



**HAL**  
open science

## In situ mapping of biomineral skeletal proteins by molecular recognition imaging with antibody-functionalized AFM tips

Benazir Khurshid, Eric Lesniewska, Luca Polacchi, Maëva L'Héronde, Daniel J. Jackson, Sébastien Motreuil, Jérôme Thomas, Jean-François Bardeau, Stephan E. Wolf, Daniel Vielzeuf, et al.

### ► To cite this version:

Benazir Khurshid, Eric Lesniewska, Luca Polacchi, Maëva L'Héronde, Daniel J. Jackson, et al.. In situ mapping of biomineral skeletal proteins by molecular recognition imaging with antibody-functionalized AFM tips. *Acta Biomaterialia*, 2023, 168, pp.198-209. 10.1016/j.actbio.2023.07.028 . hal-04272655

**HAL Id: hal-04272655**

**<https://hal.science/hal-04272655>**

Submitted on 6 Nov 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

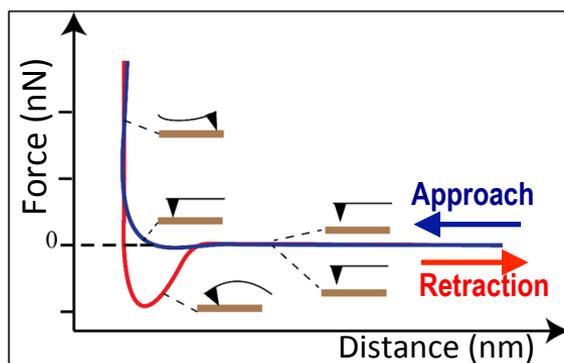
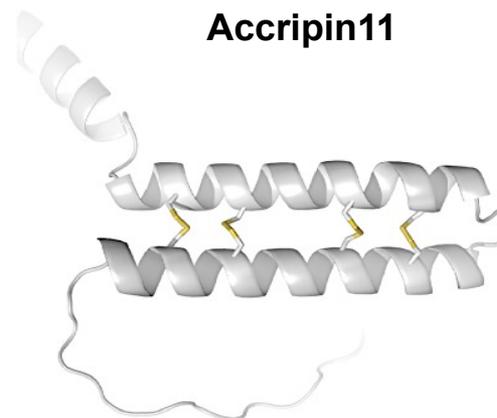
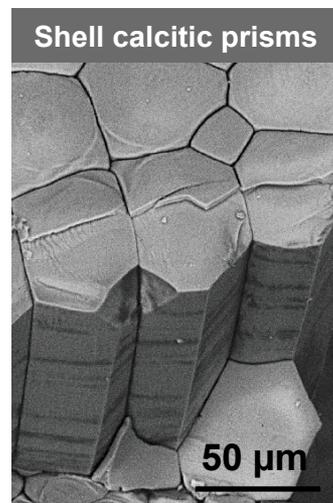
L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# Acta Biomaterialia

## In situ mapping of biomineral skeletal proteins by molecular recognition imaging with antibody-functionalized AFM tips --Manuscript Draft--

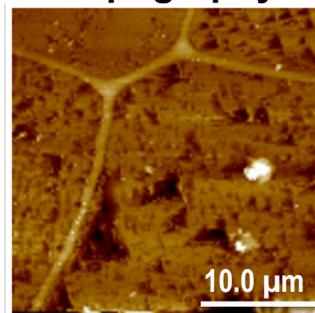
<b>Manuscript Number:</b>	AB-23-620R2
<b>Article Type:</b>	Full length article
<b>Keywords:</b>	biomineral; molluscan shell; skeletal matrix; molecular recognition; Atomic force microscopy; antibody
<b>Corresponding Author:</b>	Frederic MARIN, PhD, Habil. University of Bourgogne Dijon, France
<b>First Author:</b>	Benazir Khurshid, Ph.D
<b>Order of Authors:</b>	Benazir Khurshid, Ph.D Eric Lesniewska Luca Polacchi Maëva L'Héronde Daniel J. Jackson Sébastien Motreuil Jérôme Thomas Jean-François Bardeau Stephan E. Wolf Daniel Vielzeuf Jonathan Perrin Frederic MARIN, PhD, Habil.
<b>Abstract:</b>	<p>Spatial localizing of skeletal proteins in biogenic minerals remains a challenge in biomineralization and biomaterials research. To address this goal, we developed a novel in situ mapping technique based on molecular recognition measurements via atomic force microscopy (AFM). In order to obtain 2D maps of the distribution of a specific protein, three steps are required (1) the development and purification of a polyclonal antibody elicited against the target protein, (2) its covalent coupling to a silicon nitride AFM tip ('functionalization'), and (3) scanning of an appropriately prepared biomineral surface. We applied this approach to a soluble molluscan shell protein - accripin11 - recently identified as a major component of the calcitic prisms of the fan mussel <i>Pinna nobilis</i> [1]. Multiple tests reveal that accripin11 is evenly distributed at the surface of the prisms but is also present in the organic sheaths surrounding the calcitic prisms, which indicates that accripin11 is both an intra- and inter-crystalline protein. We also observed that the adhesion force in transverse sections is about twice higher than in longitudinal sections, suggesting that the protein is not randomly oriented in the biomineral but may exhibit preferred orientation. To our knowledge, this is the first time that a protein is localized by molecular recognition atomic force microscopy with antibody-functionalized tips in a biogenic mineral. The 'pros' and 'cons' of this new methodology in the biomineralization field are discussed in comparison with more 'classical' approaches like immunogold localization. This technique, which leaves the surface to analyze clean, might prove useful for clinical tests, on non-pathological (bone, teeth) or pathological (kidney stone) biomineralizations. Studies using implants with protein-doped calcium phosphate coating can also benefit from this technology.</p>

# Mapping of a shell protein by AFM with antibody-functionalized tip

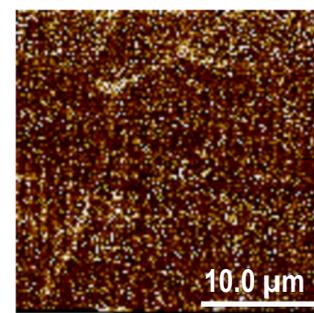


Principle of Single-Molecule-Force-Spectroscopy

Topography



Adhesion



1  
2  
3  
4 ***In situ* mapping of biomineral skeletal proteins by molecular recognition imaging with antibody-**  
5 **functionalized AFM tips**  
6  
7

8  
9 Benazir Khurshid<sup>1,2</sup>, Eric Lesniewska<sup>3</sup>, Luca Polacchi<sup>4,5</sup>, Maëva L'Héronde<sup>4</sup>, Daniel J. Jackson<sup>6</sup>, Sébastien  
10 Motreuil<sup>1</sup>, Jérôme Thomas<sup>1</sup>, Jean-François Bardeau<sup>7</sup>, Stephan E. Wolf<sup>8</sup>, Daniel Vielzeuf<sup>9</sup>, Jonathan  
11 Perrin<sup>2</sup>, Frédéric Marin<sup>1\*</sup>  
12  
13  
14

15  
16  
17  
18 Authors affiliations :

19  
20  
21 <sup>1</sup> Laboratoire Biogéosciences, UMR CNRS-EPHE 6282, University of Burgundy, Dijon, France.

