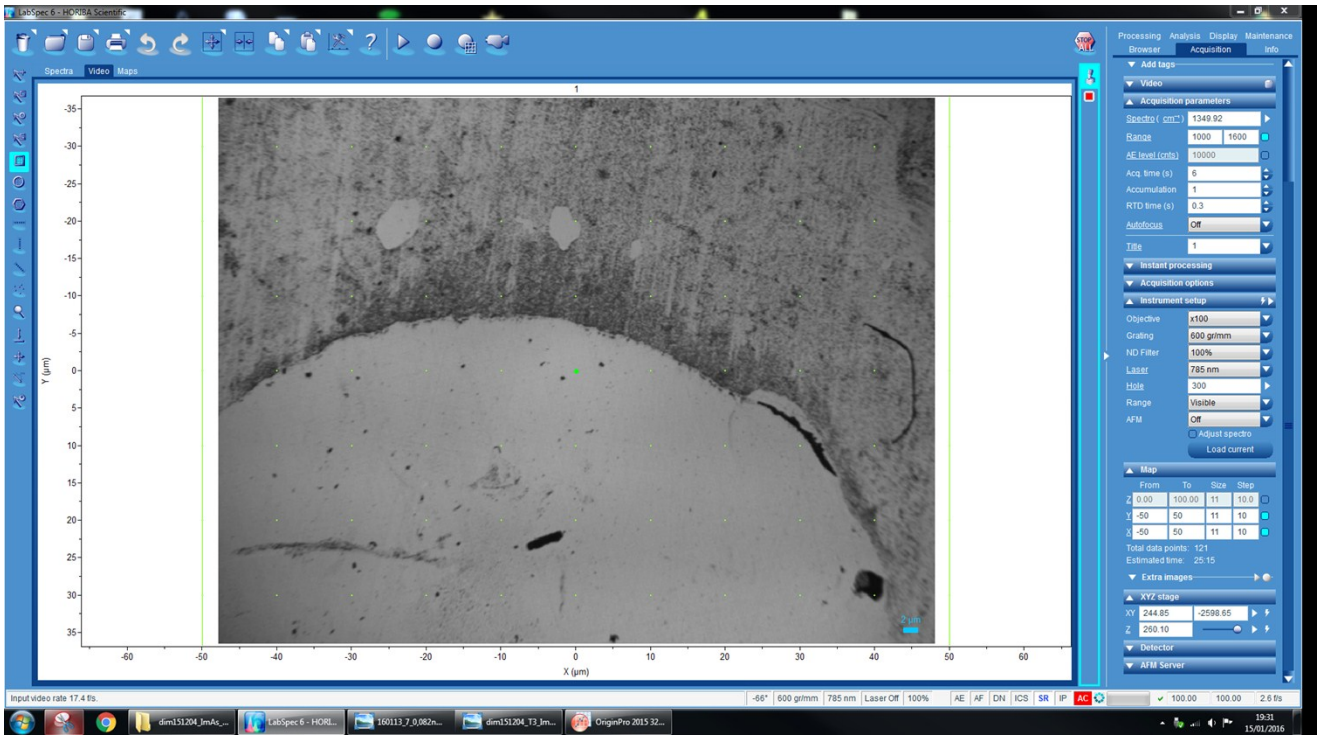


Fig. S10 – Screen photo (objective x10) before recording Raman map, in the center of this photo smaller maps (using objective x100) were recorded.

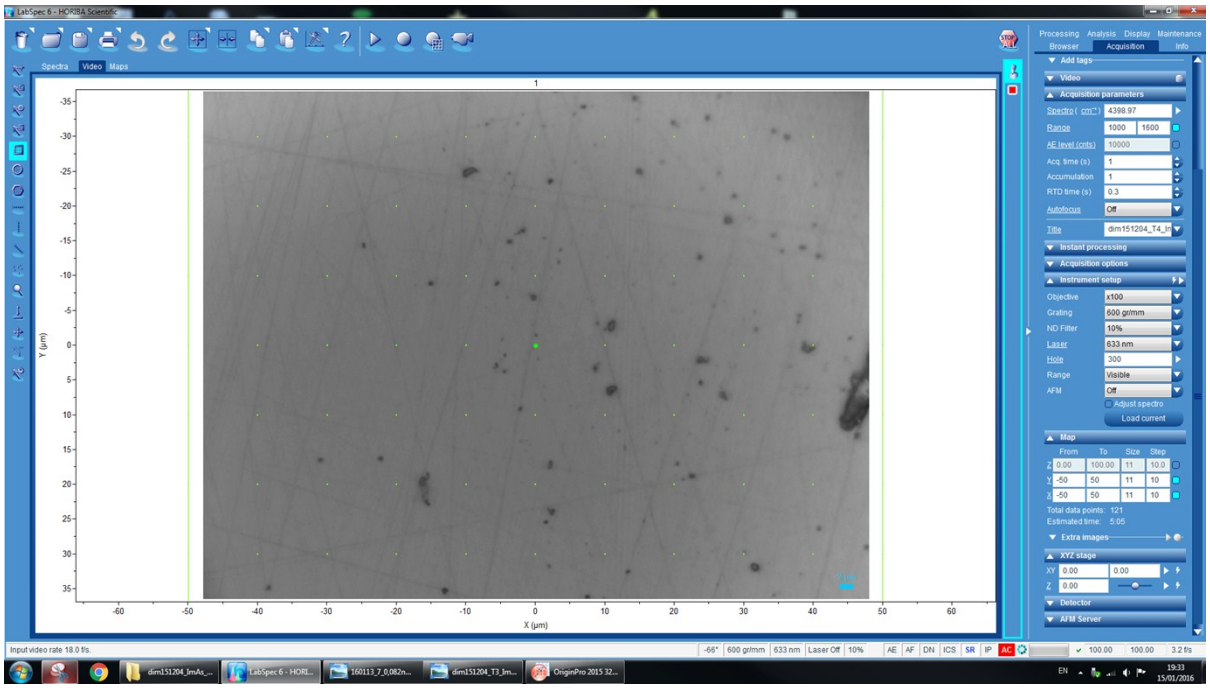


Fig. S11 Screen photo (objective x100) before recording Raman map 100x100 µm, in the same place as AFM map 100x100 µm.

