Electronic Supplementary Information

Non-covalent adsorption of neurotransmission-relevant proteins on locally laser-oxidized and pristine graphene

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Table S1: All conditions and irradiation parameters used. For each sample, each row of the sample had a different irradiation time so that the top row of the sample had the longest irradiation time, and the bottom the shortest and the columns of the sample had different laser powers in increasing order starting from the left and increasing rightwards. The rows and columns of the samples can be identified from Figure S6 as has been illustrated in Figure 1.

Protein	Sample pH	Sample concentration (µg/ml)	Irradiation times (s)	Pulse energies (pJ)
CaM	5	0.025, 0.25	0.4, 0.7, 1.0	4.5, 6.0, 9.0, 12, 14
	5	2.5	0.3. 0.7, 1.0	15, 20, 25, 30
	7	2.5	0.4, 0.7, 1.0	4.5, 6.0, 9.0, 12, 14
	9	2.5	0.4, 0.7, 1.0	10, 15, 20, 25, 30
mAChR	5	0.025	0.4, 0.7, 1.0	4.5, 6.0, 9.0, 12, 14
	5	2.5	0.4, 0.7, 1.0	4.5, 6.0, 9.0, 12, 14
	7	2.5	0.4, 0.7, 1.0	4.0, 6.0, 9.0, 10, 15, 20, 25, 30
	9	2.5	0.4, 0.7, 1.0	10, 15, 20, 25, 30

Supplementary note 1: AFM data analysis

Histograms were extracted from three different parts for each of the oxidized squares as shown in Figure S1. These areas were the surrounding pristine graphene background within a ~5 x 5 μm^2 area (Pr), the 1.5 x 1.5 μm^2 center of the oxidized square (2PO) and the whole combined ~5 x 5 μm^2 area. The histograms provided by the processing software were already normalized so that the sum of the bins equaled to 100 %. These histograms were then processed with a self-written Python script that did the steps described below.

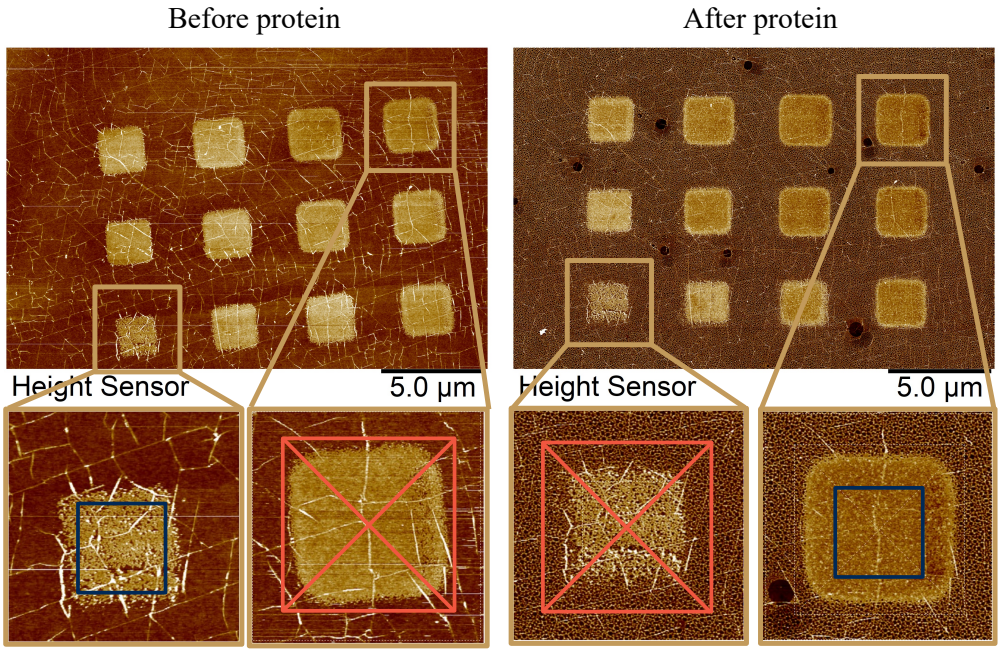


Figure S1: Illustration of where the histograms used in the analysis have been extracted. Area within the square with golden edges is the whole area (combined). The combined size was usually between 4 x 4 μm^2 and 5 x 5 μm^2. When the area crossed out with orange was removed from combined, the resulting area is the pristine. The crossed-out area was typically in the range of 3 x 3 μm^2 to 4 x 4 μm^2. The dark blue square is the area that was used as the 2PO square (always approximately 1.5 x 1.5 μm^2 in size).

The first step was to do a background correction for each of the histograms for both states of the sample (before and after protein). This was done so that both states would have their background at the same height, which was needed for comparing the different states. To do the correction, a Gaussian fit was done to the Pr histogram and the center of the fit was set to zero. (Figure S2(a)) Zero was artificially chosen as it simplified the extraction of all the other height values later in the analysis. When correcting after protein histograms, an additional Gaussian

was used in the fit to account for the protein distribution. (Figure S2(b)) The same adjustment that was done for the Pr was also made for 2PO and combined. (Figure S2(c)) A separate fit and adjustment was done for each square and state as, intrinsically, the absolute value of the background in AFM data changed based on the processing. This could, for example, be due to imperfect flattening or plane fitting.

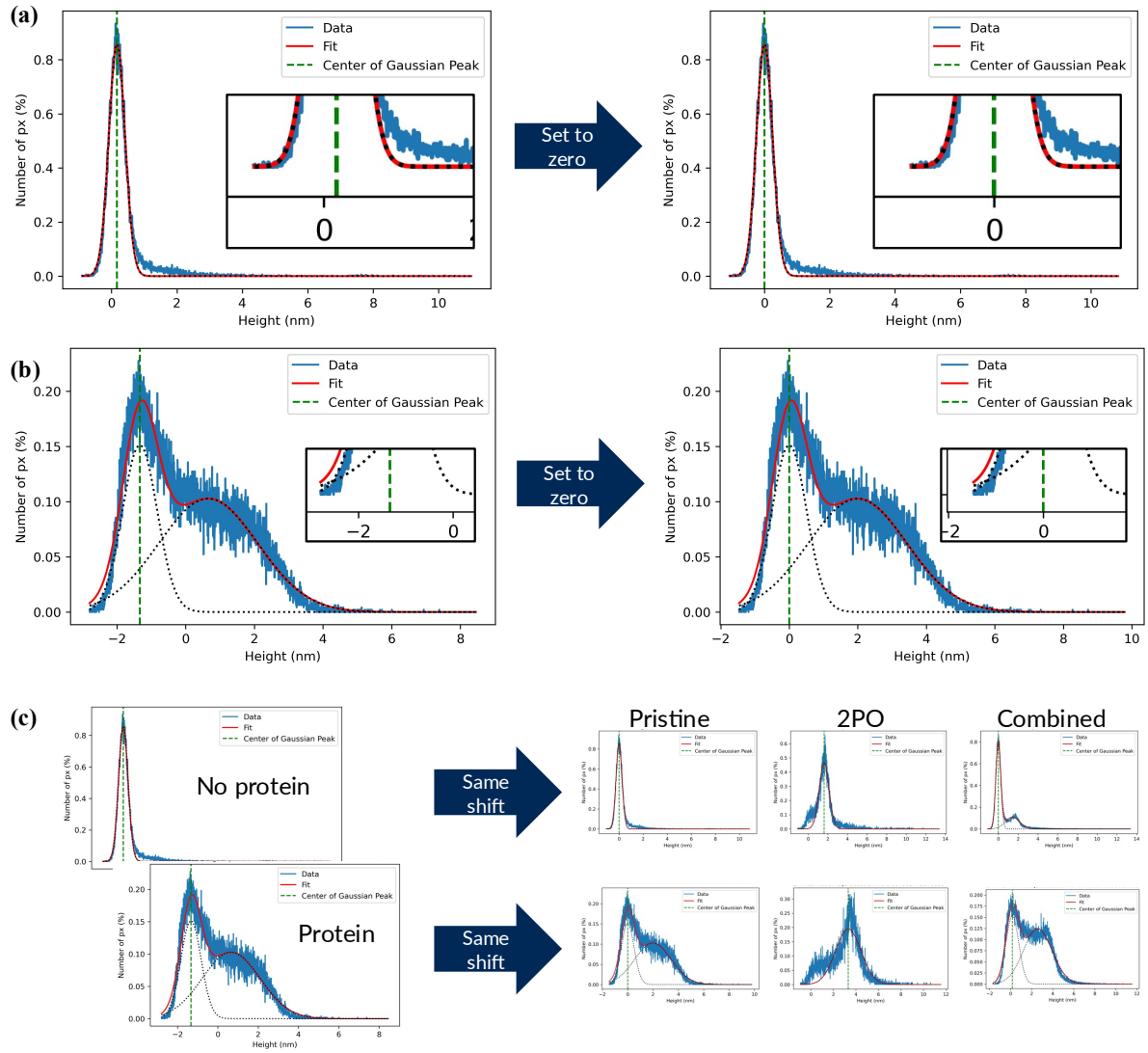


Figure S2:Illustrative graphs of the background correction. Left side of the arrows is the initial situation and right the situation after the processing step. **(a)** is the Pr graphene before incubation and **(b)** the same area after incubation. **(c)** shows all the height distributions where the background correction value gained from a single fit of the Pr is applied. (Separate fit for the surface with and without protein)

The second step was to make it possible to compare the histograms easily. This was achieved by normalizing the histograms so that they had the same bin size and that the sum of the bins was still maintained at 100 %. (Figure S3)

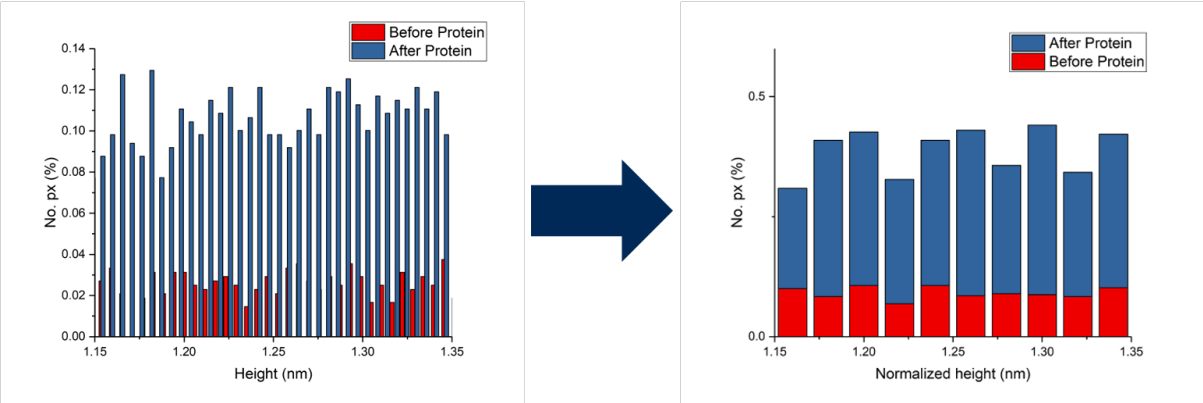


Figure S3:Illustrative graphs of the histogram normalization. On the left the histograms before normalization and on the right after the normalization.

Third, the overlap between the before and after protein histograms was calculated. The resulting histogram is marked in green in Figure S5. The sum of the bins of this new overlap histogram gives the percentage of how many pixels were not changed, as there was no protein deposition on them.

Fourth, a differential histogram was calculated from the after-protein histogram and overlap histogram (subtraction between the after and overlap histograms, Figure S4). Here we ignored the pixels that might be present at lower values than what the original histogram had prior to protein incubation. Those values were most likely due to imperfect flattening due to the two different distributions present on the pristine graphene. This differential histogram represents only the pixels that have had deposition on them. Therefore, the sum of this histogram corresponds to the coverage percentage. By performing a Gaussian fit to the histogram, the average height and standard deviation of the deposited proteins could be determined. As the background was set to zero, the values extracted here from the Pr required no further correction. However, the average determined from the differential histogram from the 2PO area needed to be corrected by the average height of the oxidized square before the protein deposition. In the cases when more than one level of height distributions was present in the 2PO (island-like oxidation), the different regions between the maxima of these distributions were treated separately.

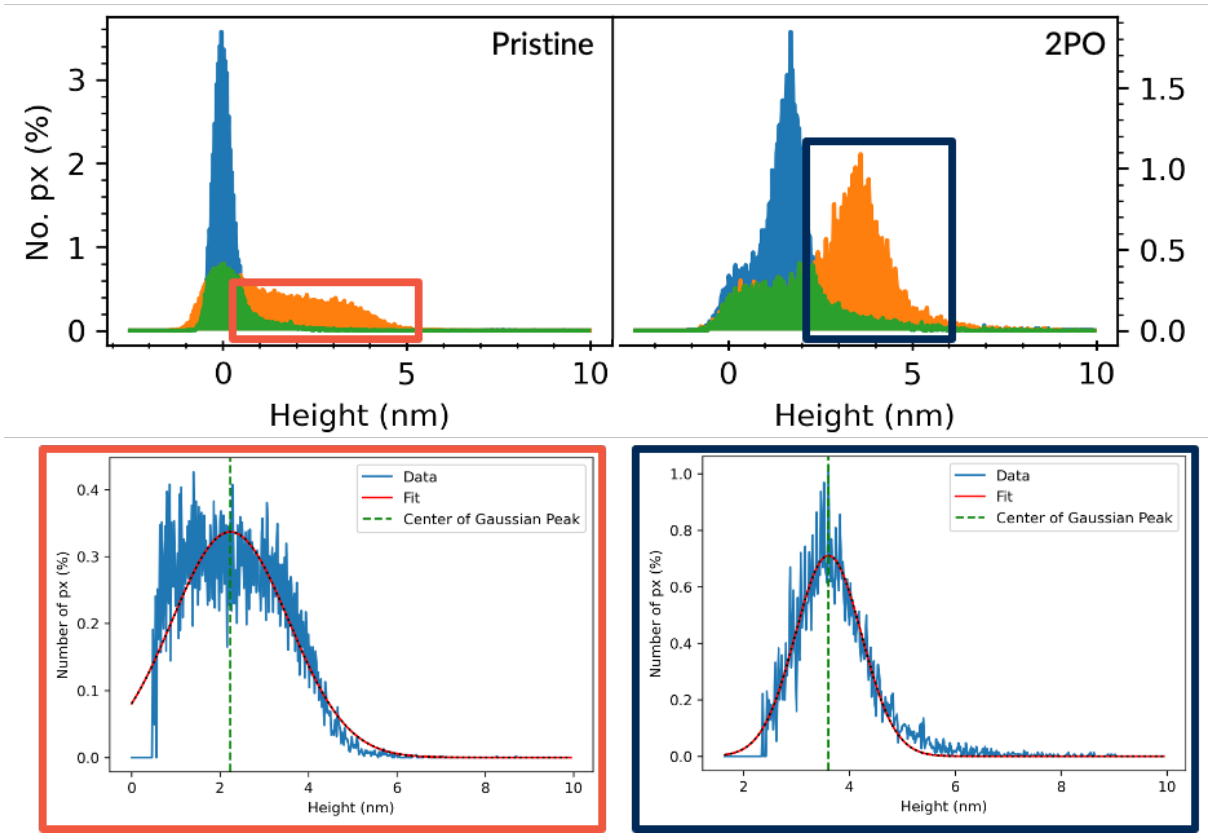


Figure S4: Illustrative graphs of the differential histogram. Top has the Pristine and 2PO histograms which are presented Figure S5 and the part of the distributions that forms the differential histogram below the graph is highlighted with a color-coded box.