22  
23 <sup>2</sup> Synchrotron SOLEIL, Beamline ANATOMIX, Saint-Aubin, Gif-sur-Yvette, France.

24  
25 <sup>3</sup> Laboratoire Interdisciplinaire Carnot de Bourgogne (ICB), UMR CNRS 6303, University of Burgundy,  
26  
27 Dijon, France.

28  
29 <sup>4</sup> IPANEMA, USR3461, CNRS/MCC, Saint-Aubin, Gif-sur-Yvette, France.

30  
31 <sup>5</sup> CR2P UMR7207, Muséum National d'Histoire Naturelle, Paris, France.

32  
33 <sup>6</sup> Department of Geobiology, Georg-August University of Göttingen, Göttingen, Germany.

34  
35 <sup>7</sup> IMMM UMR CNRS 6283, Le Mans University, Le Mans, France.

36  
37 <sup>8</sup> Institute of Glass and Ceramics, Dpt. Materials Science & Engineering, Friedrich-Alexander-University,  
38  
39 Erlangen, Germany.

40  
41 <sup>9</sup> CINAM, Aix Marseille University, CNRS, Marseille, France.  
42  
43  
44

45  
46  
47  
48  
49  
50 \* Corresponding author: Frédéric Marin: frederic.marin@u-bourgogne.fr  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61

1  
2  
3  
4 **Abstract**  
5  
6

7 Spatial localizing of skeletal proteins in biogenic minerals remains a challenge in biomineralization  
8 research. To address this goal, we developed a novel *in situ* mapping technique based on molecular  
9 recognition measurements via atomic force microscopy (AFM), which requires three steps: (1) the  
10 development and purification of a polyclonal antibody elicited against the target protein, (2) its covalent  
11 coupling to a silicon nitride AFM tip ('functionalization'), and (3) scanning of an appropriately prepared  
12 biomineral surface. We applied this approach to a soluble shell protein - accripin11 - recently identified as  
13 a major component of the calcitic prisms of the fan mussel *Pinna nobilis* [1]. Multiple tests reveal that  
14 accripin11 is evenly distributed at the surface of the prisms and also present in the organic sheaths  
15 surrounding the calcitic prisms, indicating that this protein is both intra- and inter-crystalline. We  
16 observed that the adhesion force in transverse sections is about twice higher than in longitudinal sections,  
17 suggesting that accripin11 may exhibit preferred orientation in the biomineral. To our knowledge, this is  
18 the first time that a protein is localized by molecular recognition atomic force microscopy with antibody-  
19 functionalized tips in a biogenic mineral. The 'pros' and 'cons' of this methodology are discussed in  
20 comparison with more 'classical' approaches like immunogold. This technique, which leaves the surface  
21 to analyze clean, might prove useful for clinical tests on non-pathological (bone, teeth) or pathological  
22 (kidney stone) biomineralizations. Studies using implants with protein-doped calcium phosphate coating  
23 can also benefit from this technology.  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50

51 Keywords: biomineral, protein mapping, AFM, molluscan shell, skeletal matrix, molecular recognition,  
52 antibody  
53  
54  
55  
56  
57  
58  
59  
60  
61

1  
2  
3  
4 **1. Introduction**  
5  
6

7 Biogenic calcium carbonates are the most abundant components of biominerals in nature [2].  
8  
9 They are also phylogenetically widespread across the Tree of Life [3], being produced by bacteria and  
10 protists and most metazoan phyla including sponges, cnidarians, bryozoans, brachiopods, molluscs,  
11 annelids, arthropods, echinoderms and some vertebrates, among which birds (eggshell) or teleost fishes  
12 (otoliths). Biominerals exhibit mechanical, textural, morphological or optical properties that are  
13 significantly different from their inorganic counterparts. Consequently, they present many avenues of  
14 exploration, from biomaterials science to bio-inspired chemistry and biomimicry [4-7]. In many aspects,  
15 the molluscan shell is considered as the archetypal example of a calcium carbonate biomineral for its  
16 remarkable resistance to fracture [8], the variety of crystal shapes that constitute each shell layer [9] and  
17 in some cases, its remarkable optical properties [10, 11]. These uncommon features are often considered  
18 as the result of non-classical crystallization pathways [12-16], themselves largely driven by the interaction  
19 of organic macromolecules with inorganic ionic precursors (*e.g.* calcium and carbonate/bicarbonate). The  
20 macromolecules secreted by the calcifying mollusc mantle at the mineral growth front may play multiple  
21 roles in the poorly understood transition from the initial crystallizing medium to the final calcified  
22 product. They are often occluded within the mature biomineral [17, 18].  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40

41 While the functional mechanisms by which organic macromolecules influence the form of the  
42 mature biomineral are yet to be fully described, many studies have been carried out to identify and  
43 characterize these macromolecules, comprised predominantly of both proteins and polysaccharides [17,  
44 19]. Only the first ones have received the greatest attention due to classical molecular biology approaches  
45 and to the rise of 'omics' techniques (*i.e.*, genomics, transcriptomics and proteomics) in recent years. The  
46 shell proteome, also defined as the "shellome" (*i.e.*, the set of proteins associated directly with the shell  
47 [20]) of dozens of mollusc species have now been characterized, at least partially [21, 22]. Many of these  
48 shell proteins do not have clear homologues in other model systems, and thus their precise function in  
49 biomineralization is largely unknown. To address this question, a variety of approaches are taken, which  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 include: 1) *in silico* analyses of the protein sequence, accompanied by similarity search emphasizing  
5  
6 functionally conserved domains; 2) adequate *in vitro* functional assays of recombinantly expressed  
7  
8 versions of the target protein; 3) the “wet-lab” resolution of their 3D structures (via NMR studies of the  
9  
10 crystallized proteins); 4) the knock-down of their corresponding transcripts via RNAi methods, or their  
11  
12 knock-out (or modification) via CRISPR/Cas9 gene editing; 5) and finally, the precise *in situ* localization  
13  
14 of the target protein within the biomineralized structure.  
15  
16  
17