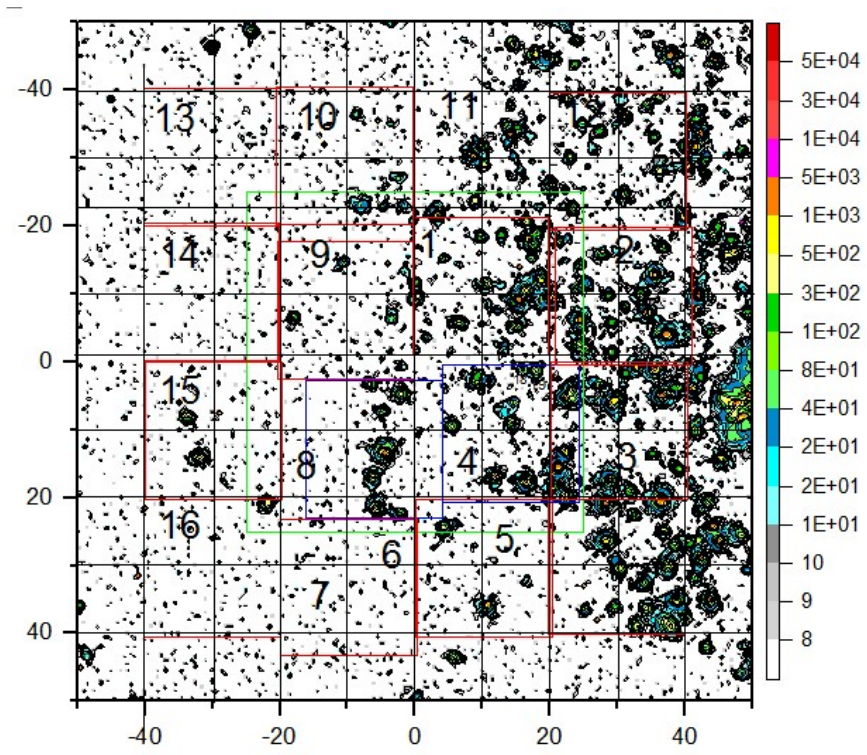


Fig. S12 Raman intensity map 100x100 µm (step 0.5 µm) for SERS signal determination from AuNPs 60 nm on Si, places marked by red squares where AFM maps 20x20 µm have been recorded

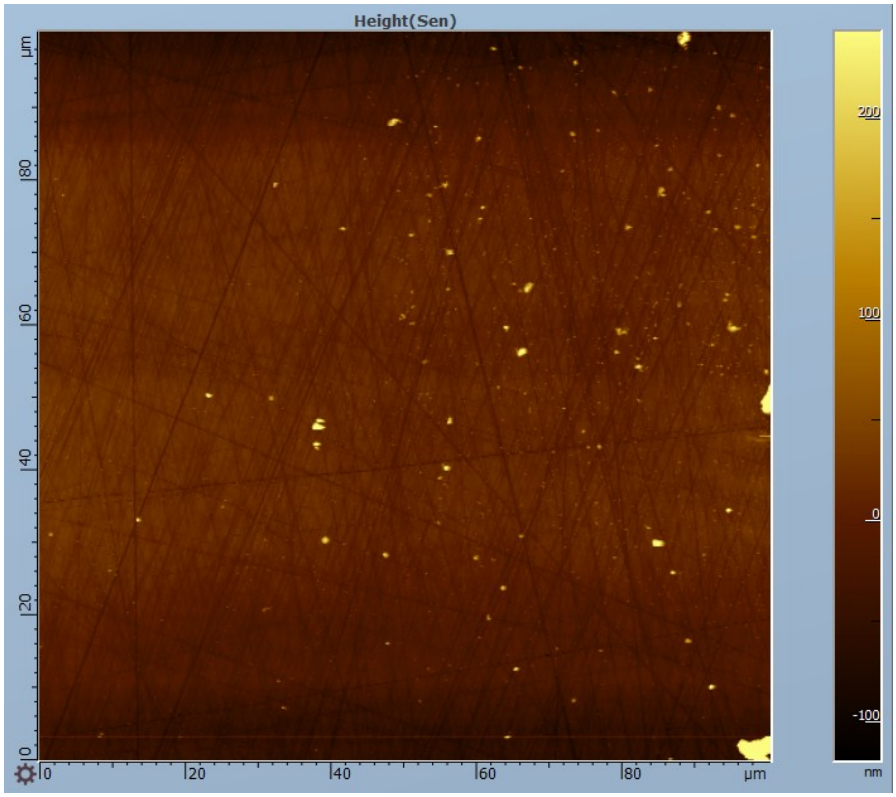


Fig. S13 Screen photo of one of the AFM maps 20x20 µm that were recorded using AFM map 100x100 µm as mask.

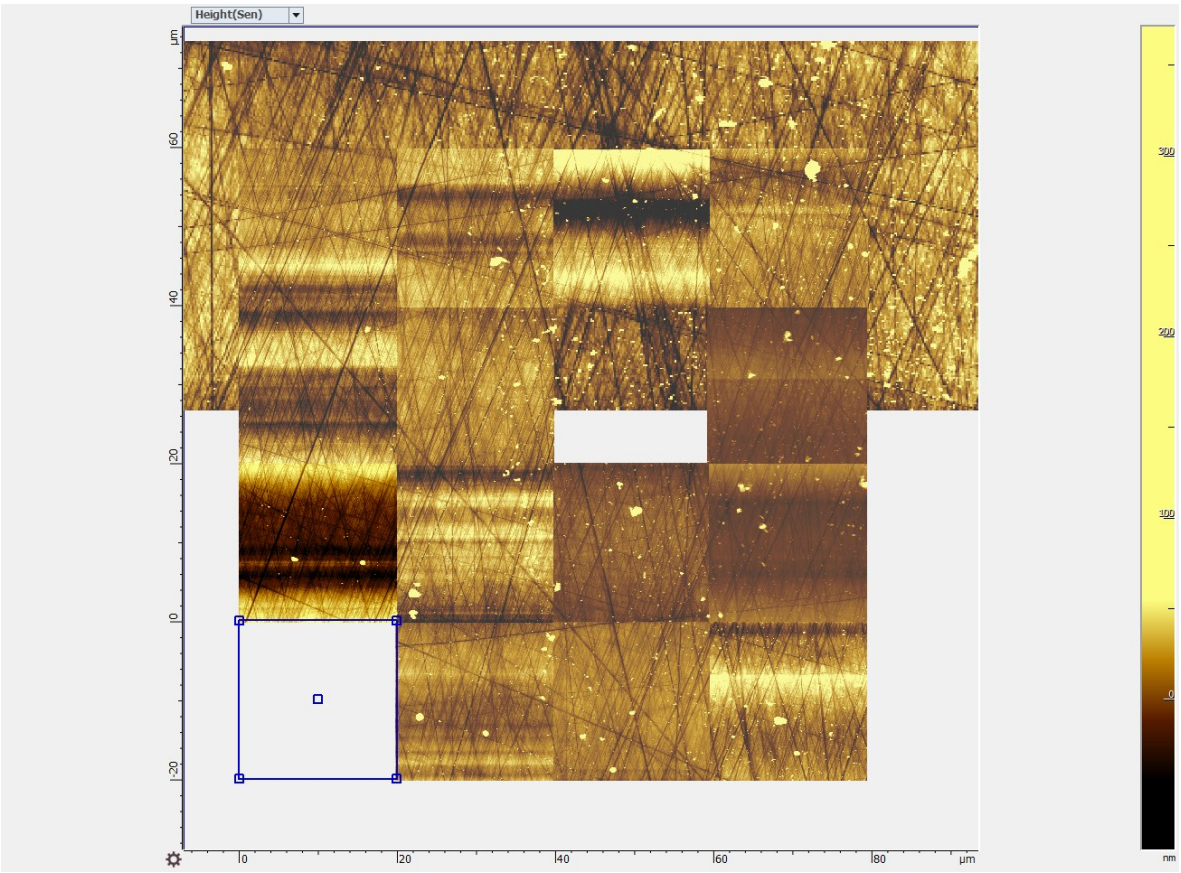


Fig. S14 Screen photo of AFM map 100x100 µm, (corresponded to Raman map), that was used as mask for recording AFM maps 20x20 µm