On the reliability of this method

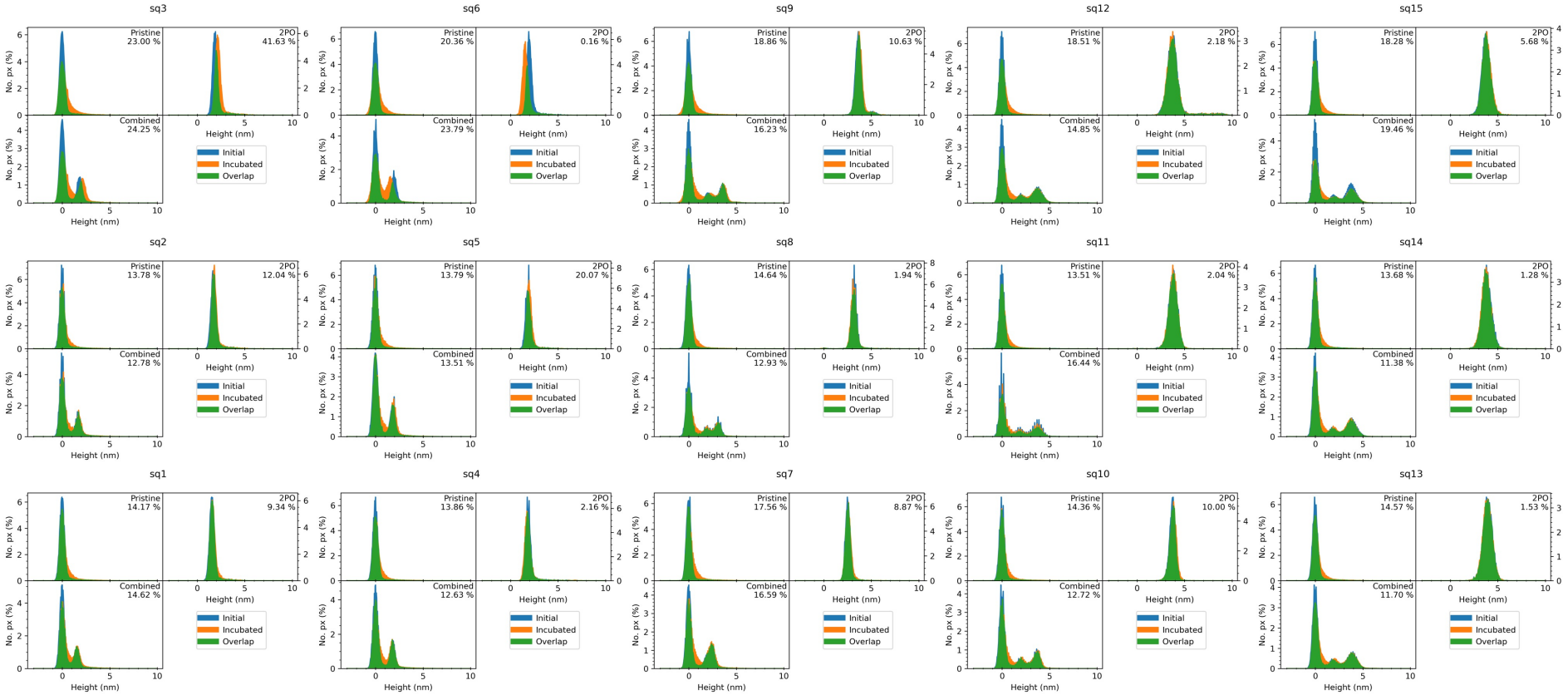
The weakness of this method is that it does not look at each pixel individually, but the chosen area as a whole. For example, it is possible that if the initial histogram has several peaks or one of the pixels on the left side of the original Pr histogram has had deposition on it, but only so little that its height is now on the right side of the area that is covered by the original Pr histogram. In this situation, the pixel would be incorrectly determined to not have changed in height and therefore, the distribution used for determining the protein height is affected and a lower amount of deposition is detected. Also, since this method uses several fits, the fit error could cause some degree of inaccuracy. For example, with a very low concentration or adsorption, it was not possible to do a reliable fit, so detecting a bad fit and manually setting the value to zero (no adsorption nor coverage) was sometimes needed.

However, if we inspect the whole histogram, this method is superior to its established alternatives in sensitivity, accuracy, and reproducibility. Using, for example, typical bearing analysis to determine the coverage percentage would introduce a significant human error and reduce the reproducibility of the analysis. Typically, the threshold from which the coverage percentage is calculated is determined by eye or some calculation based on the average heights of the background and what is on top of it. Also, this threshold is a hard vertical wall. As both the background and proteins presented a clear a gaussian distribution, using a simple threshold, one cannot distinguish the pixels where the protein distribution and the background distribution are overlapping. The method used here, instead, did take this into account, and in our belief is therefore a more reliable and accurate alternative. Additionally, bearing analysis cannot be used at all for cases where the initial histogram has more than one level of height distributions (*e.g.*, combined).

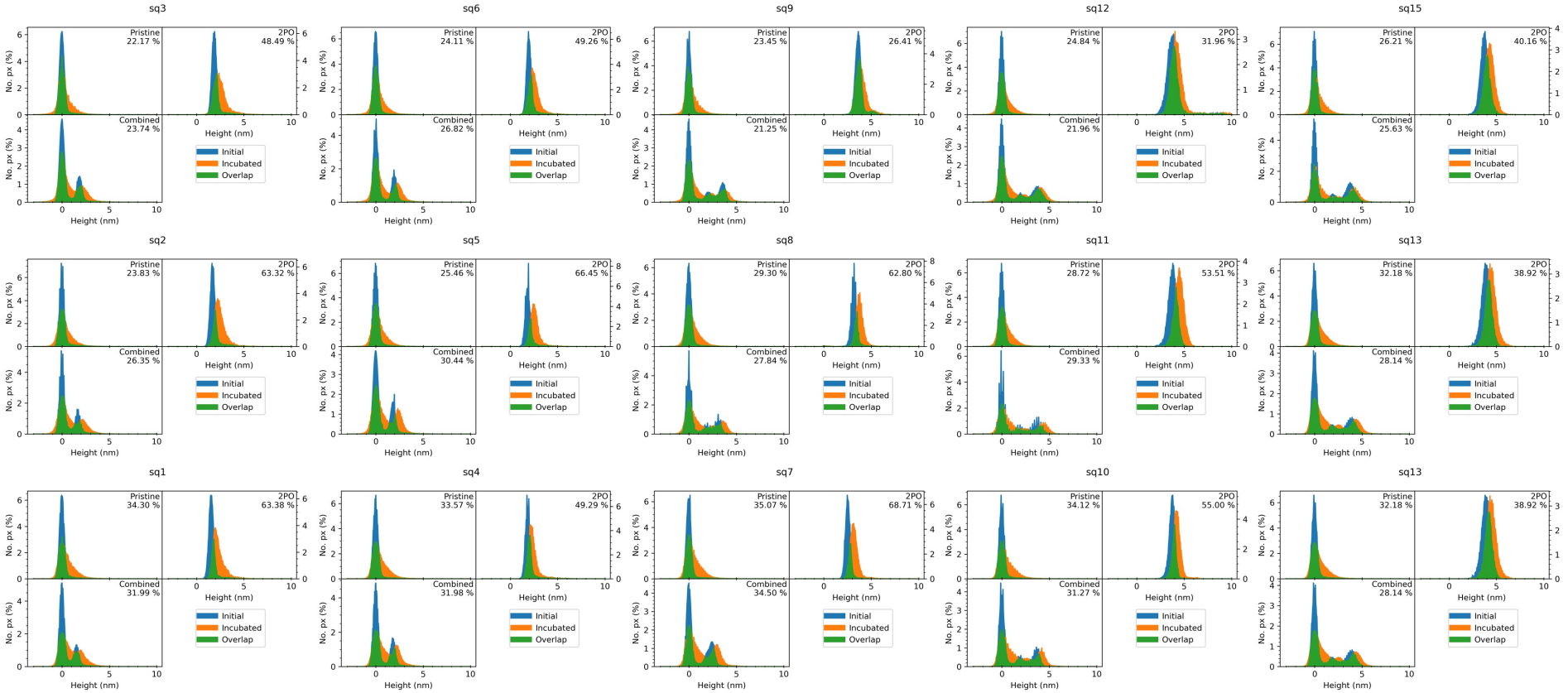
In an ideal case, a pixel-perfect aligned pair of AFM images with before-and-after states would be compared pixel by pixel. This level of accuracy in the alignment with AFM is near impossible, as many intrinsic properties of the imaging method prevent that. Minuscule drift in the piezoelectric motors controlling the scanning head, accumulation of particles onto the tip, the physical dimensions of the tip, or rotation of the sample can all cause distortion in the X and Y dimensions leading to some variation when comparing two scans at a pixel-by-pixel level. Therefore, utilizing histograms is a more realistic way to do this analysis.

All in all, the weaknesses of this method are very much related to the nature of the AFM data, and the procedures (flattening etc.) done to the data before the actual analysis. Those would be present in any analysis method utilized. But as the method used here removes many additional sources of error, we see it as a valid and valuable analysis method for these purposes. Additionally, in cases where analysis error was suspected, the histograms and their corresponding values were inspected manually to confirm the reliability of the analysis.