18  
19 Mapping the distribution of a specific shell protein with a high spatial resolution is a necessary  
20  
21 step to understand its affinity and its interactions with the mineral phase, and ultimately, its function  
22  
23 during the liquid-solid transition [12-14]. However, what may appear as trivial is singularly complicated  
24  
25 by the nature of the material, in particular its multiscale structure and the heterogeneity of the associated  
26  
27 organic matrix. Only techniques with high resolution can overcome these limitations. In that respect,  
28  
29 Atomic Force Microscopy (AFM) is a powerful tool in nano characterization because of its ability to scan  
30  
31 a surface with subnanometer resolution in air and under vacuum but also in aqueous liquids. Apart from  
32  
33 being used as an imaging tool, AFM rapidly became a multifunctional toolbox [23-27]. In  
34  
35 biomineralization, AFM has been used to observe the ultrastructure of various shells [28-30]. Recently, a  
36  
37 significant improvement was brought to the AFM technique by combining AFM imaging with the force  
38  
39 measurement, known as molecular recognition imaging [31], also defined as Force Distance (FD)-based  
40  
41 AFM. This technique relies on the interaction between two molecules: one is covalently attached to the  
42  
43 AFM tip while the other, the target molecule, is bound to the surface of interest. Approaching the  
44  
45 functionalized tip to the target molecule allows measuring the interaction force between the two  
46  
47 molecules. The technique has a great sensitivity at the piconewton scale [32, 33] and the operation can be  
48  
49 performed on multiple independent spots of the surface. Alternatively, the surface can be scanned  
50  
51 continuously, line after line, with the functionalized tip. This allows a complete 3D map to be formed  
52  
53 where each pixel is characterized by its corresponding adhesion curve. In parallel, a direct correlation  
54  
55 between the topography of a sample, with structurally localized mechanical properties, and a specific  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 interaction map can be obtained [34]. So far, molecular recognition imaging has been used on a variety of  
5  
6 biomolecular systems, including antigen-antibody complexes [35, 36], aptamer-protein [37], peptide-  
7  
8 protein [38], chromatin [39], cells [40, 41], and other ligand-receptor interactions such as avidin-biotin  
9  
10 [42]. However, to our knowledge, this approach has never been applied to the surface of biominerals.

11  
12  
13  
14 In this study, we performed AFM molecular recognition imaging of a newly discovered shell  
15  
16 protein, accripin11 [1] in the calcitic prisms of the Mediterranean fan mussel, *Pinna nobilis*. The AFM  
17  
18 probe was functionalized with a highly specific and purified polyclonal antibody, which was elicited  
19  
20 against two peptides of this protein. The probe was subsequently used to scan two orthogonal surfaces of  
21  
22 the calcitic prisms. With this approach, we successfully obtained a distribution map of accripin11. The  
23  
24 pros and cons of the technique are discussed here.

## 2. Materials and methods

25  
26  
27  
28  
29  
30  
31  
32  
33  
34 **2.1. Materials sampling** - Ten fresh shells of the Mediterranean fan mussel *Pinna nobilis*, a  
35  
36 highly protected species, were collected during a field mission in May 2017. The mission was done  
37  
38 according to ethical rules. In particular, the sampling required a specific authorization of the DDTM  
39  
40 ('Direction Départementale du Territoire et de la Mer' of the department of Alpes Maritimes, Arrêté  
41  
42 Préfectoral n° 2017-459). All the metadata describing the mission can be accessed via the dat@UBFC  
43  
44 portal of the Observatoire des Sciences de l'Univers (OSU) Theta, Besançon, France at: [https://search-](https://search-data.ubfc.fr/search.php?s=Pinna+nobilis)  
45  
46 [data.ubfc.fr/search.php?s=Pinna+nobilis](https://search-data.ubfc.fr/search.php?s=Pinna+nobilis).

47  
48  
49 **2.2. Material microstructural description** – *P. nobilis* exhibits a shell that consists of two  
50  
51 superimposed layers (Figure 1a): the internal lustrous shell layer, which is restricted to the first third of  
52  
53 the shell, is made of row stack nacre tablets of aragonite; the external brown-reddish layer exhibits a very  
54  
55 characteristic pattern, consisting of large calcitic prisms that are packed within a honeycomb organic  
56  
57 network, corresponding to the dark outlines observed in Figure 1b. This network forms insoluble sheaths  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 around each single prism adhering them together. The prisms grow inwards, 'layer-per-layer',  
5  
6 perpendicularly to the outer shell surface (blue arrow in Figure 1b). If the isolated prismatic layer is  
7  
8 treated with sodium hypochlorite, it can be entirely dissociated into 'single prisms', as shown by Figure  
9  
10  
11 1c. In this study we only considered the prismatic layer, allowing us to focus on the *in situ* localization of  
12  
13 a 'model' protein with functionalized AFM tips.

14  
15 **2.3. Choice of a model protein for *in situ* localization experiments** - Our investigations on  
16  
17 the protein composition of the prisms, combining transcriptomics and proteomics, led to the identification  
18  
19 of hundreds of proteins (Khurshid *et al.*, in preparation). We selected one candidate, the sequence of  
20  
21 which was extremely well covered by proteomics. In addition, it proved to be very abundant in the prisms  
22  
23 and specific to this microstructure. We named this novel protein accripin11 and its biochemical properties  
24  
25 have been recently described [1]. In short, accripin11 is a secreted protein of 103 amino acid residues  
26  
27 (121 residues with signal peptide). It is moderately acidic, except at its C-terminus, enriched in 10 acidic  
28  
29 residues (between positions 105 and 121) among which 7 are Asp residues (figure 2a). The protein  
30  
31 exhibits a peculiar cysteine pattern in its central domain. In computerized 3D reconstructions using  
32  
33 AlphaFold2, this pattern constrains the 3D structure to two antiparallel alpha-helices that are stabilized by  
34  
35 four disulfide bridges. The acidic C-terminus is unstructured, *i.e.*, disordered (Figure 2b). Since  
36  
37 accripin11 does not exhibit homology with any proteins of known function, its molecular role in the  
38  
39 prism's formation remains enigmatic; beyond being able to modify the shapes of calcite crystals *in vitro*  
40  
41 [1], we suggest that accripin11 can act as a linker between the mineral surface and other matrix proteins:  
42  
43 the Asp-rich C-terminus would bind the positively charged surface of growing calcium carbonate nuclei  
44  
45 via electrostatic interactions while the more hydrophobic domain, containing the two antiparallel alpha-  
46  
47 helices would be repelled and protrude from the surface, being able to bind other matrix protein partners.  
48  
49 In the present localization experiments, accripin11 constitutes our model protein.  
50  
51  
52  
53  
54