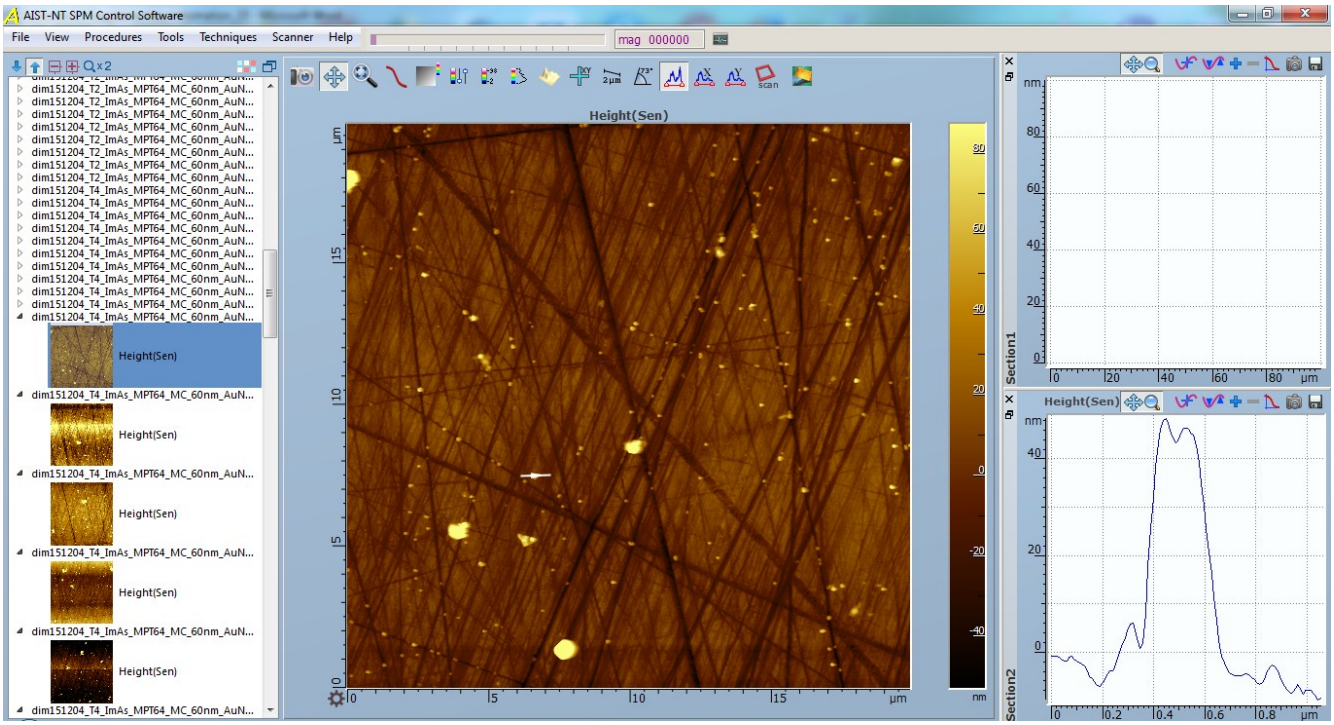


Fig. S15 Screen photo of one of 16 AFM maps 20x20 µm that were recorded using AFM map 100x100 µm as mask experiment 5; Sample: dim151204_T4

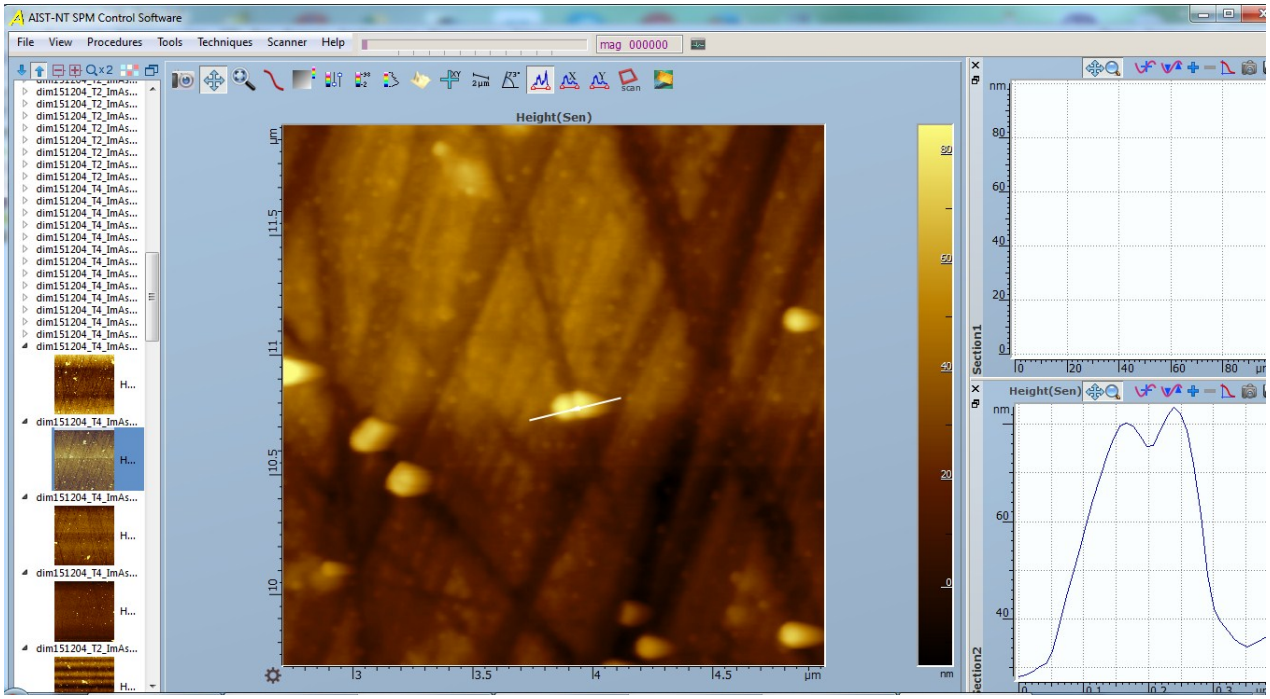


Fig. S16 Screen photo of dimer on AFM map (20x20µm), experiment 5; Sample: dim151204_T4