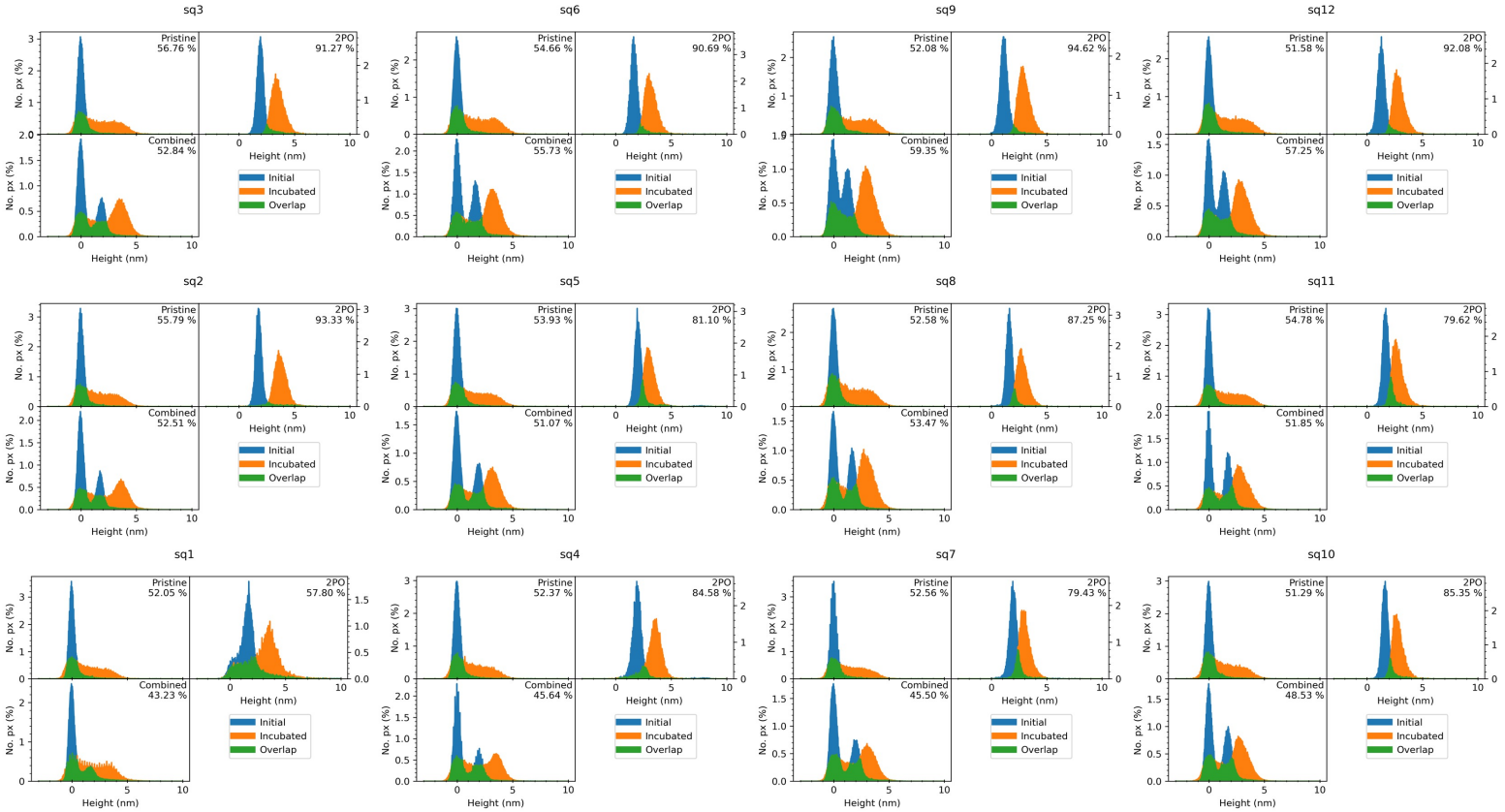
a) CaM pH 5, 0.025 µg/ml



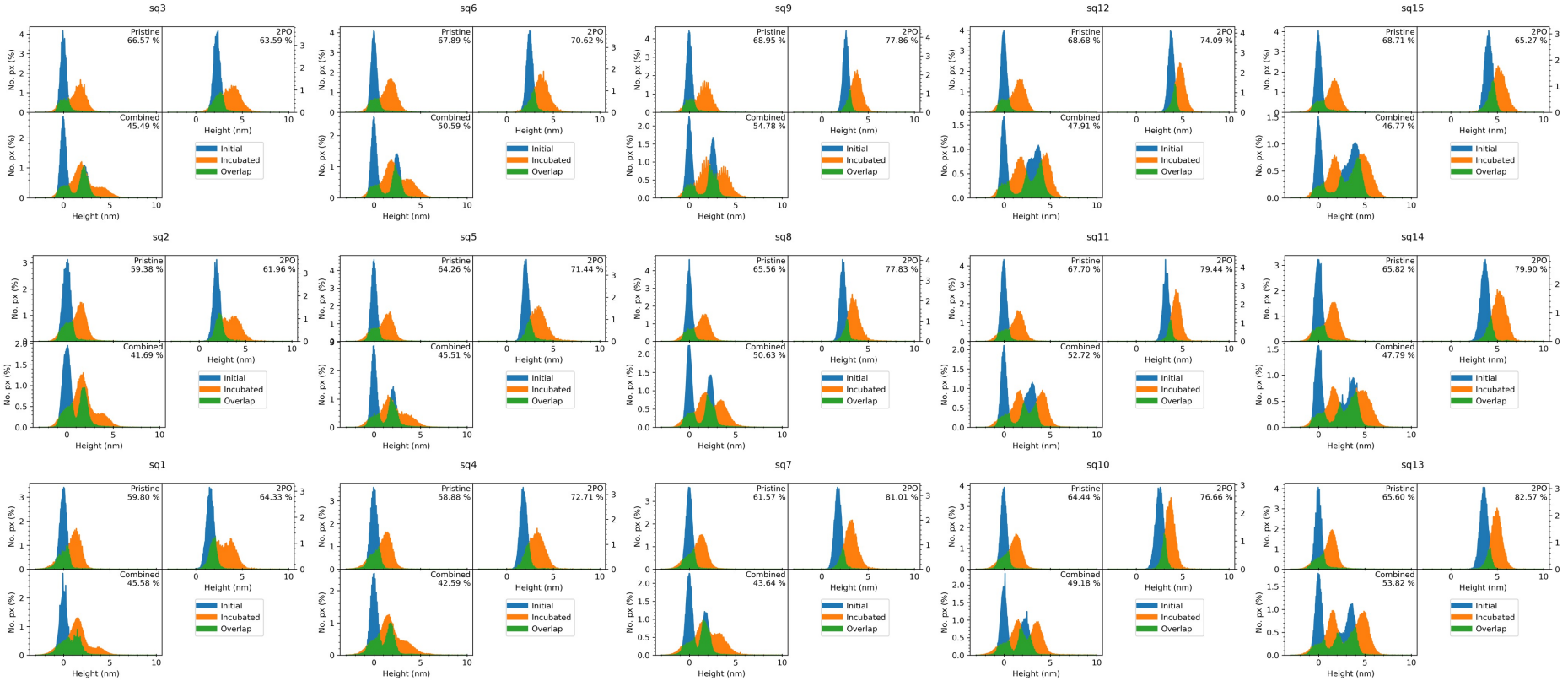
b) CaM pH 5, 0.25 µg/ml



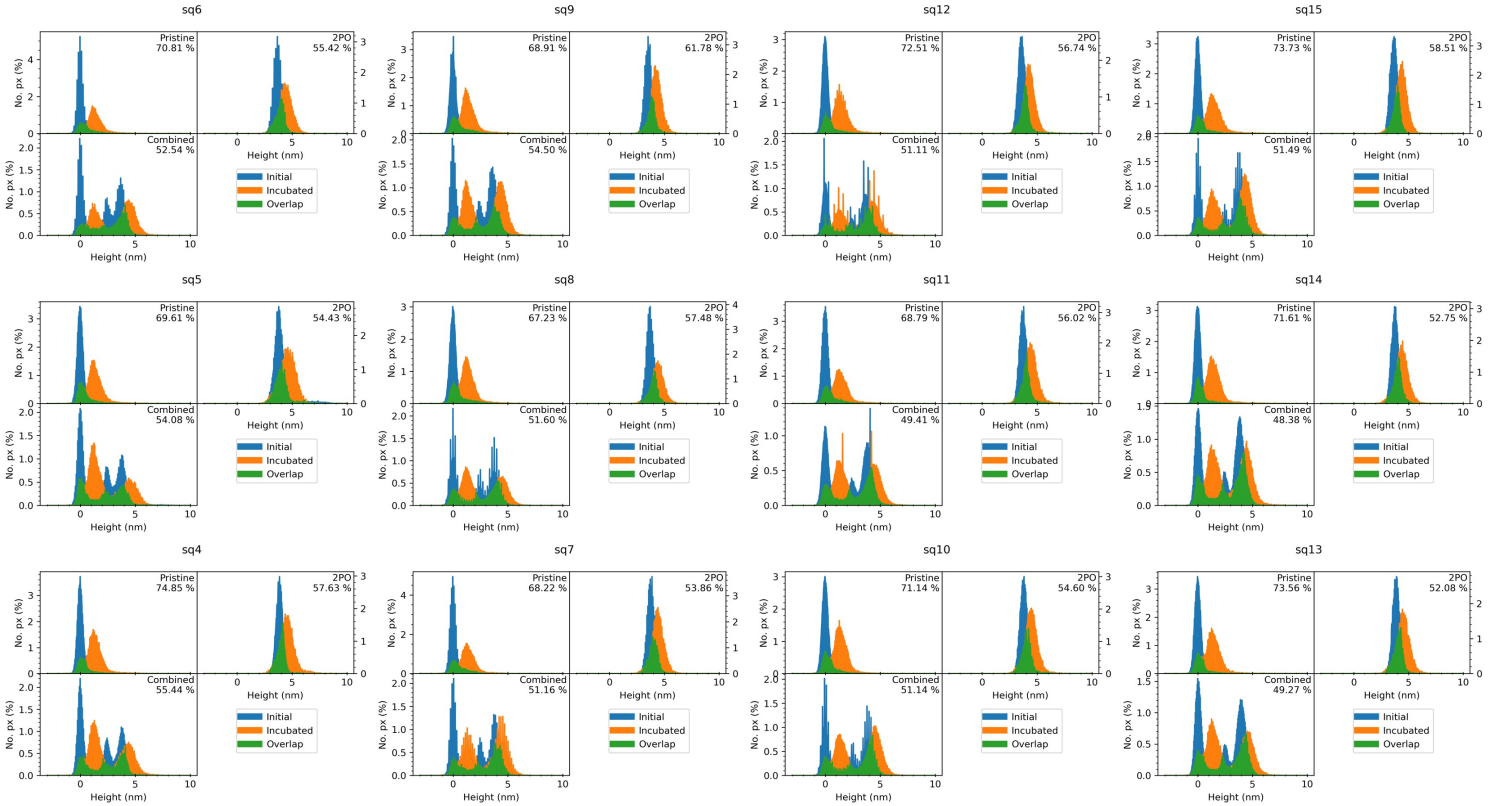
c) CaM pH 5, 2.5 µg/ml



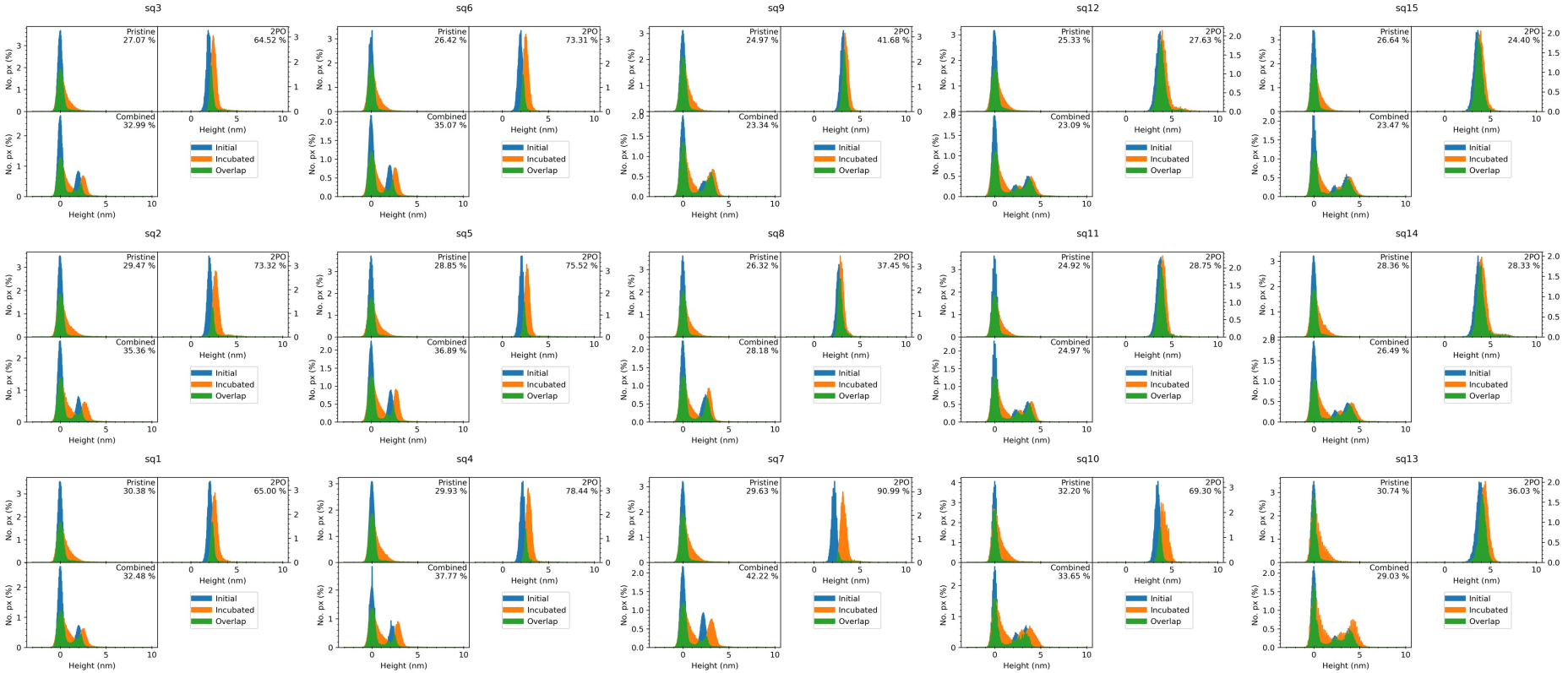
d) CaM pH 7, 2.5 µg/ml



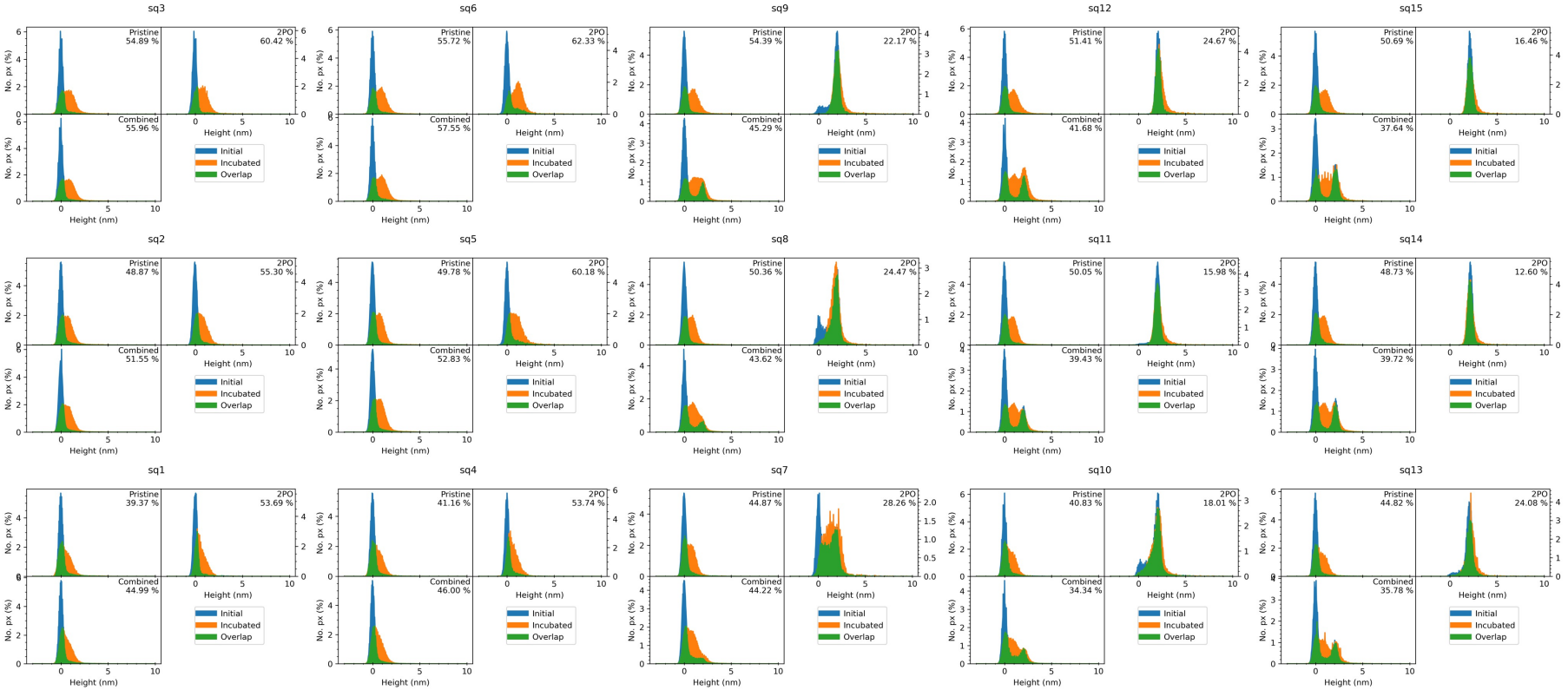
e) CaM pH 9, 2.5 µg/ml



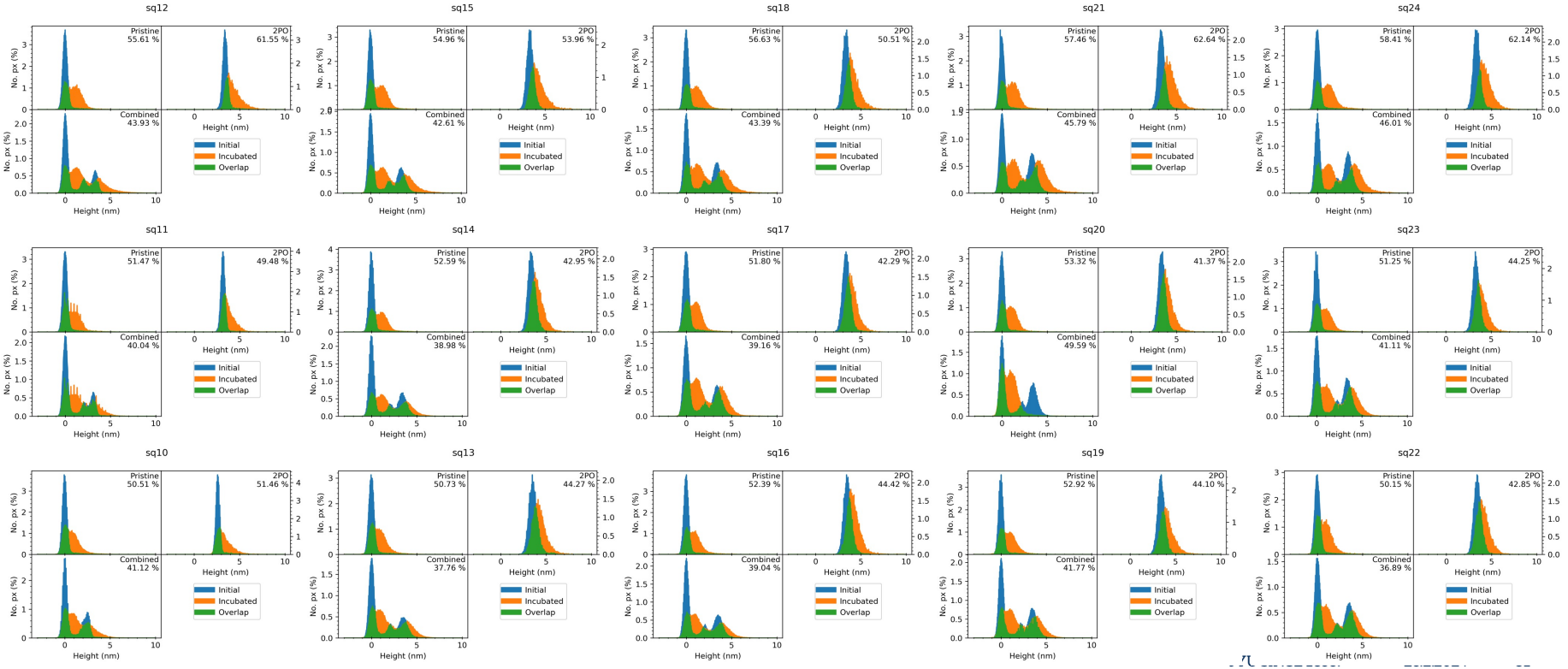
f) mAchR pH 5, 0.025 µg/ml

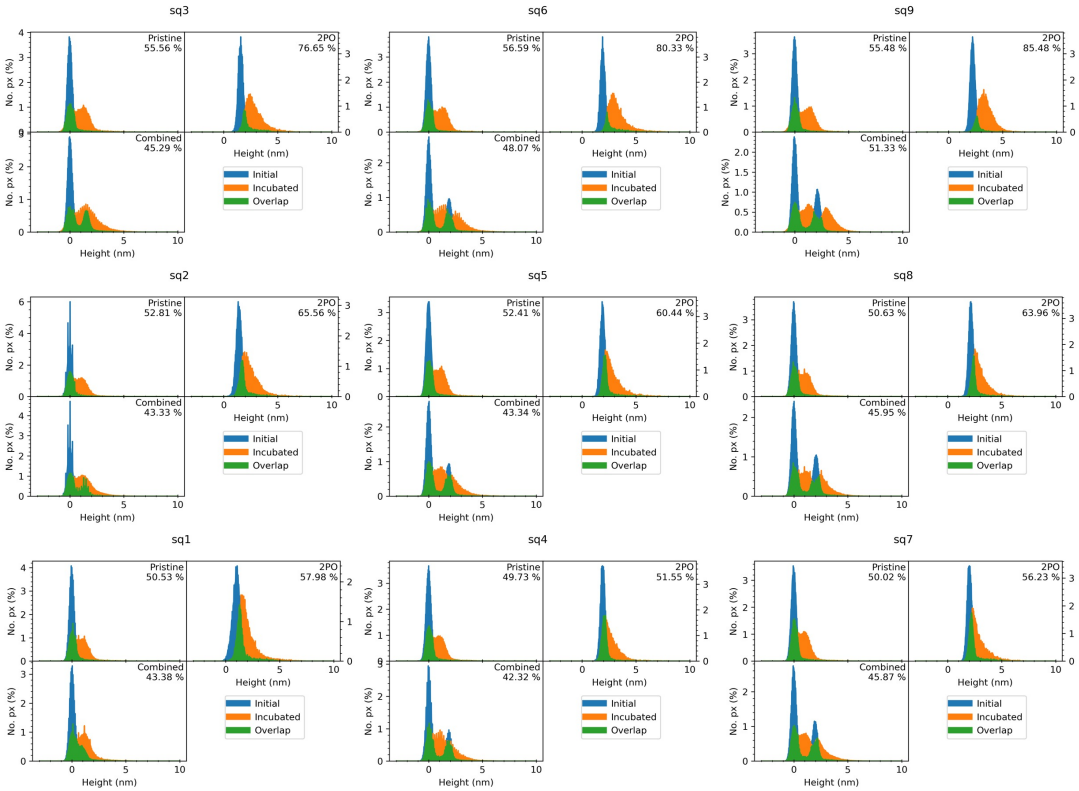


g) mAchR pH 5, 2.5 µg/ml



h) mAchR pH 7, 2.5 µg/ml




sq3
Pristine
55.56 %
2PO
76.65 %
Combined
45.29 %
sq6
Pristine
56.59 %
2PO
80.33 %
Combined
48.07 %
sq9
Pristine
55.48 %
2PO
85.48 %
Combined
51.33 %
sq2
Pristine
52.81 %
2PO
65.56 %
Combined
43.33 %
sq5
Pristine
52.41 %
2PO
60.44 %
Combined
43.34 %
sq8
Pristine
50.63 %
2PO
63.96 %
Combined
45.95 %
sq1
Pristine
50.53 %
2PO
57.98 %
Combined
43.38 %
sq4
Pristine
49.73 %
2PO
51.55 %
Combined
42.32 %
sq7
Pristine
50.02 %
2PO
56.23 %
Combined
45.87 %
No. px (%)
Height (nm)
Initial
Incubated
Overlap

i) mAchR pH 9, 2.5 µg/ml

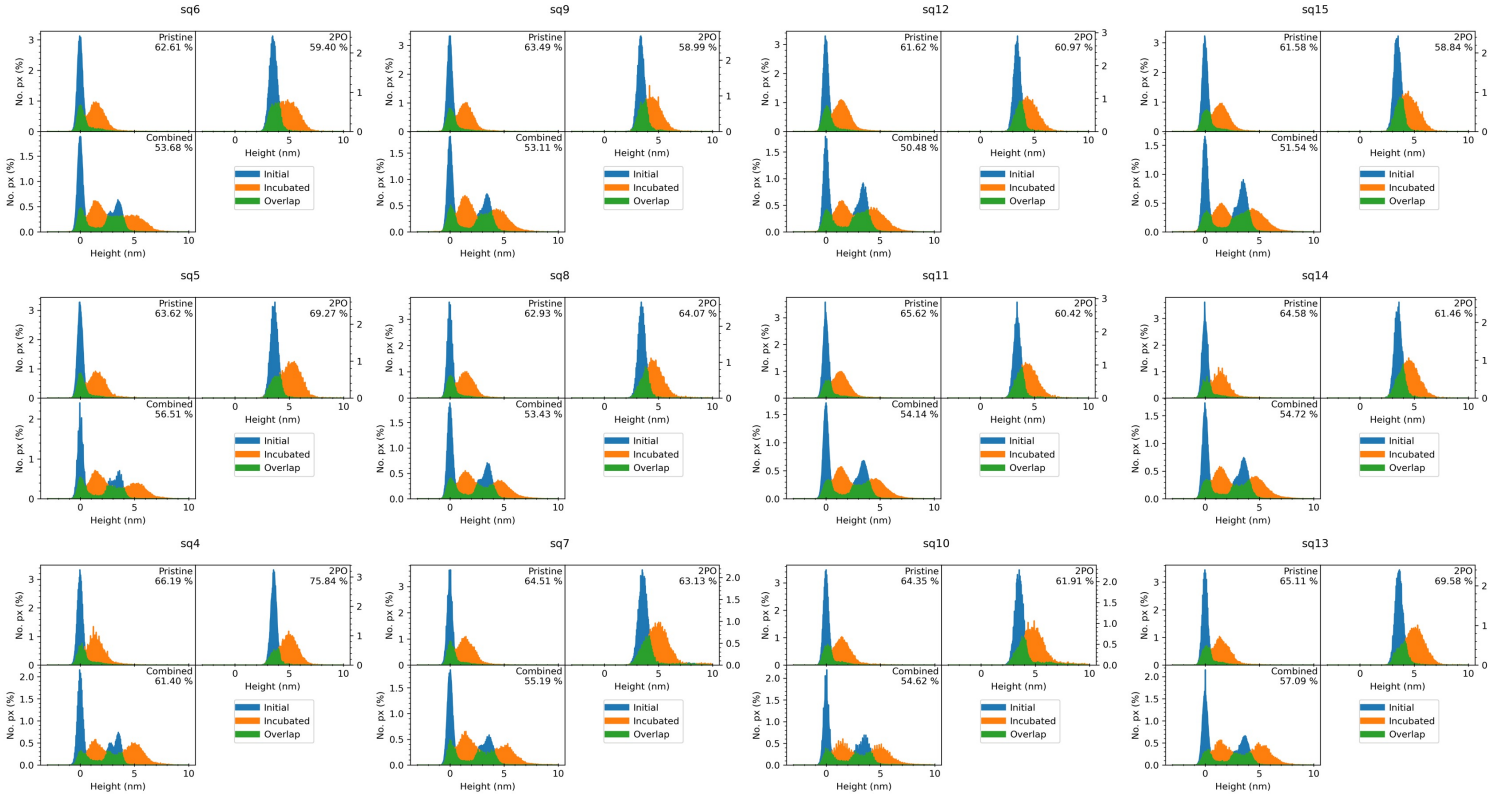
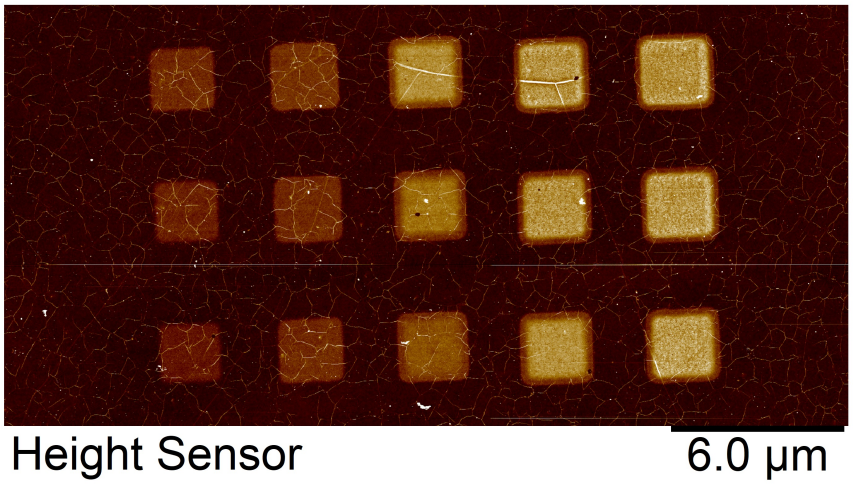
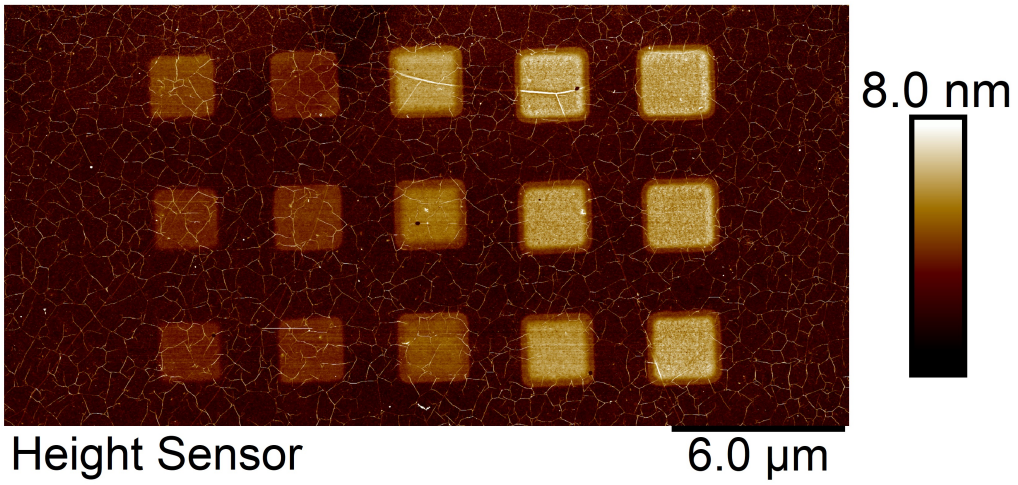


Figure S5: Height distribution histograms extracted from the AFM data for each square of each sample and each state. CaM in **a)** pH 5, 0.025 µg/ml, **b)** pH 5, 0.25 µg/ml, **c)** pH 5, 2.5 µg/ml, **d)** pH 7, 2.5 µg/ml, **e)** pH 9, 2.5 µg/ml, and mAchR in **f)** pH 5, 0.025 µg/ml, **g)** pH 5, 2.5 µg/ml, **h)** pH 7, 2.5 µg/ml, **i)** pH 9, 2.5 µg/ml. Note that **a)** and **b)** are the same sample incubated twice, one set of histograms per incubation. The groups of three figures are in the same places as their corresponding squares in the AFM scans and are labelled with their corresponding square ID number (sq#). Each group has a figure depicting the height distributions of pristine graphene surrounding the oxidized area (pristine), 2PO square, and their combined area. The combined histograms are shown to show that the background correction has been successful and to highlight that if the separation

between the pristine and 2PO areas is not done, a significant error in the corresponding values is introduced, as can be seen from the corresponding coverage percentage values. Each figure has a percentage value representing the calculated coverage percentage and three histograms that are the same area before protein incubation (Initial), after the incubation (Incubated) and the calculated overlap between these two histograms (Overlap).