55  
56 **2.4. Polyclonal antibody production, testing and purification** – In our previous paper [1], a  
57  
58 recombinant accripin11 was obtained in insufficient amounts for developing functional assays and, in  
59  
60 parallel, raising antibodies. Consequently, while most of the recombinant protein was utilized for  
61  
62  
63  
64  
65

1  
2  
3  
4 functional assays, the following procedure was performed by Eurogentec SA (Liège Science Park,  
5 Seraing, Belgium) to generate polyclonal antibodies, according to the protocol called the “double X  
6 option”: in short, two immunogenic peptides were designed and synthesized: the first corresponding to  
7 the central domain (KDCAQQCTRDRETCFG, residues 65 to 80); the second to the C-terminal region  
8 (TAAPKPAKEPSSADD, residues 97 to 111) as shown by the two domains underlined in brown and in  
9 grey of Figure 2a. These peptides were coupled to carriers (KLH for the first peptide, and OVA for the  
10 second) and injected into rabbits, to generate polyclonal antibodies according to a speedy 28-day  
11 immunization protocol (contract FR10162), including injections at 0, 7, 10 and 18 days, and bleedings at  
12 0 (pre-immune serum), 21 (medium), and 28 (final) days. The sera (2nd and 3rd bleeding) containing the  
13 antibodies were tested by Enzyme linked ImmunoSorbent Assay (ELISA) against the whole acetic acid  
14 soluble prism matrix, for the determination of their respective titer. In parallel, they were tested on  
15 Western blot against the same prism extracts to check their specificity [1].  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30

31 Following the results of the Western blot, we selected the 3<sup>rd</sup> bleed serum, which was found to  
32 combine both high titer and high specificity. The antibodies were purified from their serum by an  
33 antibody purification kit (ref. ab109209, Abcam France, Paris) based on the binding of antibodies (*i.e.*,  
34 immunoglobulins) to Protein A fixed to resin beads. The purification was performed according to the  
35 manufacturer's instruction: the serum containing the antibodies was mixed, first with the 10x binding  
36 buffer, then with the Protein A resin, and the mixture was incubated for 3 hours at room temperature with  
37 gentle agitation on a rotary shaker, to optimize binding of the antibody to the Protein A grafted resin. The  
38 suspension was poured and packed in a separating column, which was washed 3 times with 7 mL of  
39 washing solution to elute unbound serum macromolecules (including proteins), metabolites and inorganic  
40 ions contained in the serum. The antibody was then eluted and collected in a 2 mL Eppendorf tube by  
41 pouring 1 mL elution buffer in the column. This operation was repeated five times in total, each time with  
42 a new Eppendorf tube. 0.25 mL neutralizing buffer was added and mixed immediately to each of the 5  
43 collected eluents. The 5 preparations were then placed on ice before testing.  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 Aliquots (20  $\mu$ L) of the five eluents were then sampled, mixed to 20  $\mu$ L of 2x Laemmli buffer,  
5  
6 heat-denatured for 5 min., and tested on a 12% acrylamide hand cast SDS-PAGE gel, as previously  
7  
8 described [43]. The gel was stained with silver nitrate [44]. In parallel, a second identical gel was electro-  
9  
10 blotted on nitrocellulose according to the standard Western blot protocol [45]. As the antibody  
11  
12 preparation (IgGs) cross-reacts with the secondary antibody (*i.e.*, GAR/AP, Sigma-Aldrich A3687), the  
13  
14 membrane was then simply blocked with gelatin (0.5% wt/vol. in Tris buffered saline) and treated directly  
15  
16 with GAR/AP (diluted 30,000 times), before being rinsed five times with TBS/Tween, revealed with  
17  
18 NBT/BCIP and dried. The Eppendorf tubes containing the highest concentration of antibodies were  
19  
20 pooled together and this solution was dialyzed (SpectraPor 7, cutoff 1 kDa) against 2L ultrapure water,  
21  
22 with two water changes, in order to remove the salt of the neutralizing buffer, which may interfere with  
23  
24 subsequent AFM tip functionalization. The solution was further concentrated in a Vivaspin 20 mL  
25  
26 centrifugal concentrator tube (Sartorius, Germany, ref. VS2002, cutoff 10 kDa) to a final volume of 1.5  
27  
28 mL (3 times concentration) and stored at 4°C for quick use, or aliquoted and frozen for long term storage.  
29  
30  
31  
32

33 **2.5. Functionalization strategy** - The strategy for functionalizing AFM tips relies on the  
34  
35 covalent binding of the antibody to the silicon nitride tip, as shown in Figure 3. We used a direct coupling  
36  
37 procedure known as the 'glutaraldehyde pathway' [46-48], which does not require intermediate linker  
38  
39 ('spacer') like polyethyleneglycol (PEG) [49]. The pathway is a four-step procedure, interspersed with  
40  
41 short rinsing steps. The first step is the silanization, required to activate the tip by introducing amine  
42  
43 groups. The interest of this step is that the probe can be functionalized directly, without prior surface  
44  
45 preparation [50]. The silanization reaction occurs between silanol groups of the tip surface and a  
46  
47 trichlorosilane group in the silane reagent, leading to the formation of an organosilane layer [51]. The  
48  
49 second step is the glutaraldehyde incubation, which substitutes active aldehyde groups to amines of the  
50  
51 APTES modified surface. In the third step, the antibody covalently binds to the active aldehyde groups  
52  
53 [52]. In the last step, bovine serum albumin (BSA) blocks the remaining active sites where the antibody is  
54  
55 not present.  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4       **2.6. AFM tip functionalization** - We used silicon nitride AFM tip (ScanAsyst-Air probe with  
5  
6 a nominal spring constant of 0.4 N/m, Bruker Nano). The functionalization was performed according to  
7  
8 the protocol described in [52]. All steps were performed at room temperature. The tip was first silanized  
9  
10 with 2% v/v 3-aminopropyltriethoxysilane (APTES, ref. 440140-100ML, Sigma-Aldrich) in acetone (ACS  
11  
12 reagent, ref. 179124-500 ML, Sigma-Aldrich) for 10 minutes to render the AFM probe NH<sub>2</sub>-activated.  
13  
14 The AFM tip was then briefly rinsed with ultrapure water and immersed for 30 min. in glutaraldehyde  
15  
16 (grade II, ref. G6257-100ML, Sigma-Aldrich) solution (0.5% v/v glutaraldehyde in ultrapure water). The  
17  
18 tip was again rinsed with ultrapure water and incubated in a solution of purified anti-acripin11 antibody  
19  
20 (200–300 ng/μl) for 30 min, before being briefly rinsed in ultrapure water. Finally, the remaining  
21  
22 aldehyde groups on the probe surface were blocked by treating the tip for 1 min with 100 μg/L bovine  
23  
24 serum albumin (BSA, 25 g, ref. RPN412V, GE Healthcare), filtered sterile (0.22 μm) in Tris buffered  
25  
26 saline (TBS). The functionalized tip was stored in phosphate buffered saline (PBS) at 4°C or immediately  
27  
28 mounted on the AFM tip holder for scanning.  
29  
30  
31  
32