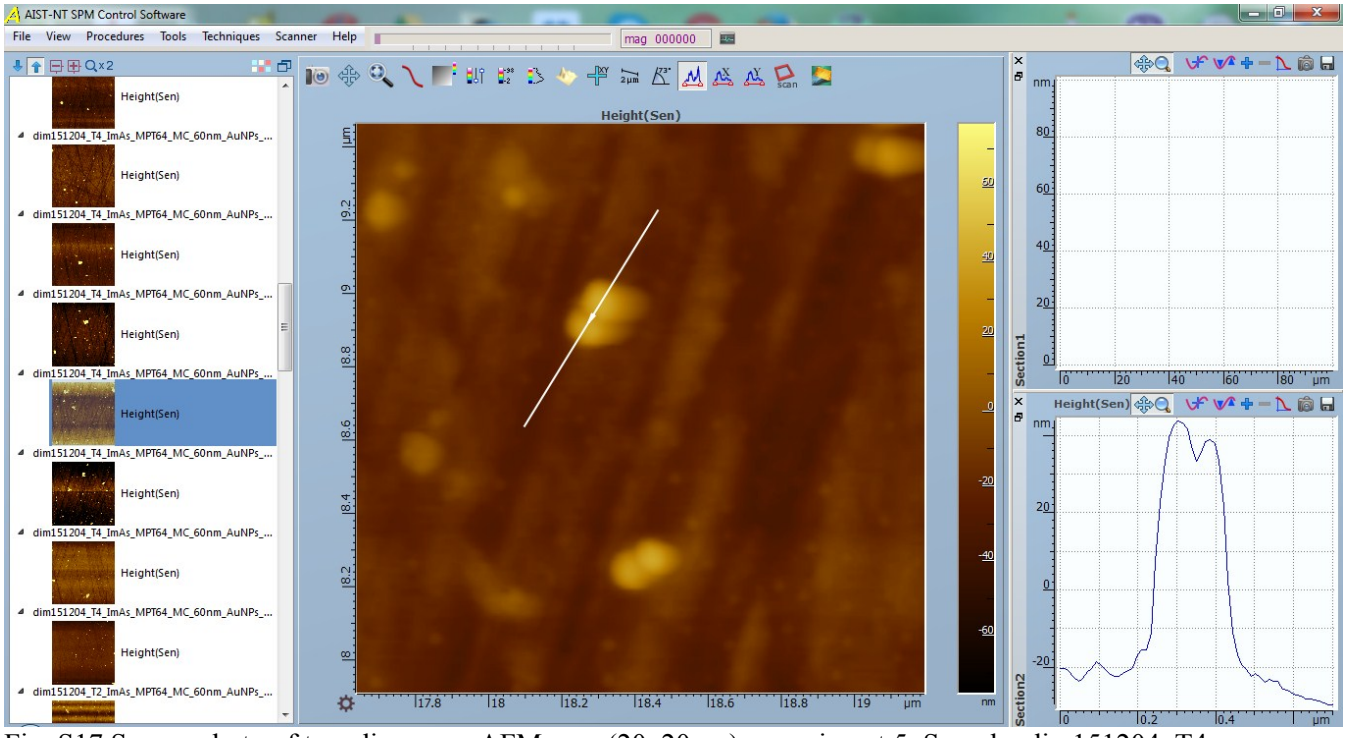


Fig. S17 Screen photo of two dimers on AFM map (20x20µm), experiment 5; Sample: dim151204_T4

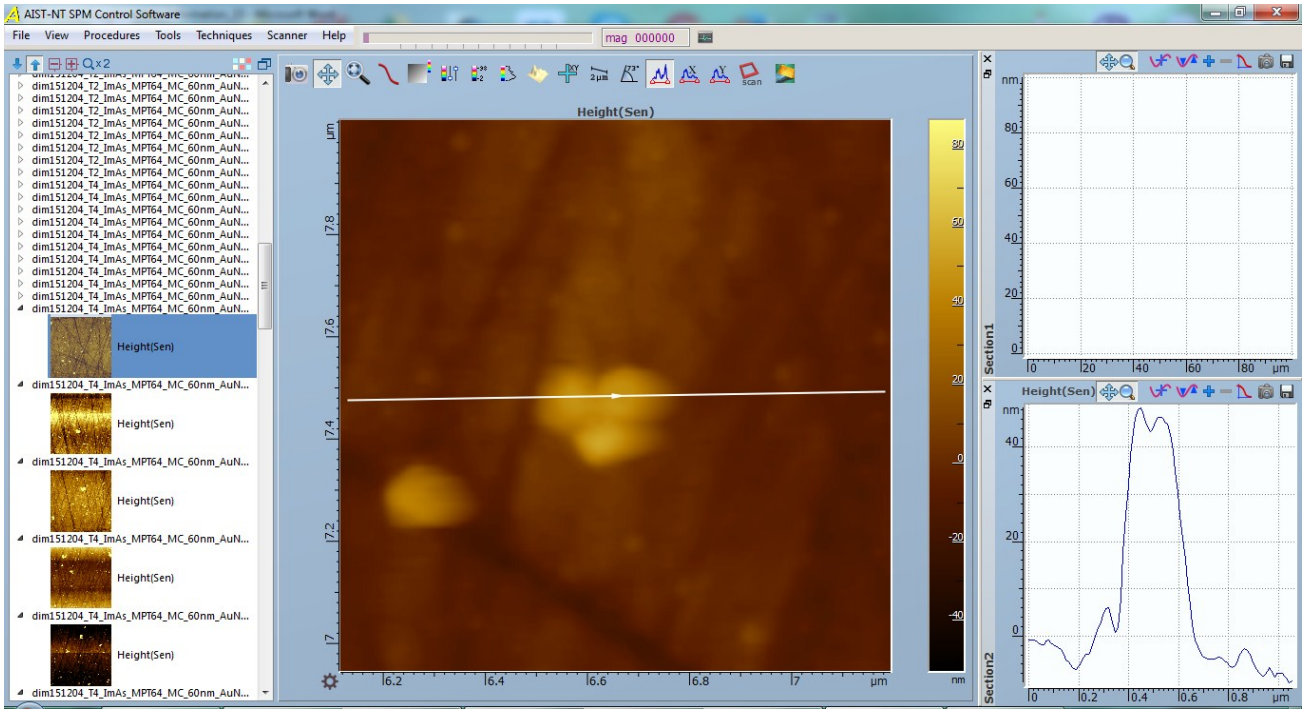


Fig. S18 Screen photo of trimer on AFM map (20x20µm), experiment 5; Sample: dim151204_T4