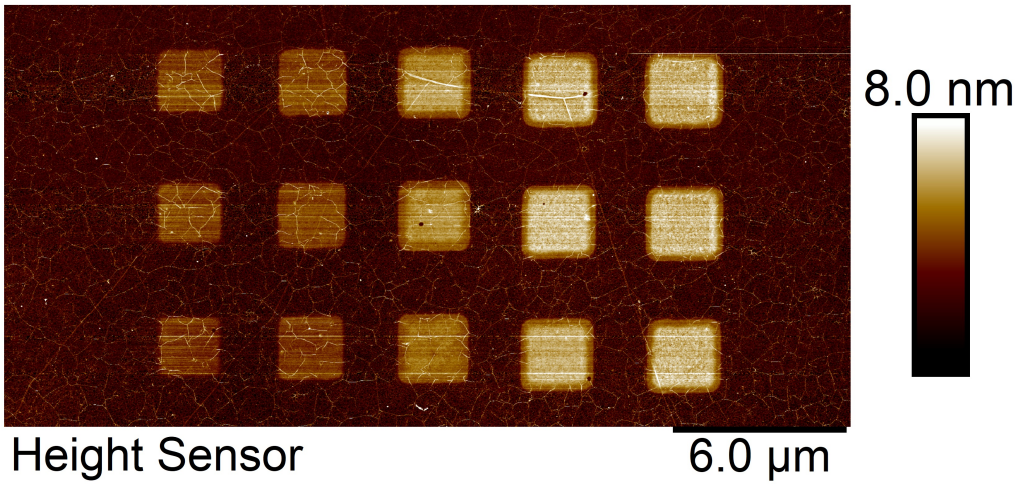
a) CaM pH 5, 0.025 µg/ml



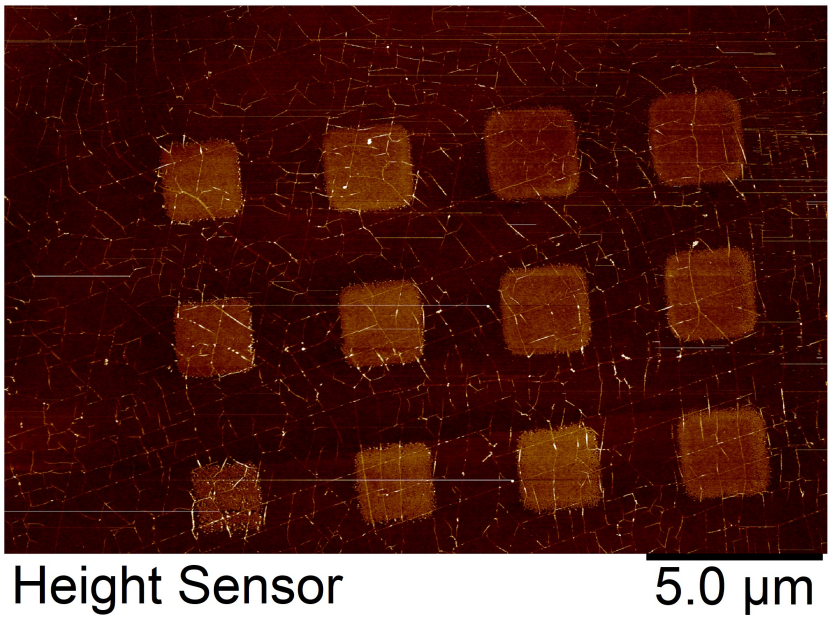


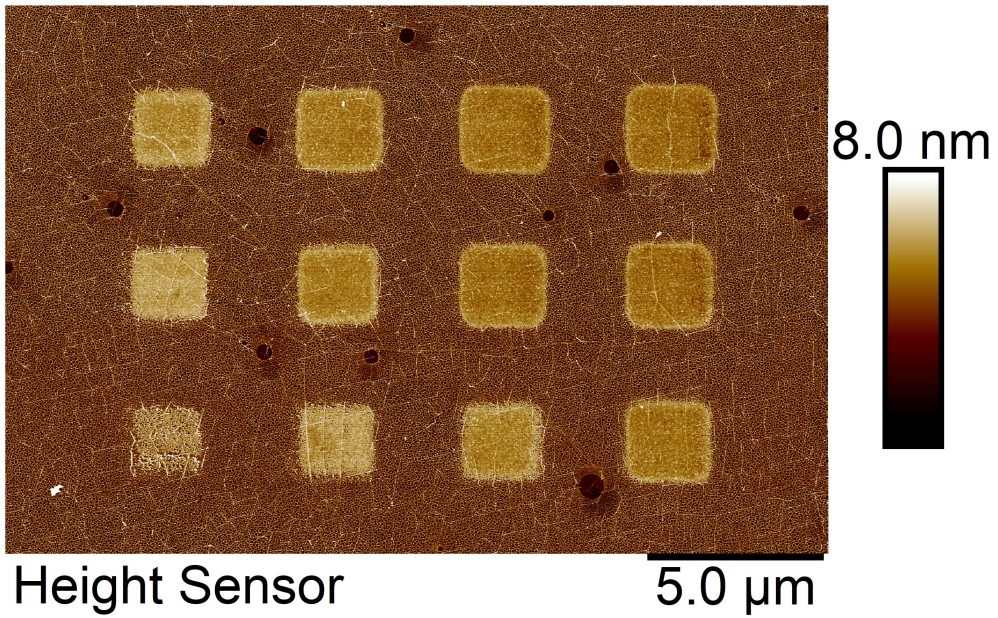
b) CaM pH 5, 0.25 µg/ml

Same as above

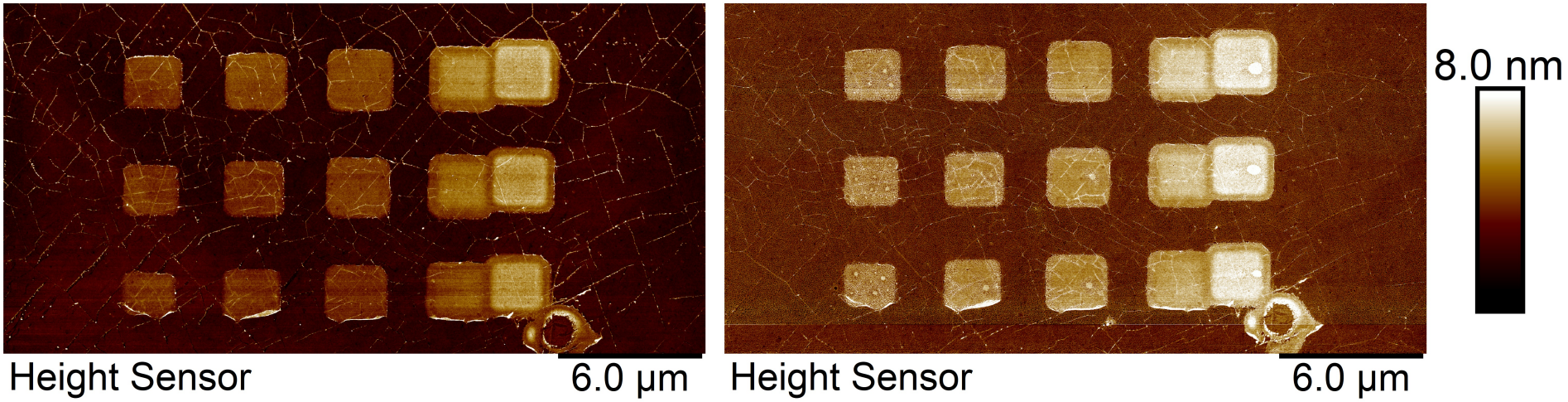


c) CaM pH 5, 2.5 µg/ml

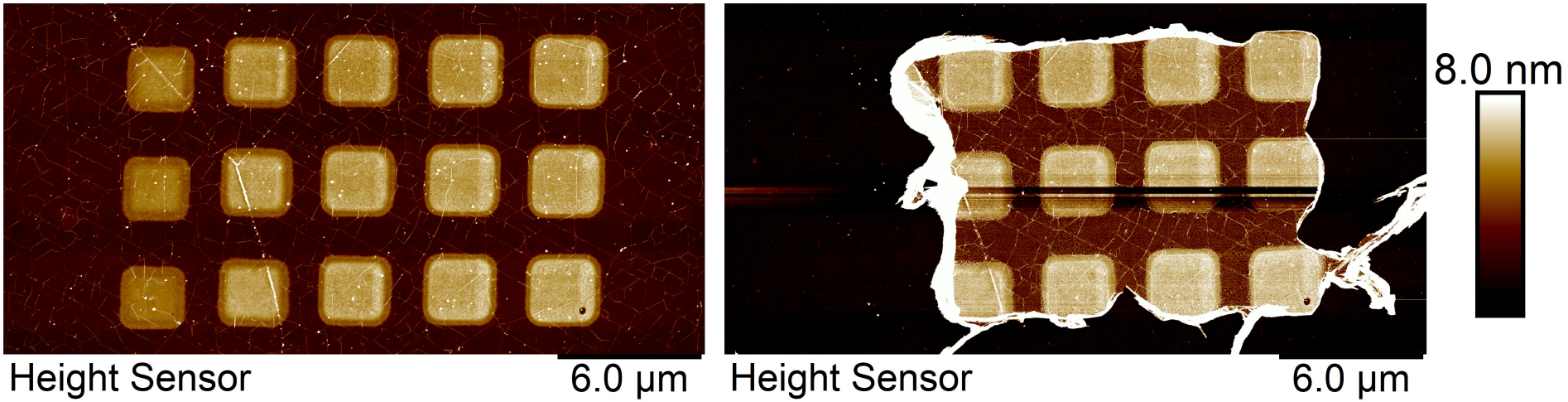




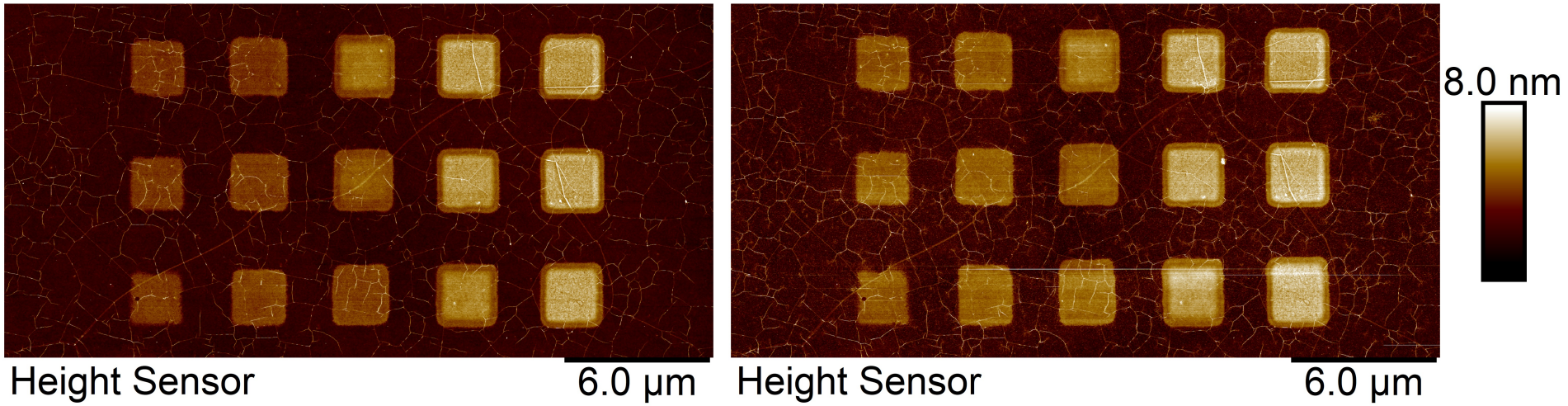
d) CaM pH 7, 2.5 µg/ml



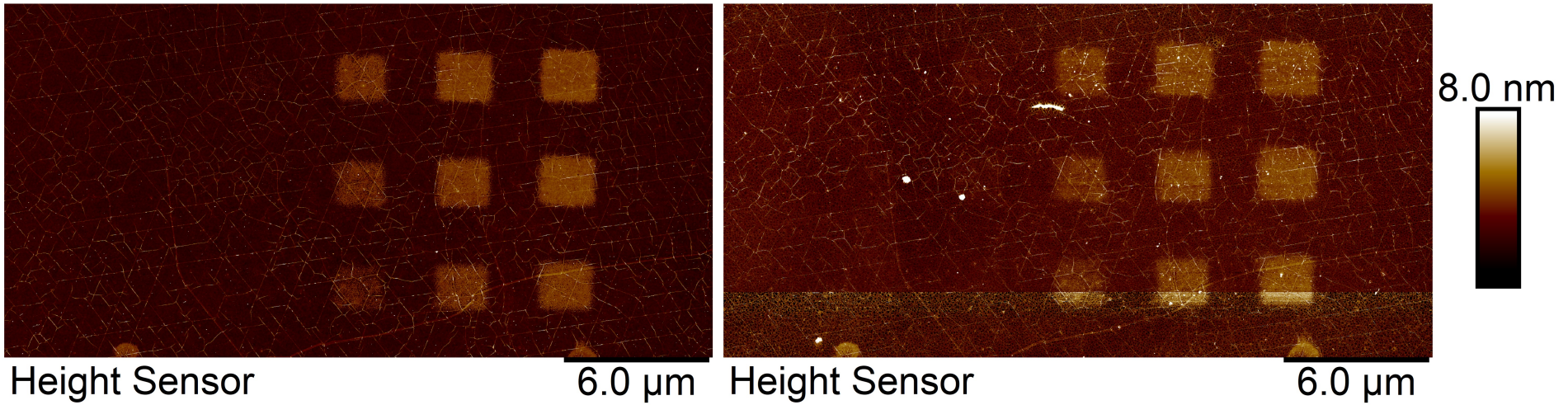
e) CaM pH 9, 2.5 µg/ml



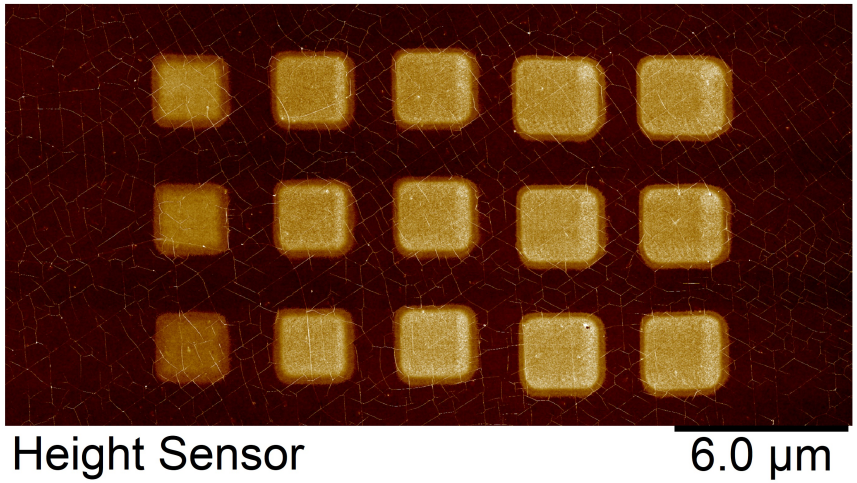
f) mAchR pH 5, 0.025 µg/ml

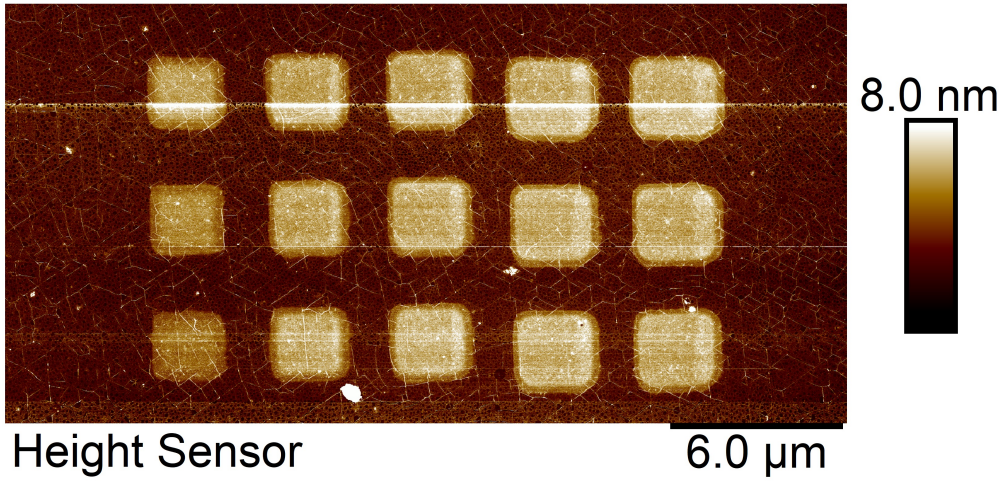


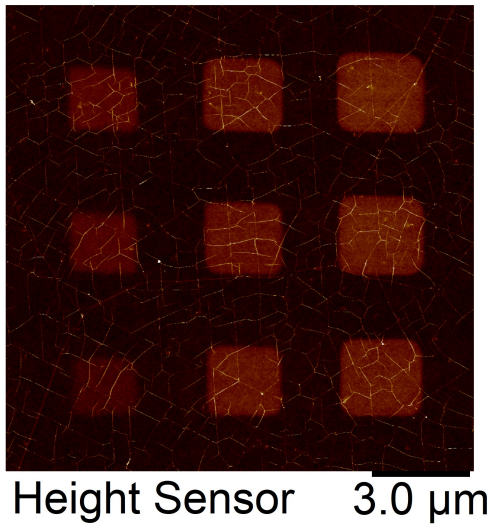
g) mAchR pH 5, 2.5 µg/ml

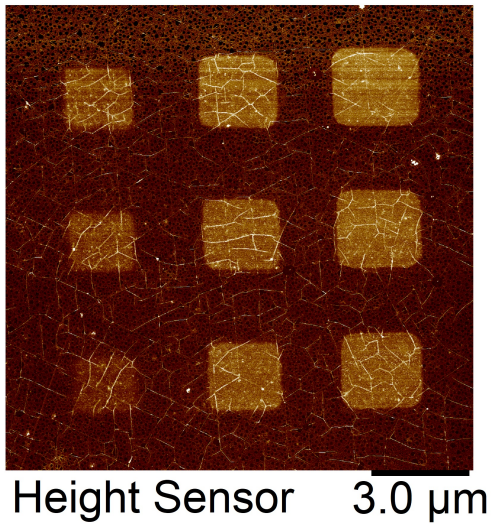


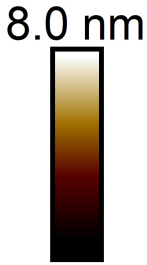
h) mAchR pH 7, 2.5 µg/ml











i) mAchR pH 9, 2.5 µg/ml

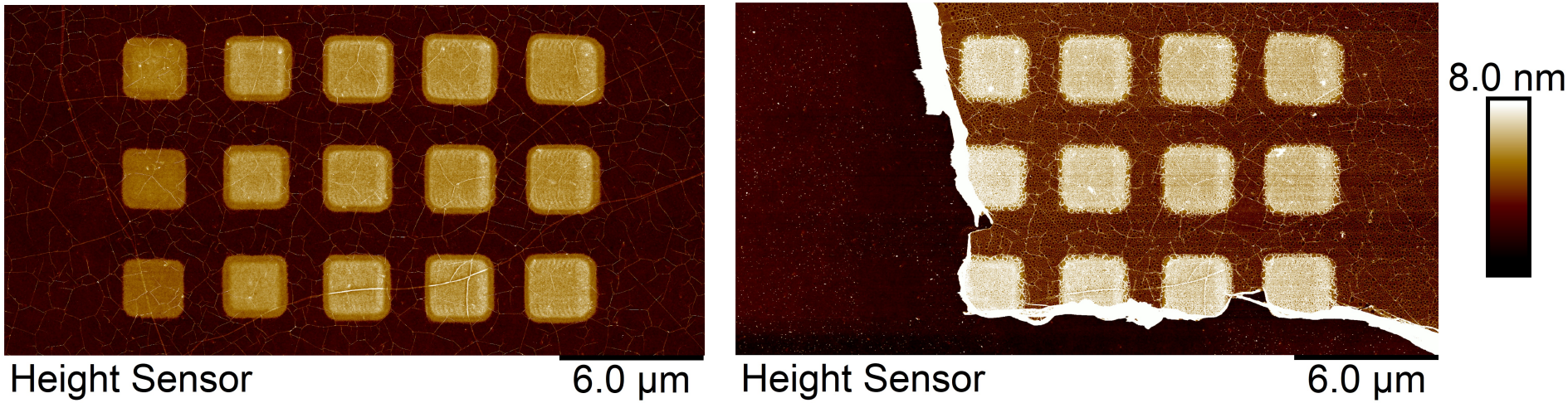
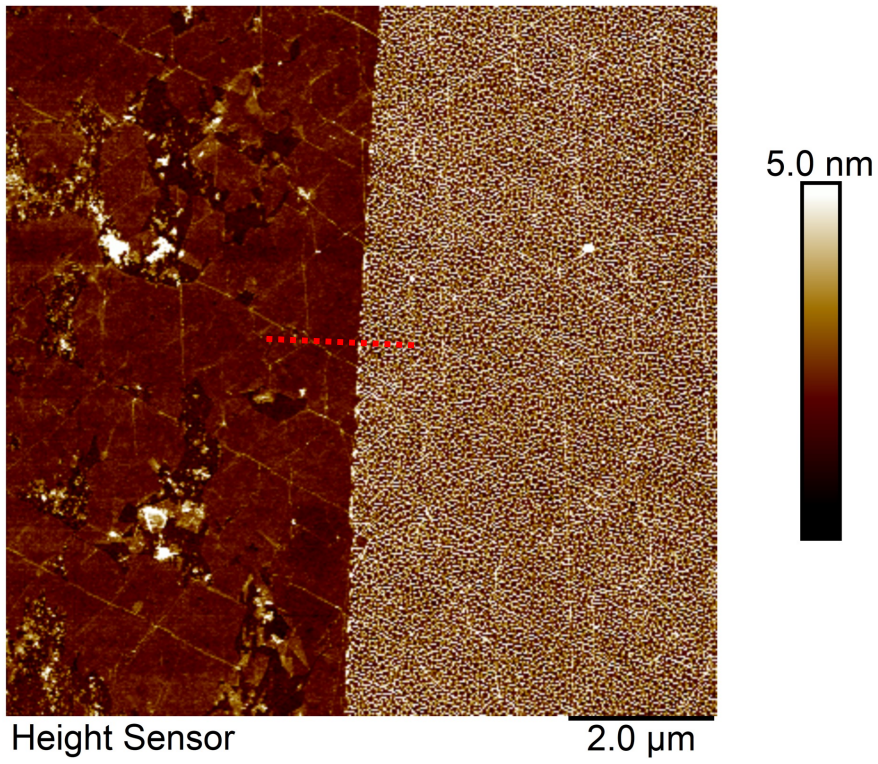
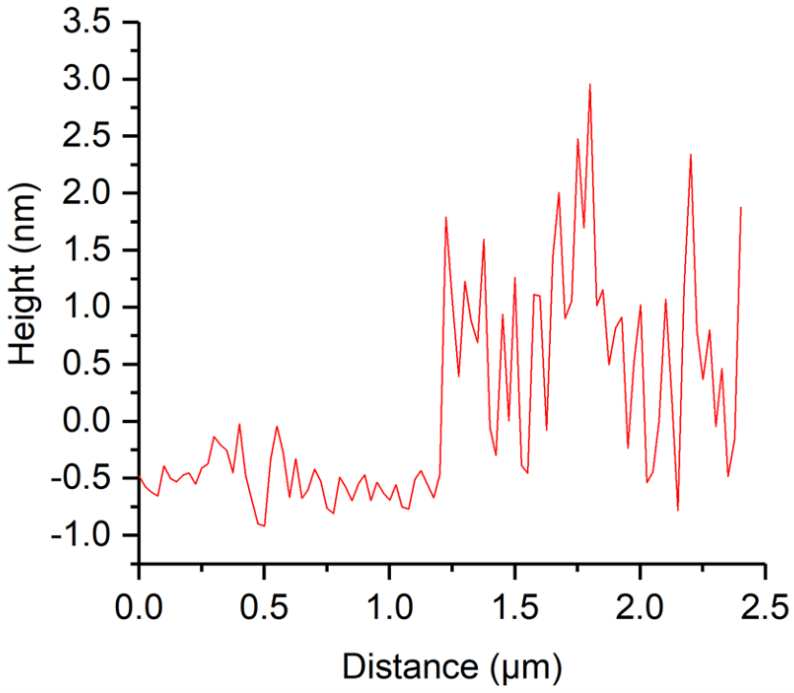


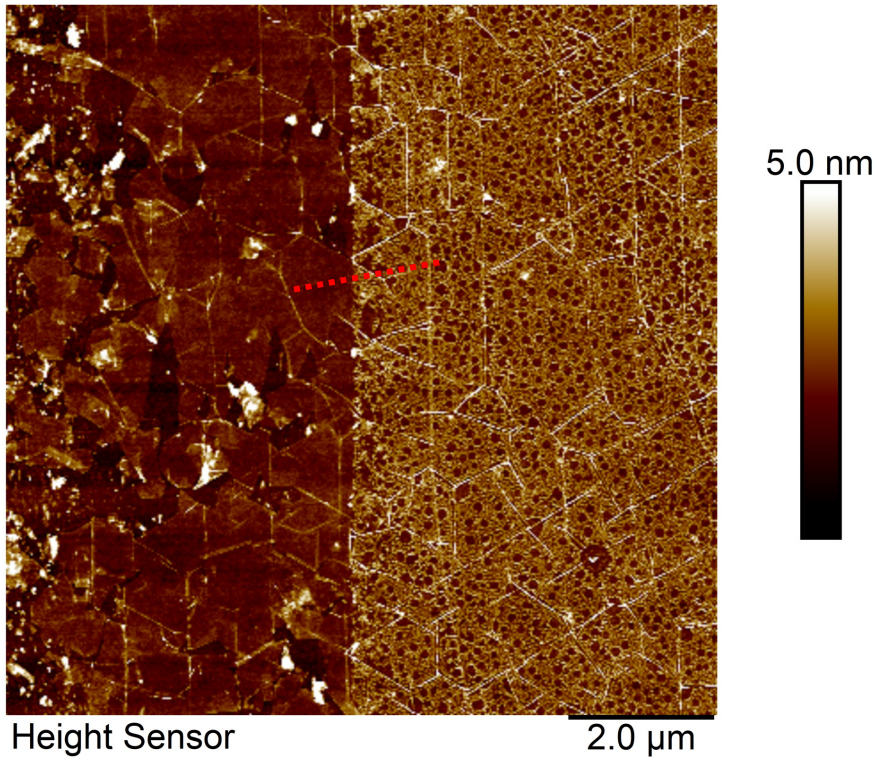
Figure S6: AFM scans of all the samples used in the study in different conditions and for both proteins. CaM in **a)** pH 5, 0.025 µg/ml, **b)** pH 5, 0.25 µg/ml, **c)** pH 5, 2.5 µg/ml, **d)** pH 7, 2.5 µg/ml, **e)** pH 9, 2.5 µg/ml, and mAchR in **f)** pH 5, 0.025 µg/ml, **g)** pH 5, 2.5 µg/ml, **h)** pH 7, 2.5 µg/ml, **i)** pH 9, 2.5 µg/ml. Left image is before protein incubation and right after the incubation. Note that **a)** and **b)** are the same sample, which was incubated twice, once per concentration.