33       **2.7. Sample surface preparation for AFM study** - We prepared two prismatic shell  
34  
35 surfaces, each orthogonal to the other: transversal sections perpendicular to the elongation axis of the  
36  
37 prisms; and longitudinal sections parallel to their elongation axis. These preparations were realized by  
38  
39 using an ultramicrotome for obtaining flat, freshly exposed, surfaces. To this end, we used a Leica EM  
40  
41 UC6 ultramicrotome (Leica, Austria), available at IPANEMA laboratory, Gif-sur-Yvette, France. First,  
42  
43 the sample was cut by means of a wire saw (Well Diamond Wire Saws, model 3032 mounting a diamond  
44  
45 wire of 0.3 mm diameter) into cubes with a surface of approximately 3x3 mm, which were then fixed on  
46  
47 an AFM specimen holder with 2-4 mm clamping range by Leica. The small fragments of the sample were  
48  
49 cut directly without embedding in resin. The sample holder was fixed on a flat XYZ scanning  
50  
51 piezoelectric stage mounted on the ultramicrotome arm. The sectioning was performed by a vertical  
52  
53 movement of the specimen over the extremely sharp blade of a fixed knife. Firstly, the surface of the  
54  
55 sample was flattened by removal of several (1 μm thick) layers with a triangular tungsten carbide knife  
56  
57 (Electron Microscopy Sciences, 40° cutting angle) adjusted at 10° clearance angle to the sample surface.  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 Finally, thinner sections (500, 200 and 100 nm layers) were trimmed off the surface with a diamond knife  
5  
6 (Diatome Histo, 45° cutting angle) adjusted at 6° clearance angle to the sample surface. The prepared  
7  
8 surface from ultramicrotome was used for AFM measurement without any further surface treatment.  
9

10 **2.8. AFM measurements and data analysis** - A Dimension ICON-2 FastScan Bio Atomic  
11  
12 Force Microscope (Bruker Nano, Santa Barbara, CA, USA) was operated in the Force Volume mode to  
13  
14 conduct single-molecule force spectroscopy (SMFS) with ScanAsyst powerful AFM control associated to  
15  
16 Nanoscope V controller. The AFM was equipped with a FastScan Head 35  $\mu\text{m}$  scan range system with  
17  
18 temporal resolution of up to 3 frames-per-second for live sample observations using FastScan probe  
19  
20 models. Triangular oxide sharpened silicon cantilevers of nominal spring constants of 0.25-0.45 N/ m  
21  
22 (ScanAsyst-Air probes, Bruker Nano) were used for single-molecule force spectroscopy. Measuring the  
23  
24 resonant frequency and the quality factor of the cantilever in air by the AFM thermal method allowed us  
25  
26 to get the spring constant of the cantilever to standardize our AFM force measurements. In single-  
27  
28 molecule force spectroscopy (SMFS) experiments, the AFM tip is continuously approached to and  
29  
30 retracted from the biological sample, while monitoring the interaction force. The obtained force-extension  
31  
32 curves provide key insight into the molecular elasticity and localization of single molecules, either on  
33  
34 isolated systems or on cellular surfaces. The cantilever sensitivity was estimated from 50 forces curves, at  
35  
36 least, on fused silica calibrate sample ( $E_{\text{modulus}} = 72.9$  GPa for cantilever spring constant  $K < 10$  N/m)  
37  
38 prior SMFS experiments. Overview images ( $25 \times 25 \mu\text{m}^2$ ) were recorded at imaging forces of 150 pN, the  
39  
40 AFM tip was oscillated vertically at 8 kHz applying a 15 nm amplitude, the sample was scanned using a  
41  
42 line frequency of 10 Hz, and 512 pixels were scanned per line (512 lines). Best high-resolution AFM  
43  
44 topographs and interaction maps were found at imaging forces  $\geq 150$  pN, oscillation frequency  $\leq 8$  kHz,  
45  
46 oscillation amplitudes between 10 and 30 nm, and line scanning frequencies  $\leq 2.5$  Hz. All FD-based AFM  
47  
48 images were recorded in standard PBS buffer. For each SMFS experiment, a 250 nm ramp size and an  
49  
50 interaction time from 0.1 to 10 s were used. We varied the loading rate from 10 nm/s to 1740 nm/s on a  
51  
52 series of different transversal junctions, for selecting the optimal value for the loading rate. Identical scan  
53  
54 rates were selected for the "extend and retract" motion of the functionalized probe. We also selected a  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 contact point duration of 100 ms for all experiments. The control measurement was performed, by  
5  
6 recording the adhesion maps from a non-functionalized tip in the same area than that scanned with the  
7  
8 functionalized tip. Alternately, tips functionalized with biofunctionalized estrogen receptor probes were  
9  
10 used as negative controls [53] to scan the surface of *Pinna nobilis* prismatic layer. No specific interactions  
11  
12 were noticed, particularly along the periprismatic membranes. All experiments were performed in a  
13  
14 temperature-controlled environment (25 °C).  
15  
16

## 17 **2.9. Statistical analysis**

18  
19 At least thirty areas were scanned per sample type. The adhesion force distribution for a loading  
20  
21 rate of 500 nm/s was quantified in the two sections (transversal, longitudinal) by performing statistics  
22  
23 over 128x128 points per area; the data (average, standard deviation) were treated with Excel and a  
24  
25 histogram was generated.  
26  
27  
28  
29  
30