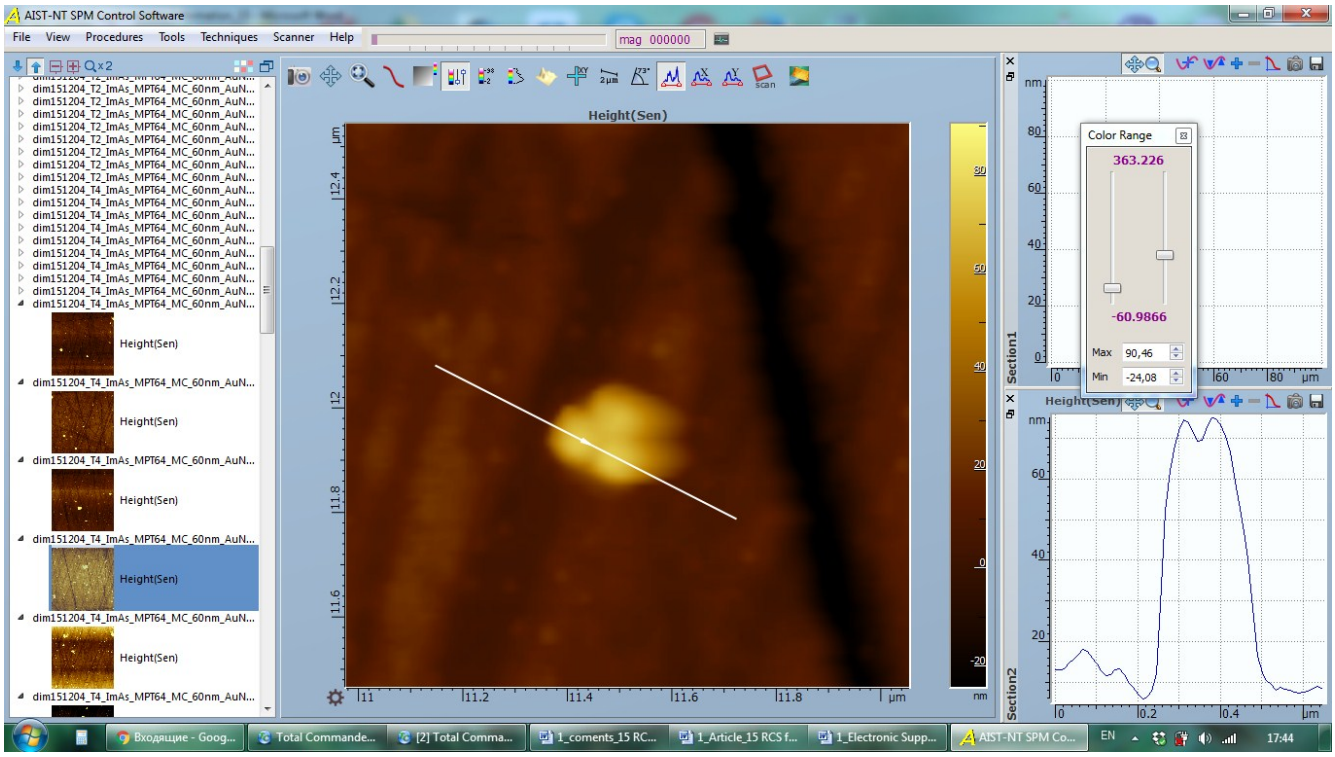
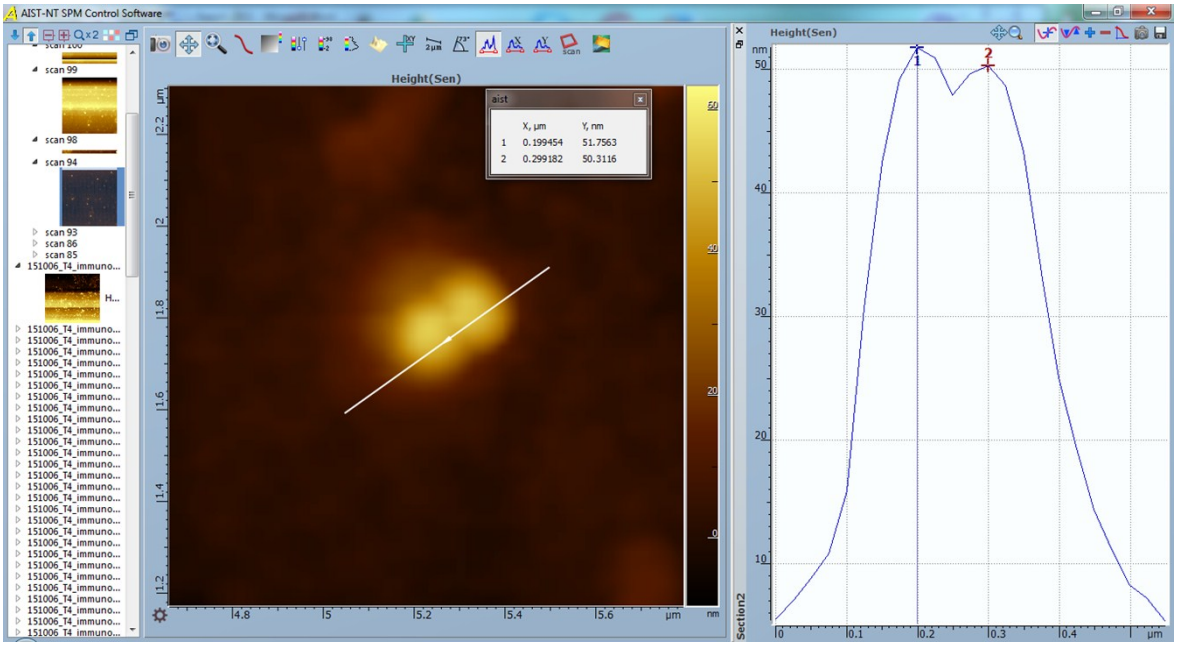


Fig. S19 Screen photo of trimer on AFM map (20x20µm), experiment 5; Sample: dim151204_T4

A



B

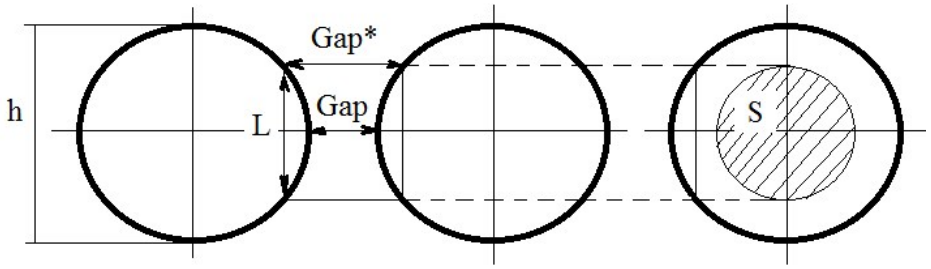


Fig. S20 Determination of the particle size and the distance between NPs (Gap), (A) using AIST-NT Software, (B) using TEM and calculation of mean hot spot cross section (S) between two NPs in dimers calculated from the length L (diameter) of the contact zone between NPs.

Here the interparticle gap is calculated from AFM cross section (on the right) as the difference in x-coordinates of two peaks for each nanoparticle minus the sum of heights of each nanoparticle. Under spherical approximation each height is assumed to be equal to the diameter of each nanoparticle.

UV-vis absorption spectra of prepared sols of NPs.