a)


5.0 nm
Height Sensor
2.0 µm


Height (nm)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
0.0
0.5
1.0
1.5
2.0
2.5
Distance (µm)

b)



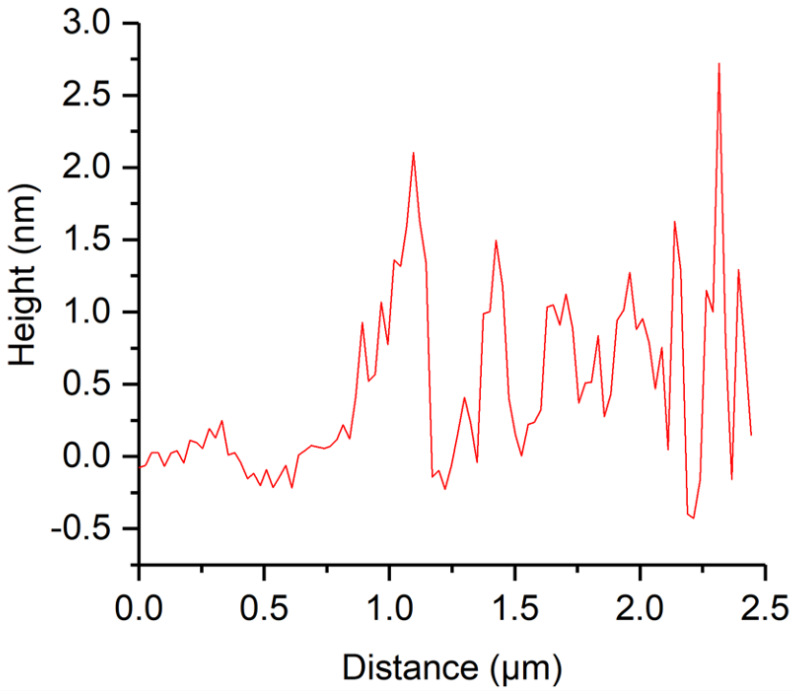


Figure S7: Examples of scratch tests for **a)** CaM and **b)** mAchR to see how deep the holes in the protein network are and if the graphene surface is visible from underneath. The dotted line in the AFM scan (top) shows where the cross-section (bottom) has been taken from.

Supplementary note 2: s-SNOM negative control experiment

To verify that the signal in the protein-resonant s-SNOM optical phase image (Figure 3(b), 1645 cm^{-1}) originates from the protein, an off-resonant image was taken in the same area at 1730 cm^{-1} (Figure S8 b)). At this frequency, the protein did not absorb the light as strongly as at 1645 cm^{-1}. While the oxidized areas are clearly visible in both the protein resonant and non-resonant images, the fine network structure of the protein is missing in the negative control image (Figure S8 b)). This is further supported by comparing the profiles of the topography and the optical phase (Figure S8 c)): The profiles do not correlate. Thus, we assume that the signal detected at 1645 cm^{-1} originated from the mAchR protein fragment.