## 31 **3. Results**

32  
33 **3.1. Polyclonal antibodies against accripin11** – A polyclonal antibody was obtained against  
34  
35 two accripin11 peptides. This unpurified antibody preparation represents a mix of immunoglobulins that  
36  
37 target specifically each of the designed peptides of accripin11, according to the double X option  
38  
39 immunization program developed by Eurogentec. In a first step, the antibody preparation was tested  
40  
41 unpurified, *i.e.*, in the whole serum form, against the unfractionated acetic acid soluble prism matrix,  
42  
43 which electrophoretic migration pattern is shown in Fig. 4A, lane 2, on the silver-stained gel. This matrix  
44  
45 contained several discrete and non-discrete bands. When this matrix was blotted on a nitrocellulose  
46  
47 membrane and tested by Western blot with the unpurified anti-accripin11 antibody preparation, the results  
48  
49 revealed a single reactive band, as shown in Figure 4a, lane 4. Given the apparent molecular weight of the  
50  
51 band, this corresponds to accripin11. This clearly demonstrated that the antibody preparation was  
52  
53 accripin-specific and did not cross-react with other macromolecules of the prism matrix. This conclusive  
54  
55 prerequisite step allowed us to go to the next step.  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 In a second step, the antibody was purified from its serum and the five eluted fractions obtained  
5  
6 by affinity chromatography were partly denatured and tested on silver-stained gel (Figure 4b) and  
7  
8 Western blot (Figure 4c). As shown by these two figures, antibody preparations on gel are characterized  
9  
10 by three bands on a gel: one of very high molecular weight, corresponding to the full-size  
11  
12 immunoglobulin molecule (IgG, between 130 and 170 kDa); the second intermediate band corresponds to  
13  
14 the IgG heavy chain between 43 and 55 kDa, while the third lower band corresponds to the IgG light  
15  
16 chain, slightly above 34 kDa. These results are in conformity with earlier finding on IgGs on SDS-PAGE  
17  
18 gels [54]. The result of the simplified Western blot (single incubation with the secondary antibody, Figure  
19  
20 4c) confirms the identity of these three bands, as they all hybridize with the secondary goat-anti-rabbit  
21  
22 antibody (GAR/AP). According to Figure 4b, eluates 3 and 4 (respectively, lanes 4 and 5) contain most of  
23  
24 the purified antibody while eluates 2 and 5 (respectively lanes 3 and 6) contain traces, and eluate 1 (lane  
25  
26 2) is devoid of IgG. We pooled together the content of eluates 2 to 5 (lanes 3 to 6), to maximize the  
27  
28 amount of usable antibody, before desalting the pooled eluates by dialysis against ultrapure water,  
29  
30 followed by a spin concentration.  
31  
32  
33  
34

35 **3.2. Mapping of the biomineral surface** - Prior to biomineral surface scanning, we performed  
36  
37 series of tests with loading rates varying from 10 nm/s to 1740 nm/s on a series of different periprismatic  
38  
39 membranes, as shown by the graph of Figure 5. We obtained an increasing exponential curve. We  
40  
41 selected 500 nm/s (2.7 in logarithmic scale) as the reference for the loading rate for the reported  
42  
43 experiments. This represents a good compromise between acquisition speed and the risk of erosion of the  
44  
45 functionalized tips. One has to consider that the interaction values are relative and depend on the  
46  
47 acquisition speed, on the "extend and retract" motion of the probe, and on the contact duration between  
48  
49 the functionalized probe and the specimen.  
50  
51  
52  
53

54 The results of the mapping experiments with functionalized tips acquired from prismatic layer  
55  
56 samples prepared via ultramicrotomy are illustrated in Figures 6, 7 and 8. Figure 6 shows successively the  
57  
58 3D (Figure 6 a, d), 2D (Figures 6b, e) images and line profiles (Figures 6c, f) of the preparations, in  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 transverse (Figure 6a-c) and longitudinal (Figure 6d-f) sections, respectively. In both sections, the  
5  
6 periprismatic sheath appears slightly in relief, particularly in transverse section (Figure 6a). The mineral  
7  
8 surface is not completely flat or uniform but exhibits a certain nano-rugosity and nano-porosity. However,  
9  
10 all things considered, the differences of heights are minimal between the peri-prismatic sheath and the  
11  
12 calcite surface as shown by the two profiles of figures 6c and 6f: at maximum, a difference of 160 nm is  
13  
14 recorded in transverse section (Figure 6c) and 130 nm for figure 6c (longitudinal). The AFM tip can  
15  
16 easily accommodate such height differences. Our multiple attempts showed that the ultramicrotomy  
17  
18 procedure generates flatter surfaces than the mirror polishing followed by surface cleaning (see  
19  
20 supplementary Figure 1c and d). We consider that a low height difference (below 200 nm) guarantees the  
21  
22 quality of the results by excluding artifact signals such as non-specific adhesion due to topography.  
23  
24

25  
26 Figure 7 presents adhesion maps with unfunctionalized tip (Figure 7a and c, negative control) and  
27  
28 with the same tip that was functionalized with anti-accripin11 antibody (Figure 7b and d). The views  
29  
30 represent transversal (Figures 7a and b) and longitudinal (Figure 7c and d) sections of the prisms. The  
31  
32 adhesion forces varied between 0 to 2 nanonewtons in transverse section (Fig. 7b) this latter relative value  
33  
34 corresponding to the antigen-antibody binding force. Therefore, white pixels in the adhesion maps shown  
35  
36 in Figure 7b represent spots where the antigen-antibody binding-induced adhesion is recorded, in other  
37  
38 words, where accripin11 is present. The control experiments (with unfunctionalized tips) show very few  
39  
40 unspecific adhesion spots in transverse section (Figure 7a). For some unknown reasons, we recorded a  
41  
42 higher background in the negative control of longitudinal sections (Figure 7c). However, one notices that  
43  
44 a high density of white spots is observed on Figure 7d. In both 'positive' surfaces, on the mineral surface  
45  
46 excluding the periprismatic sheath, accripin11 seems to be homogeneously distributed everywhere,  
47  
48 without drawing any particular adhesion pattern. Prior to force spectroscopy measurements, topography in  
49  
50 Peak Force QNM mode showed a high density of skeletal proteins along the ridges, typically about 100 to  
51  
52 150 proteins/ $\mu\text{m}$ . In addition, where the periprismatic sheaths are located, we observe a denser pattern of  
53  
54 accripin11, suggesting that this protein is more concentrated at that particular site than on the mineral  
55  
56 surface, *i.e.*, at the interface between the mineral itself and the periprismatic sheath. However, it is not  
57  
58  
59  
60  
61