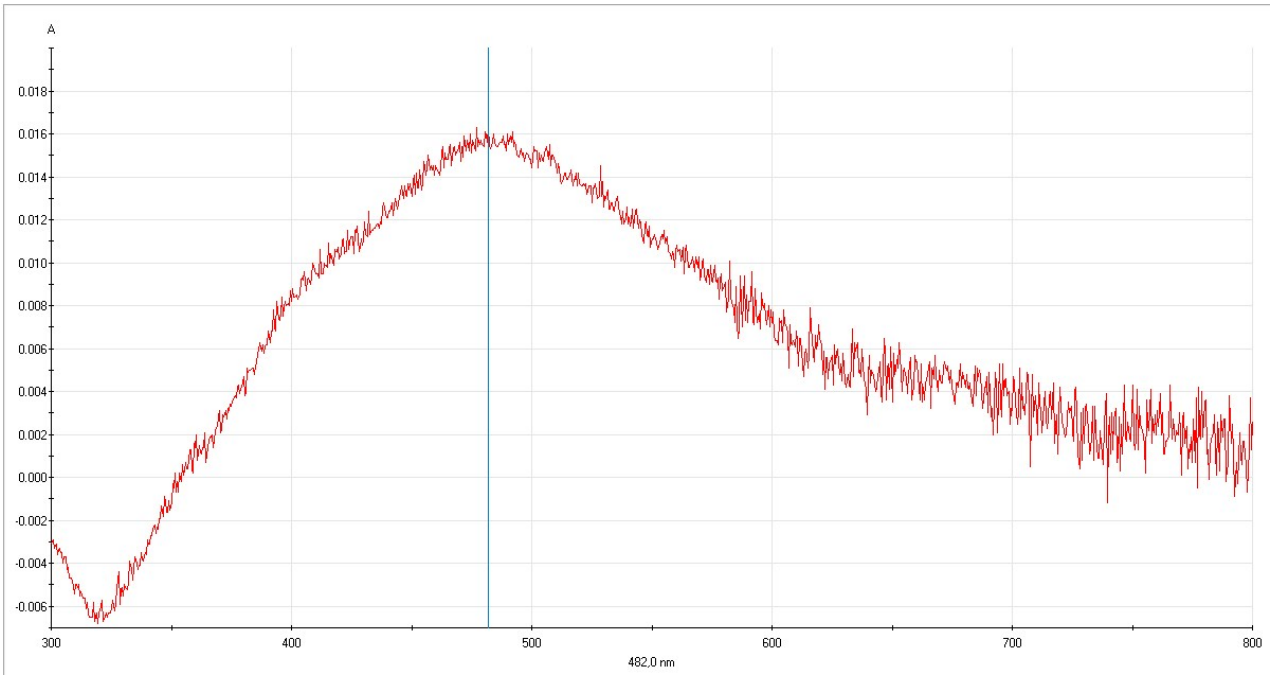


Fig. S21 UV-vis absorption spectra of prepared sol of Ag NPs 100 nm.

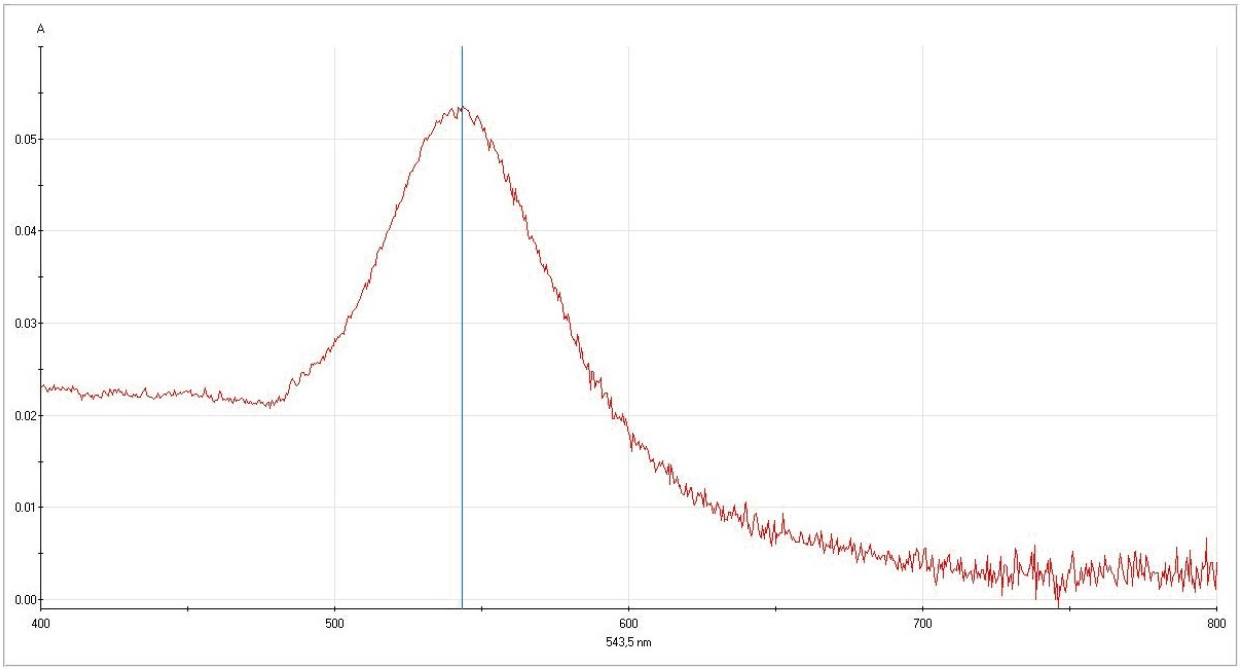


Fig. S22 UV-vis absorption spectra of prepared sol of Au NPs 60 nm.

Measuring of particles size distribution in suspension using dynamic light scattering (DLS).

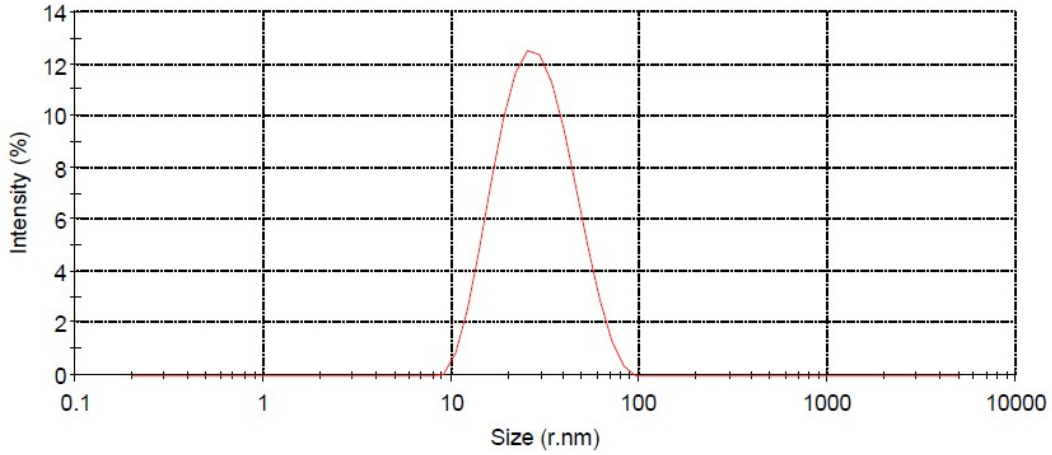


Fig. S23 Size distribution by intensity for suspension Au NPs 60 nm.