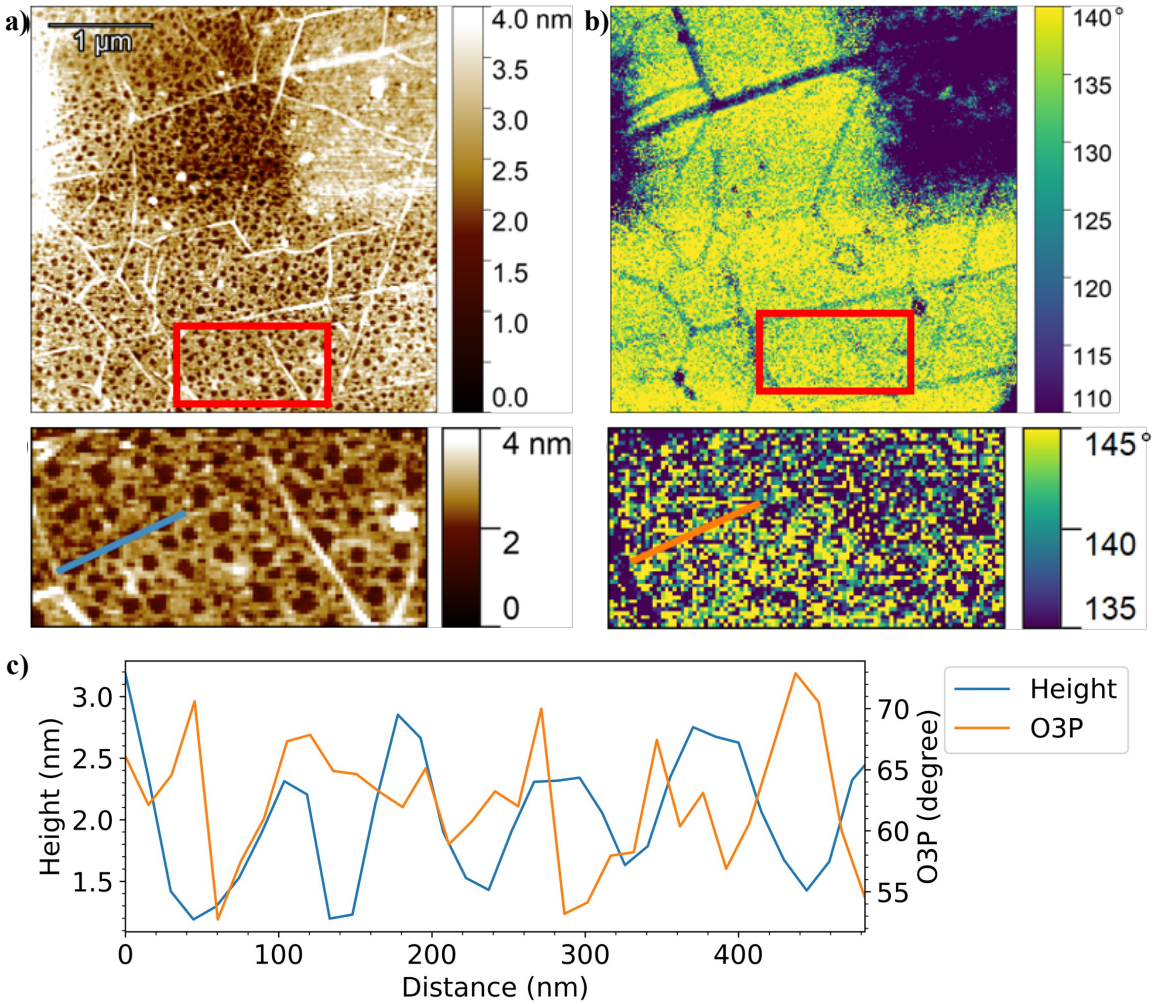
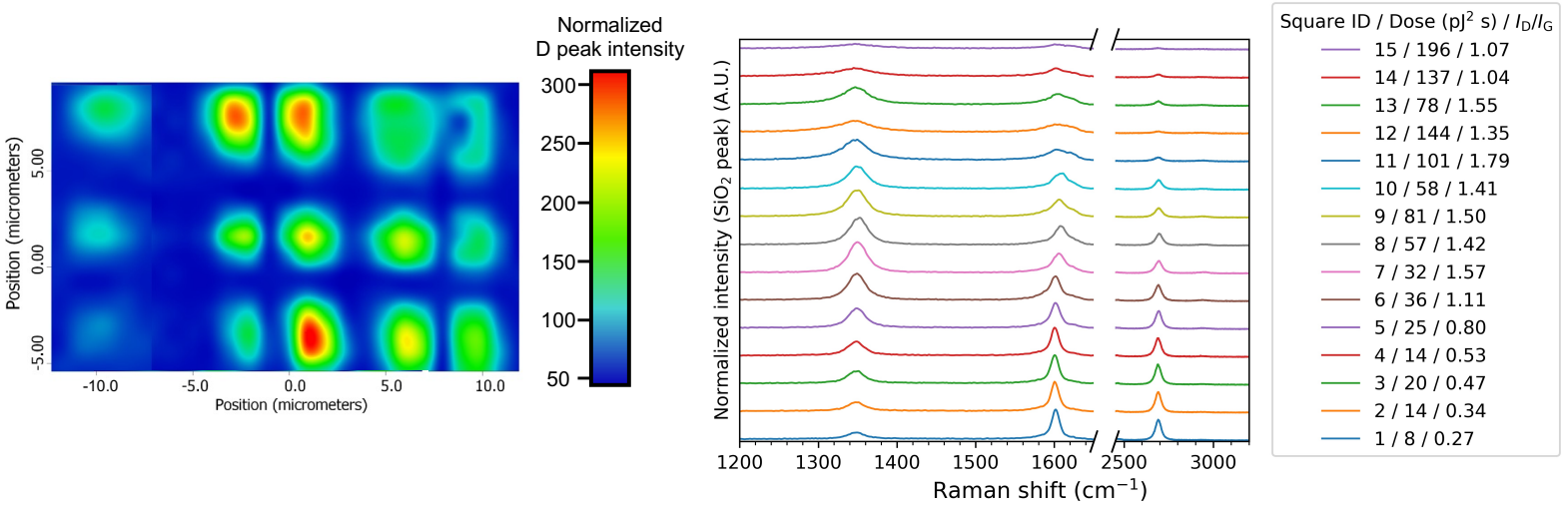
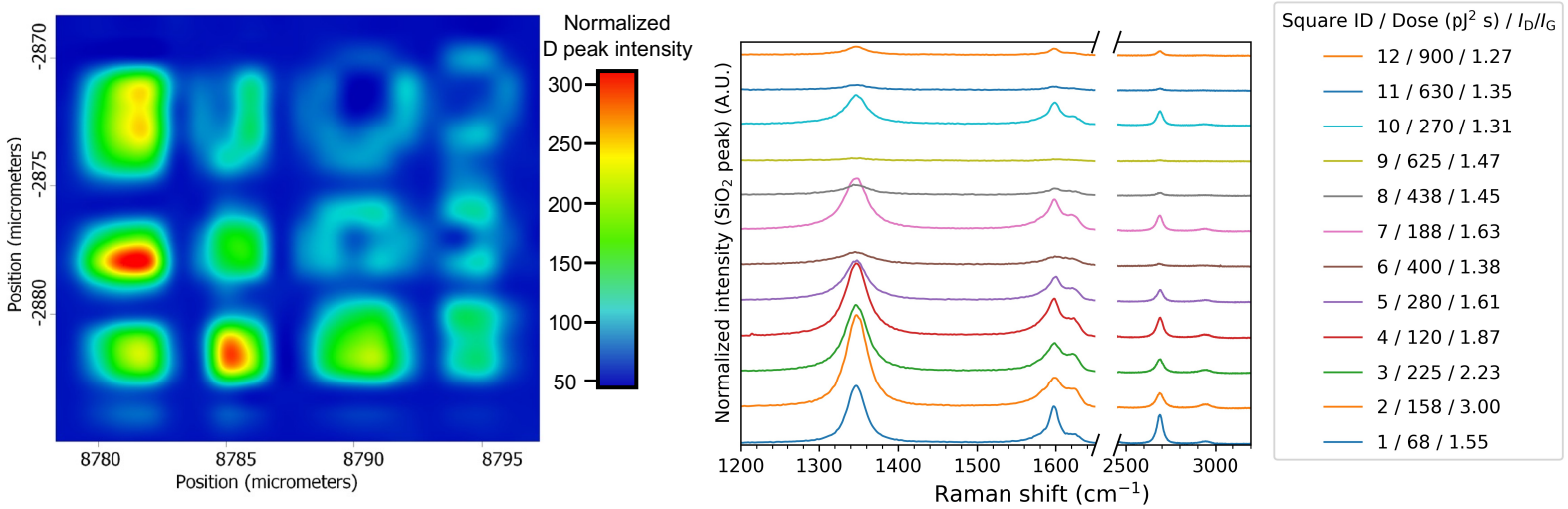


Figure S8: s-SNOM imaging of a sample previously incubated in mAchR solution (2.5 µg/ml, pH 5). **a)** Topography and **b)** optical phase (O3P at 1730 cm^{-1}, not resonant with the protein) images of the same area. The highlighted areas are shown enlarged below their corresponding images. The profiles along the blue and orange line in the enlarged images of **a)** and **b)**, respectively, are plotted in **c)**.