1  
2  
3  
4 clear whether the alignment of accripin11 along the sheaths is homogeneous or whether it may exhibit  
5 some variations. In Figure 7b, the sheath is hardly underlined, and the locations where the density of  
6 white spots is the highest are triple junctions of the sheath while, in Figure 7d, the sheath that crosses the  
7 picture from left to right is intensely marked.  
8  
9

10  
11  
12  
13 Interestingly, in our attempt to quantify the interaction between the functionalized tip and the prism  
14 surface, we found out that in transverse section (perpendicular to the elongation axis of the prisms), the  
15 adhesion force is about twice higher (56 pN +/- 30pN) than in longitudinal sections (26 pN +/- 17 pN)  
16 parallel to the elongation axis, as shown by the histogram of Figure 8. A possible reason of this difference  
17 is discussed below. We have not considered multiples of the single average force. This is because our  
18 probe can, in some areas, probe two or three proteins at the contact point. Its multiple interactions have  
19 been documented in previous publication [36]. As negative control, as expected, negligible adhesion  
20 forces were recorded for spots with no accripin11, in both sections.  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33

#### 34 **4. Discussion**

35  
36 In this paper, we describe a novel methodology to localize biomineralization proteins on the  
37 surface of biominerals. To our knowledge, this is the first time that this technique is applied in the field of  
38 biomineralization. Until recently, there have been a number of attempts to visualize the distribution of  
39 organics throughout a biomineral, but each of them has significant limitations: Muyzer and Westbroek  
40 [55] used immunohistological techniques to visualize bulk organic matrix in shells via optical  
41 microscopy. Their approach based on mixtures of macromolecules for eliciting the polyclonal antibody  
42 lacked specificity and resolution. These two pitfalls were partly overcome (1) by raising more specific  
43 antibodies against single shell proteins, and (2) by using gold-coupled secondary antibodies to amplify the  
44 signals and allow SEM observations in back-scattered electron mode [56, 57]. The technique was pushed  
45 to its limits by double labelling for a target protein and chitin [58]. While the immunogold approach  
46 undoubtedly represents a milestone, it is limited to intermediate magnifications, typically between 1,000x  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 to 20,000x, and requires most of the time a final silver enhancement step to increase the signal by  
5  
6 precipitating silver around the gold nanoparticles. However, this step can induce false positive signals if  
7  
8 the shell contains traces of heavy metals that can also react with silver [59]. Another drawback is that the  
9  
10 surface of the sample, covered by blocking agent (BSA, gelatin or carbohydrate-free reagent), antibodies,  
11  
12 gold and silver particles, cannot be reused for other purposes, like EDX or WDS investigations.  
13  
14 Histochemical staining is another method used [60] to map reactive chemical groups on the surface of  
15  
16 nacre tablets. However the technique does not give structural information on the macromolecules that  
17  
18 carry the reactive groups. Atom probe tomography has been applied at the nanoscale [61], but here again,  
19  
20 the interacting organic molecules cannot be identified. Finally, mapping by micro- or nano-FTIR or  
21  
22 Raman spectrometry are useful, but these techniques are limited to chemical reactive groups [62, 63] or  
23  
24 pigments [64]. In addition, in spite of reaching the 'nano' level, they lack the lateral resolution required for  
25  
26 precise localization of the reactive groups. The AFM approach that we use here overcomes many of these  
27  
28 limitations. In particular, it can answer the question of affinity of the protein of interest with the mineral  
29  
30 phase. This in turn has a direct implication on which non-classical crystallization pathway is selected for  
31  
32 building the studied biomineral. For example, 'inter-crystalline' proteins (*i.e.*, proteins that constitute the  
33  
34 sheath 'surrounding' a given crystal unit) tend to exhibit hydrophobic properties due to their enrichment in  
35  
36 aliphatic amino acids. At the scale of a single crystal (micrometric to sub-micrometric scale), the location  
37  
38 of such proteins is governed by a 'segregation mechanism' (*i.e.*, a phase separation) from the aqueous  
39  
40 mineral front, which grows from a center and progresses radially. Consequently, these proteins condense  
41  
42 (by cross-linking) into a soft and flexible but water-insoluble barrier surrounding the crystal, as soon as  
43  
44 this latter enters into confluence with its immediate neighbor crystals. In contrast, 'intracrystalline'  
45  
46 proteins, which are more hydrophilic, stay disseminated, at the nanoscale, in the vicinity of the nascent  
47  
48 (usually amorphous) nanograins that will become welded to their neighbor nanograins according to a  
49  
50 nanoscale assembly process [65]. The nanograin welding leads to the trapping of these hydrophilic  
51  
52 proteins, which can consequently be considered as truly occluded ('intracrystalline') in the forming  
53  
54 crystal.  
55  
56  
57  
58  
59  
60  
61

1  
2  
3  
4 In the present case, we obtained mapping of accripin11 at the surface of the calcitic prisms of  
5  
6 *Pinna nobilis*, in two sections perpendicular to each other, the transverse section and the longitudinal  
7  
8 ones. For each of these surfaces, we obtained an even distribution of accripin11, implying that this protein  
9  
10 should be considered primarily as intracrystalline. Moreover, we observed that accripin11 was slightly  
11  
12 more concentrated along the periprismatic sheaths, suggesting that it may be intercrystalline as well. A  
13  
14 possible explanation of this duality might originate from the amphiphilic nature of accripin11 sequence,  
15  
16 with a hydrophobic N-terminus and a hydrophilic C-terminus. In our scanning experiments, we noticed  
17  
18 that the adhesion force in transverse section was twice as high than that of longitudinal section. This  
19  
20 finding is particularly interesting because it means that the accessibility of the antibodies to the target  
21  
22 accripin 11 differs, depending on the orientation of the section: in other words, the antigen-antibody  
23  
24 recognition seems to be hindered in longitudinal sections in comparison to transverse sections. This  
25  
26 decrease of recognition may have something to do with the very close environment of the complex,  
27  
28 including both the mineral phase and other surrounding matrix macromolecules. Going further, if  
29  
30 accripin11 was randomly oriented on the surface, there should be no statistical difference between the  
31  
32 longitudinal and transversal sections. But since the adhesion forces are significantly different, this  
33  
34 suggests obviously that accripin11 is not randomly oriented at the surface of the prism section, but has  
35  
36 probably preferential orientations in the biomineral. This is the most likely explanation of the adhesion  
37  
38 difference between the two sections. At this stage, our assertion is speculative, but if supported by other  
39  
40 approaches such as solid-state Nuclear Magnetic Resonance (ss-NMR) and structure prediction [66], we  
41  
42 have an elegant – although indirect - manner to detect preferential orientations of some matrix  
43  
44 macromolecules in biominerals.  
45  
46  
47  
48  
49  
50