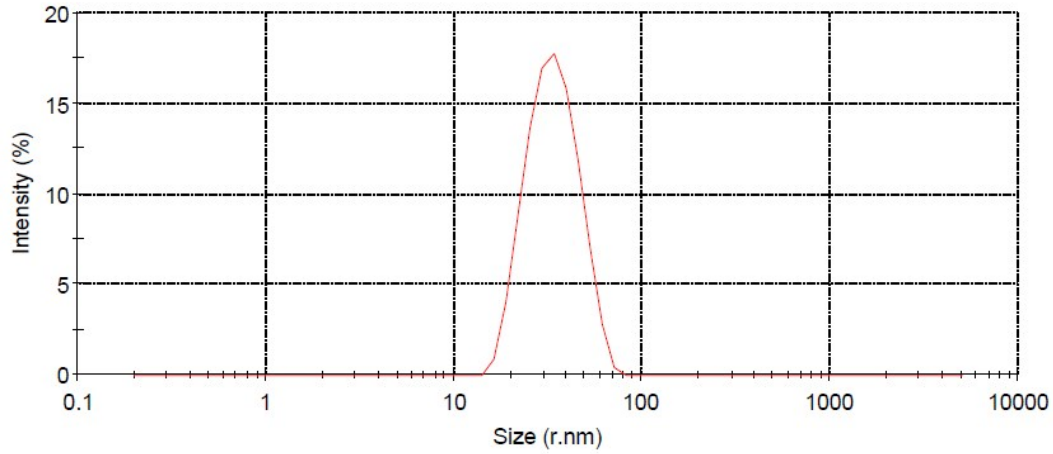
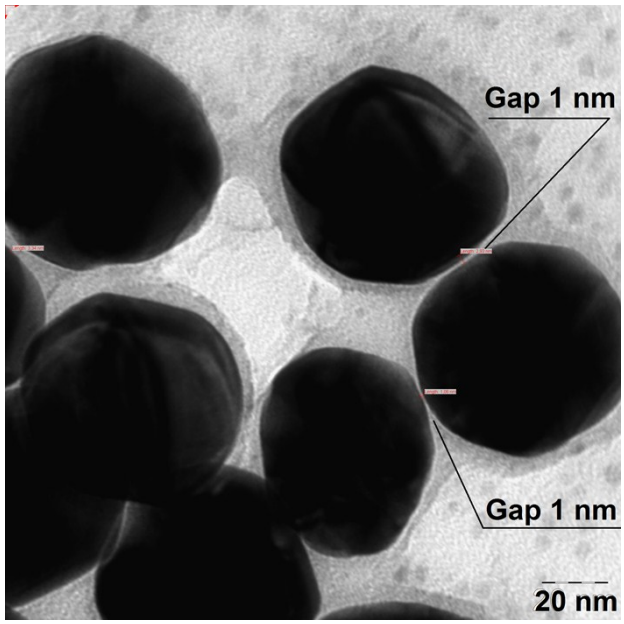


Fig. S24 Size distribution by intensity for ERL suspension (Au NPs 60 nm covered with antibody).

A 

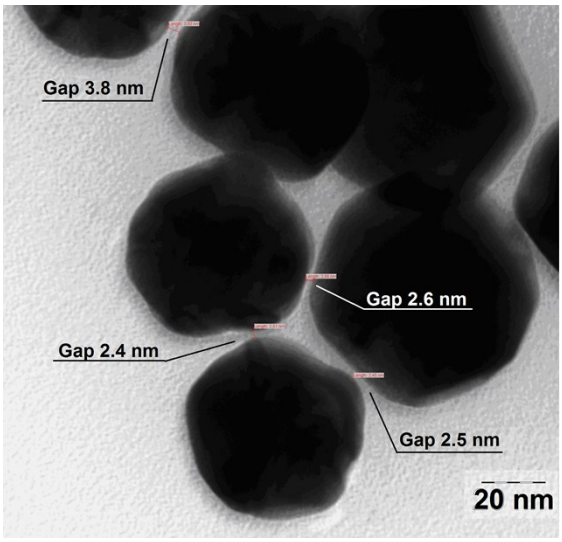
B 

Fig. S25 (A, B) – TEM photo of Au NPs (60 nm) used in immunoassay as ERL on TEM grids.

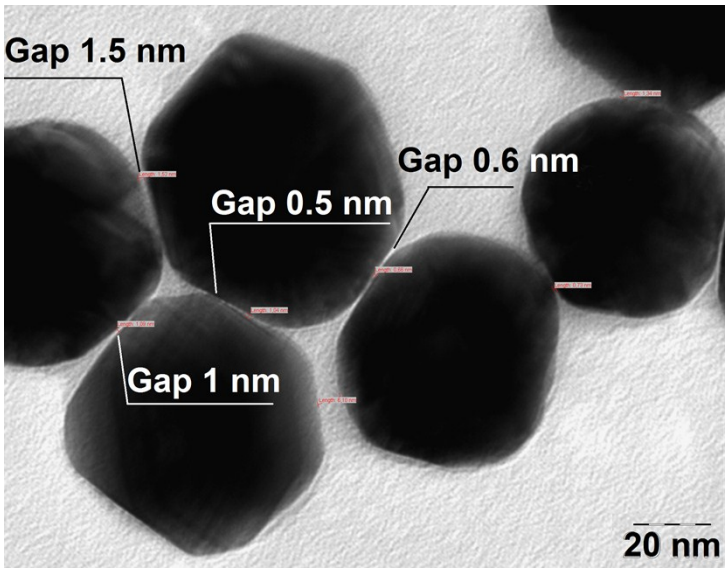


Fig. S26 TEM photo of Au NPs (60 nm) with analite 4-ATP on TEM grids.

More NPs TEM photos can be downloaded from:
https://www.dropbox.com/sh/7zpavdfitarpvbp/AAC7yWdfoUxZ2FkcbqUu0wUsa?dl=0

Table S7. Datasheet for Experiment One (remade with 10% power) observing 2-MOTP modified AuNPs and AgNPs (80 nm) on Au and Ag film.

AuNPs_80nm_Au	Hight(Average)	Intensity	AuNP_80nm_Ag	Hight(Average)	Intensity
M1	77	384	**M1**	58	288
M2	64	775	**M2**	76	740
M3	57	125	**M3**	70	354
M4	76	371	**M4**	72	655
M5	56	135	**M5**	58	871
M6	77	667	**M6**	68	409
M7	66	384	**M7**	71	441
M8	76	329	**M8**	71	355
M9	76	820	**M9**	63	807
M10	85	404	**M10**	61	595
M11	77	836	**D1**	70	1024
M12	75	230	**D2**	78	2249
M13	83	719	**D3**	85	2932
M14	101	848	**D4**	70	2370
M15	84	726	**D5**	65	2539
D1	75	1363	**D6**	72	2088
D2	80	1408	**D7**	85	589
D3	78	2049	**D8**	103	1685
D4	80	2744	**D9**	77	1024
D5	58	610	**D10**	60	2222
D6	70	1154			
D7	73	1275			
D8	93	825			
D9	80	1825			
D10	70	1275			
Substrate	**Gold**			**Silver**	
AuNPs_80nm_Au	**MON**	**DIM**	**AuNPs_80nm_Ag**	**MON**	**DIM**
NPs	15	10	**NPs**	10	10.00
INTENSITY	516	1452	**INTENSITY**	551	1872
STDEV	262	617	**STDEV**	210	764
Background	7.5			5.4	
I BG corrected	509	1445		**546**	1866
Signal per 1mW	807	2269		861	2925
Signal per 1mW BG corrected, I	795	2258		853	2916
Enhancment Factor(EF)	8.70E+06	1.23E+07	**Enhancment Factor(EF)**	9.30E+06	1.60E+07
Conclusion		**Gold**		**Silver**	
I SERS dimer/I SERS monomer		2.84		3.42	