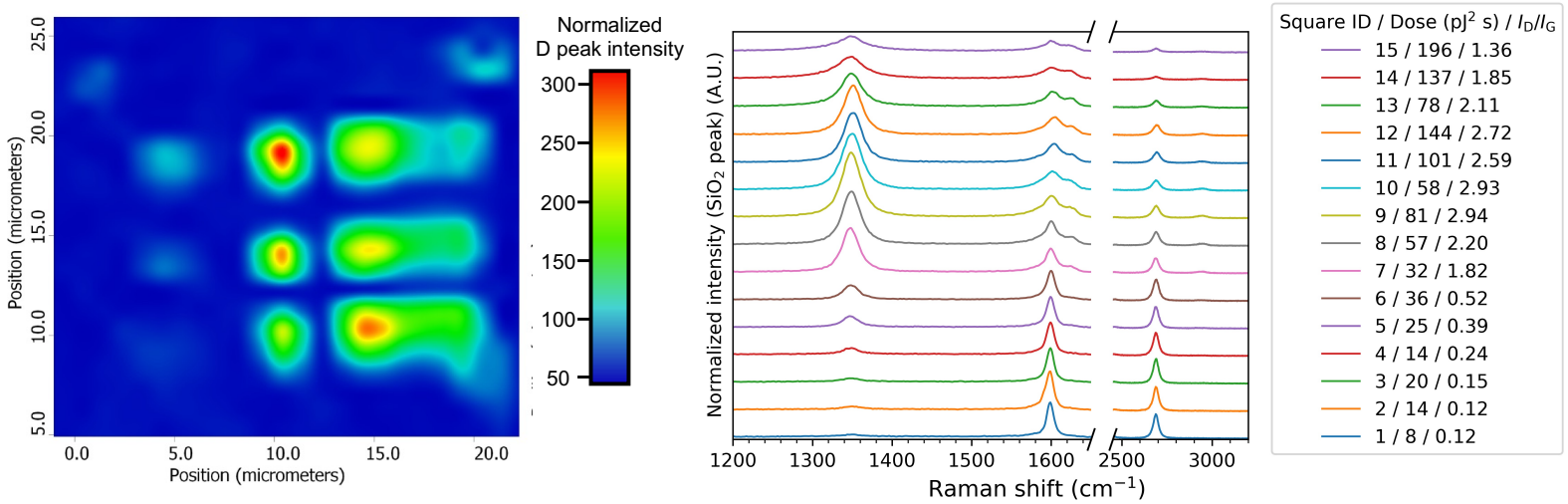
a) CaM pH 5, 0.025 µg/ml and 0.25 µg/ml



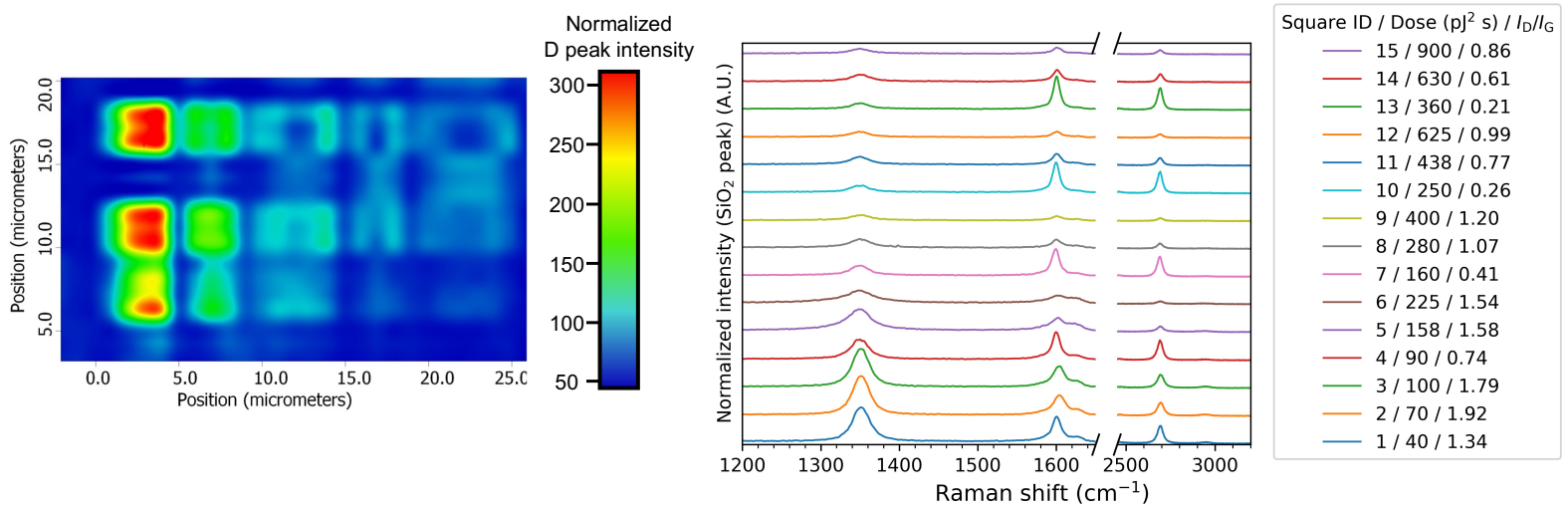
b) CaM pH 5, 2.5 µg/ml



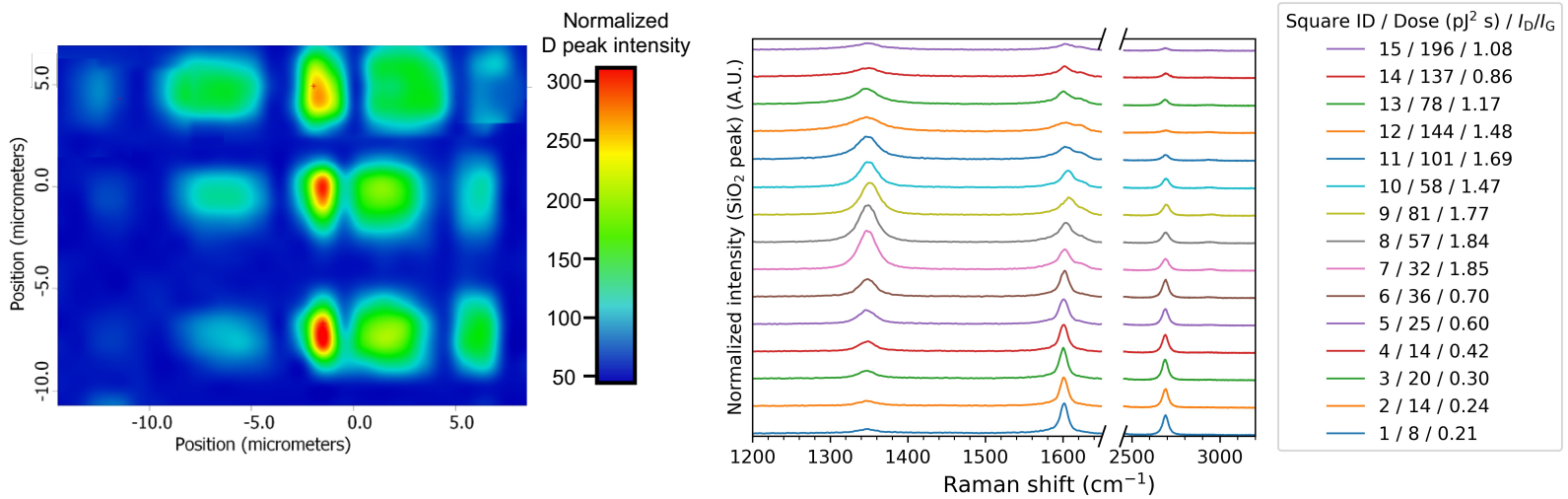
c) CaM pH 7, 2.5 µg/ml



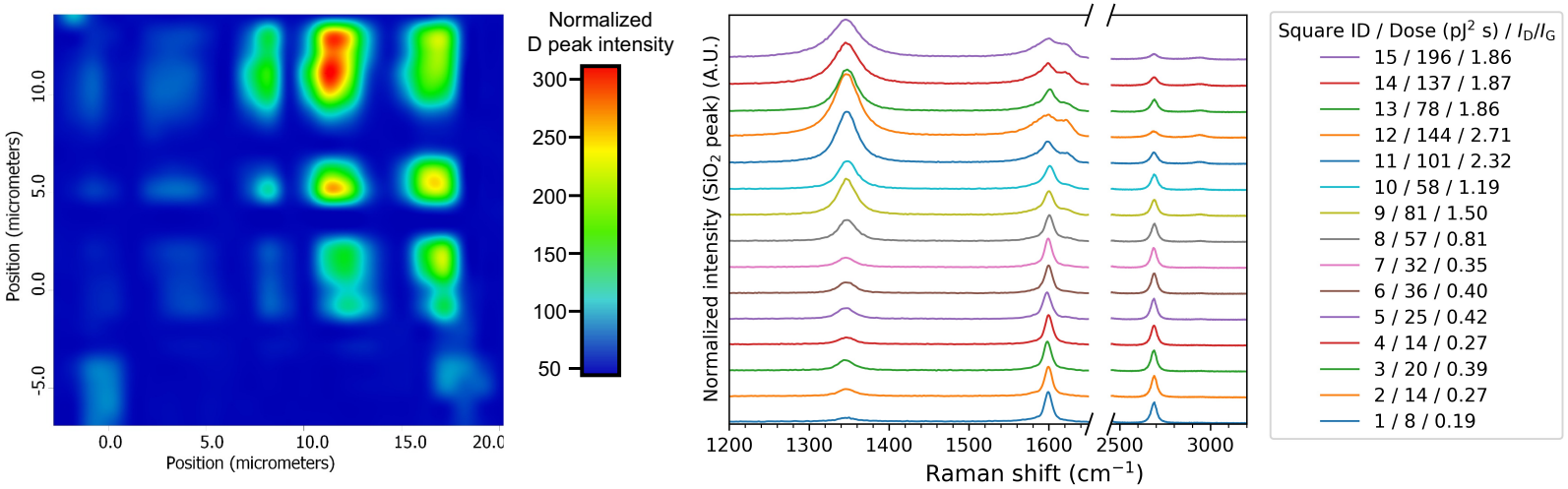
d) CaM pH 9, 2.5 µg/ml



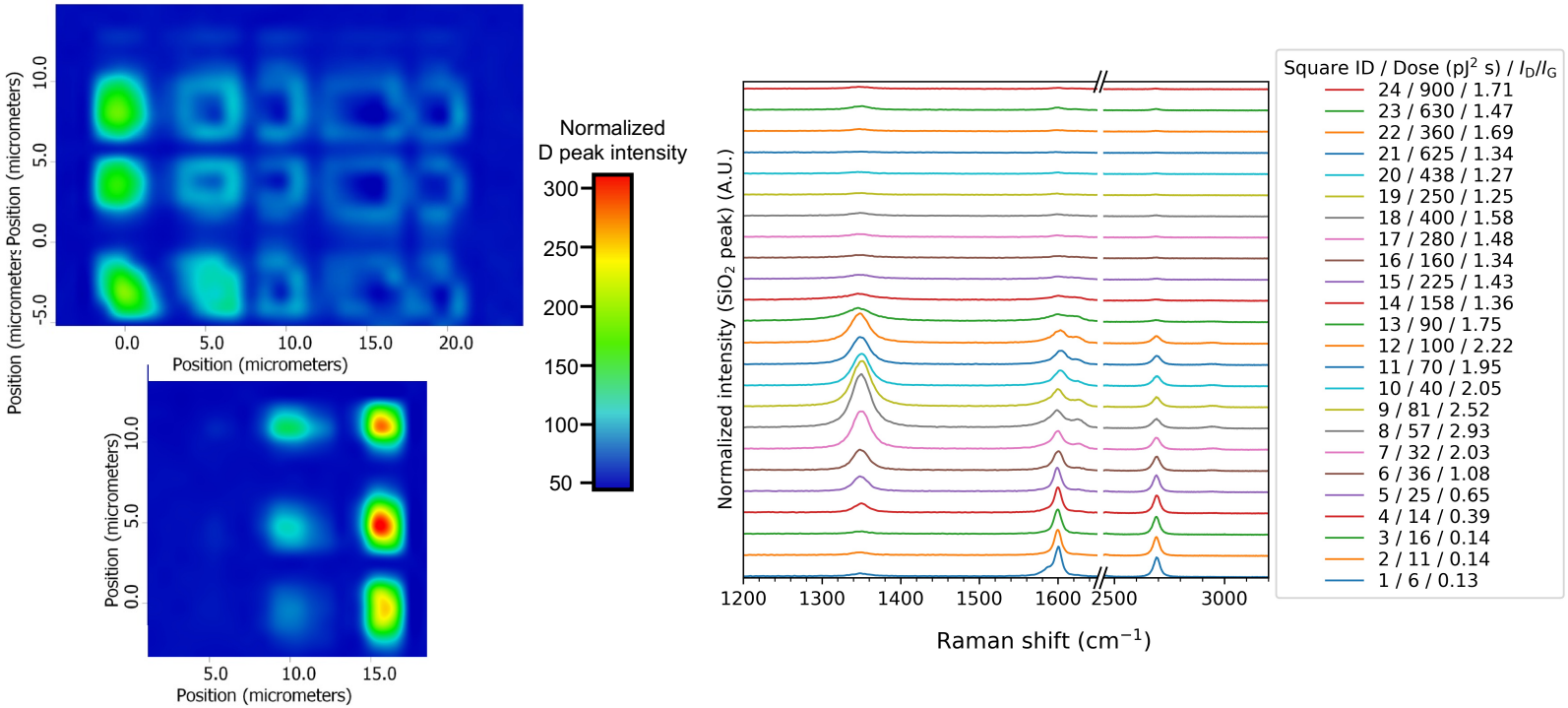
e) mAchR pH 5, 0.025 µg/ml



f) mAchR pH 5, 2.5 µg/ml



g) mAchR pH 7, 2.5 µg/ml



h) mAchR pH 9, 2.5 µg/ml

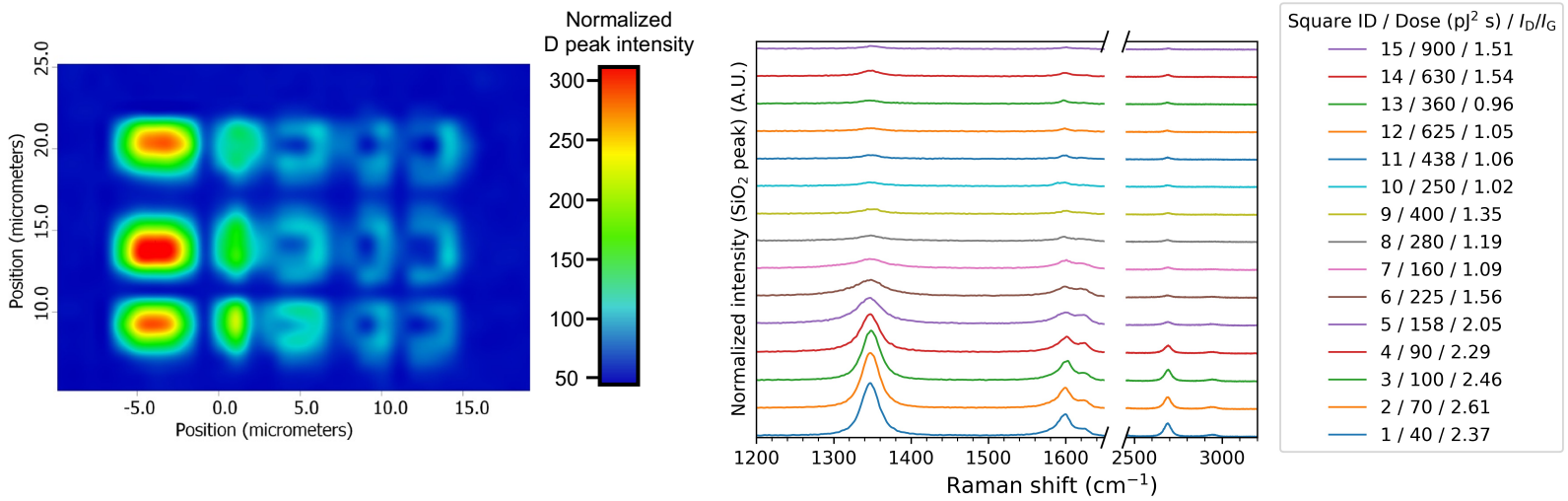


Figure S9: Raman maps collected for all the samples before incubation, plotted with the intensity of the D peak (I_D, 1349 cm^{-1}) as the plot color accompanied by the corresponding Raman spectra chosen from the centers of each square labelled with their corresponding square ID number, irradiation dose for 2PO, and I_D/I_G ratio. The maps have been normalized so that the highest I_D has been set to 300. The spectra have been normalized by setting the SiO_2 peak (900 – 1000 cm^{-1}) maximum intensity to the same value for each spectrum. These samples were used for the following conditions: CaM in **a)** pH 5, 0.025 µg/ml and 0.25 µg/ml, **b)** pH 5, 2.5 µg/ml, **c)** pH 7, 2.5 µg/ml, **d)** pH 9, 2.5 µg/ml, and mAchR in **e)** pH 5, 0.025 µg/ml, **f)** pH 5, 2.5 µg/ml, **g)** pH 7, 2.5 µg/ml, **h)** pH 9, 2.5 µg/ml. With the highest doses, the graphene has already been ablated (the center of the square is darker) and here the Raman signal can mostly come from the surrounding less-irradiated graphene.

Supplementary note 3: Outlier low concentration results

The sample used for 0.25 µg/ml CaM and pH 5 (Figure 5(a)) was first incubated at the lowest concentration of 0.025 µg/ml (Figure S6(a)) but the AFM data showed only very small changes in the height distributions after the incubation. Therefore, many of the height values had to be set to zero as there was not enough deposition to do a reliable fit to the differential histogram. Where a height was possible to define, the values were very low: 0.65 to 0.75 nm (Pr) and 0.2 to 0.5 nm (2PO). As the deposition seemed so low that the analysis did not seem reliable, an additional incubation in a ten times higher concentration was performed immediately after the first post-incubation-AFM-image had been taken with the same sample. With 0.25 µg/ml (Figure 5 (a)) the analysis was more reliable. Still the average protein heights (~0.9 nm (Pr) and 0.56 – 0.9 nm (2PO)) are below the reported dimensions of the protein (6.1 x 2.6 x 2.1 nm^3)[1], which indicated the limitations of this analysis method as discussed above in the Supplementary note 1. Therefore, it is likely more reliable to emphasize only the coverage in these lower concentrations and give less value to the height values extracted, as was done in the main article.

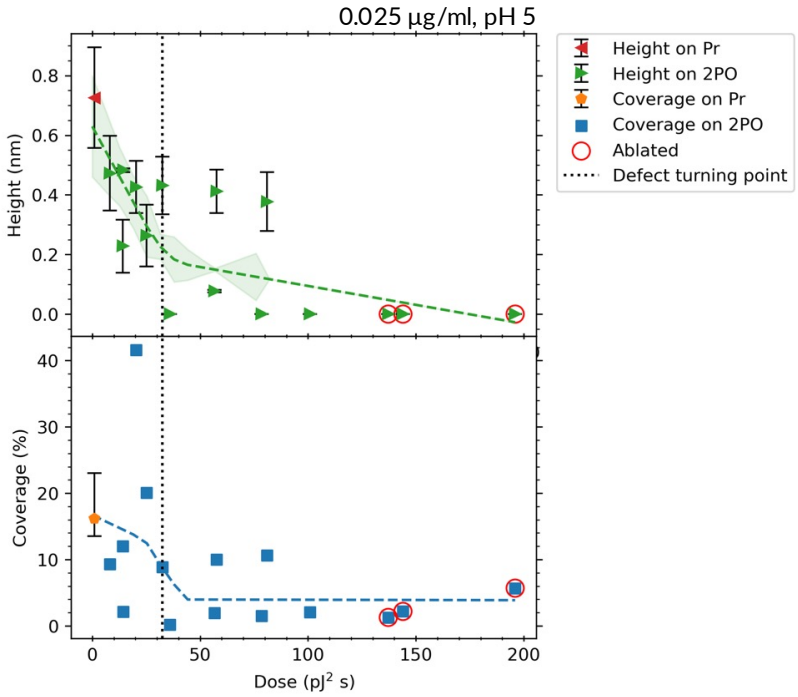


Figure S10: Adsorption results for CaM at 0.025 µg/ml concentration and pH 5 in a 50 mM PBS solution as shown in Figure 5 for other incubation conditions used for CaM.

Supplementary references

1 S. T. Rao, S. Wu, K. A. Satyshur, M. Sundaralingam, K. -Y Ling and C. Kung, *Protein Science*, 1993, **2**, 436–447.