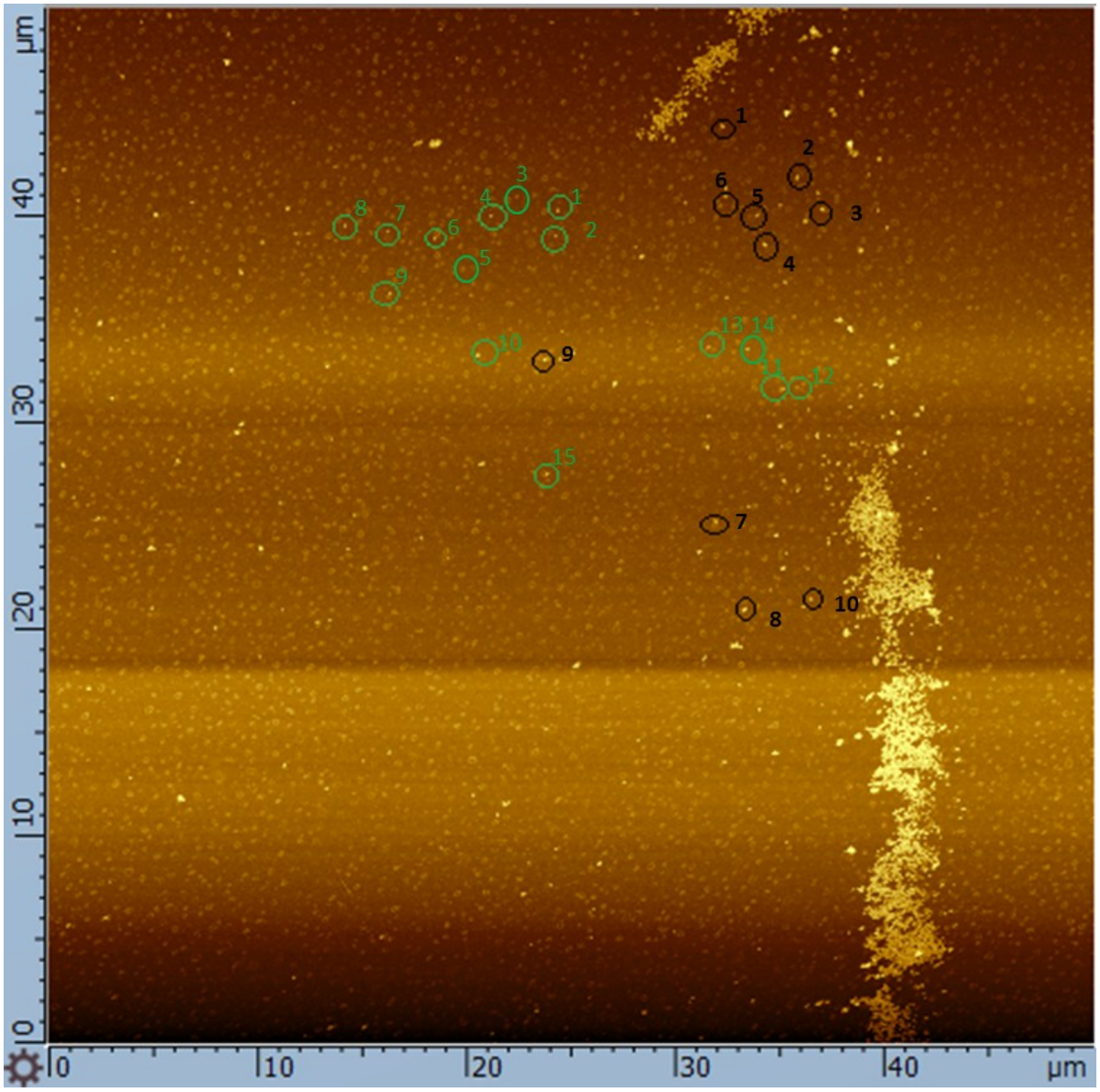


Fig. S27 AFM image AuNPs_80 nm /Au film (experiment One data in Table S6). Monomers are circled in green color, dimmers are circled in black color. This area will be shown on Raman map on the next page

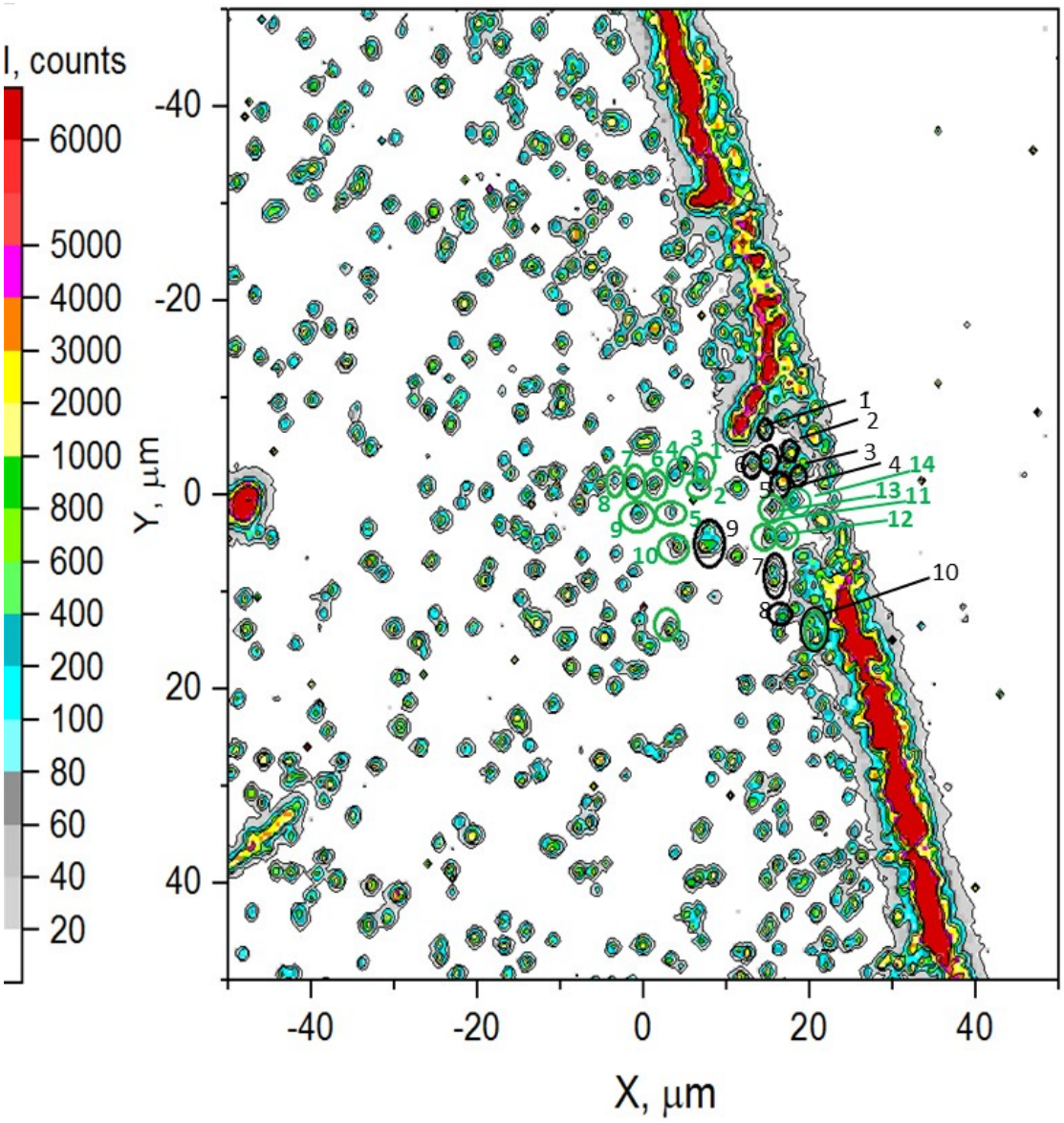


Figure S28. Raman map image (performed by ORIGIN PRO2016) AuNPs_80/Au from Experiment One. Zoomed AFM image is shown on Fig. S27 . Monomers are circled in green color, dimmers are circled in black color.