51 In order to be utilized optimally, Single Molecule Force Spectroscopy (SMFS) has some  
52  
53 specific requirements: a highly purified and specific antibody, an efficient functionalization of the AFM  
54  
55 tip and an appropriate mineral surface preparation. The antibody must be pure and should recognize only  
56  
57 one target-protein in a mixture of proteins. It is well known that antibodies that are supposedly specific of  
58  
59 one given antigenic determinant can give unexpected cross-reactivity [67] or can cross-react with other  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 proteins that exhibit similar determinants as the target protein [68, 69]. The gold-standard way to check  
5  
6 antibody specificity is via Western blot and we highly recommend performing this prerequisite check in  
7  
8 order to simplify the interpretation of AFM scans. In addition to antibody specificity, purifying the  
9  
10 antibody from its serum is a mandatory step to get rid of all contaminating serum proteins (serum  
11  
12 albumin, fibrinogen, transferrin, fetuin, lipoproteins). This step can be achieved in few hours with a kit.  
13  
14 At last, purified antibody preparations contain salts that come from the eluent in addition to ionic  
15  
16 components of the neutralizing buffer. These components can impair subsequent AFM tip  
17  
18 functionalization steps. Therefore, before use in AFM experiments, we recommend dialysis of the  
19  
20 purified antibody solution, to remove these salts, followed by concentration of the antibody in a  
21  
22 centrifugal concentrator tube.  
23  
24  
25

26  
27 Functionalization of the AFM tip is another critical step. The antibody must be covalently  
28  
29 bound, for maintaining a stronger bond to the tip than between the antibody and its target antigen. We  
30  
31 adopted an extremely simple tip functionalization procedure [50, 52], which required a limited number of  
32  
33 steps and reactants. In our hands, this maximized the rate of success. As the functionalization was made  
34  
35 'blind', its effectiveness was checked *a posteriori* by performing the positive control experiment with the  
36  
37 functionalized tip. We recommend preceding this positive control by a negative control performed with  
38  
39 the same unfunctionalized tip, in the same area of the sample. At last, we also recommend preparing  
40  
41 always fresh solutions for functionalization: attempts made with old solutions led to complete failure of  
42  
43 the experiment.  
44  
45

46  
47 The third key element of our approach concerns sample preparation. In conventional AFM  
48  
49 mapping, samples are prepared by mirror polishing. Over several pilot trials, we found that preparation of  
50  
51 the surface by ultramicrotomy provided more consistent results than by mirror polishing, mainly because  
52  
53 of the lower roughness (around 100-150 nm) and the absence of any surface treatment (see supplementary  
54  
55 figure 1). Although the polishing/cleaning approach could be optimized with 30 nm colloidal silica, the  
56  
57 post-polishing treatment - including desorption with a surfactant like Tween20 to remove surface  
58  
59 contaminants, 'bleaching' with dilute sodium hypochlorite to clean the surface or slight etching (1 min.)  
60  
61

1  
2  
3  
4 with dilute EDTA (1% w/vol) to expose occluded proteins - induced higher topography (EDTA  
5 treatment) and/or swelling of the periprismatic sheaths due to the Tween20 desorption step. So far,  
6 ultramicrotomy without any further treatment appears to be the optimal way to prepare biominerals  
7 surfaces for mapping of organics with functionalized AFM tips.  
8  
9

10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

Despite the good resolution we achieved in mapping accripin11 across the surfaces of these calcitic prisms, our approach is not without its limitations. Using glutaraldehyde to functionalize the AFM tip poses a challenge in controlling the density of proteins on the tip surface. Furthermore, antibody attachment to the tip is achieved randomly, *i.e.*, the antigen binding sites of the antibody are not all optimally oriented for surface scanning. Thus, only a part of the antibody-occupied sites on the AFM tip will be correctly oriented to interact with their corresponding target antigen [50]. As underlined before, the functionalization is made 'blind' and is empirically optimized; its effectiveness is assessed *a posteriori* during data acquisition. The tip, once functionalized, is also not stable. A functionalized AFM tip can only last a maximum of 3 days in PBS buffer at 4 °C [52], with the best scanning results achieved directly after functionalization of the tip. Specific and non-specific interactions between the tip and the surface can also be difficult to distinguish. The use of a spacer, like PEG, may partly solve this problem, however tip functionalization becomes more complicated and tedious to implement with these additional steps [36, 70]. As we emphasize above, surface preparation is critical, and if target antigens are not firmly bound to the sample surface, then they can adhere to the tip during data acquisition and thereby pollute it. The end result is an inactivated tip. Finally, surface preparation requires an ultramicrotome for hard tissues, a sophisticated and costly device.

In spite of these limitations, we assert that the technique brings substantial improvements to alternative approaches. For instance, the resolution of the technique is higher than that of immunolabelling approaches; proteins can be localized at the nanometer scale and does not require any labels to be fixed to the biomineral surface. As a consequence, scanning a surface with a functionalized tip leaves the mineral surface clean in contrast to immunogold staining, and so the surface can be re-used for other physical investigations such as EDX, WDS, FTIR or Raman spectroscopies. These characterizations are

1  
2  
3  
4 not possible after immunogold staining. Tip functionalization also allows for the estimation of antigen-  
5  
6 antibody interactions with piconewton accuracy. When performed via the glutaraldehyde pathway, the  
7  
8 process is quite simple and does not require complex chemical modification of the tip. The procedure is  
9  
10 rather rapid (2 hours), which means that the functionalization of few tips can be tested in a day. An  
11  
12 efficient way of operating consists in performing a set of negative controls with different unfunctionalized  
13  
14 tips, and to start the functionalization of the first tip during while the second and following tips are tested  
15  
16 in negative controls. Finally, the use of ultramicrotome to prepare the flat sample surfaces for  
17  
18 measurement eliminates the chances of spreading protein or contamination and thus, eliminates the  
19  
20 requirement of surface treatment that may induce artifacts by increasing surface topography.  
21  
22  
23

24 To conclude, the technique, as exposed here, is adapted to skeletal proteins, the most abundant  
25  
26 macromolecules identified in calcium carbonate biominerals. While our study employs a mollusc shell  
27  
28 and may appear to be fringe case, we are confident that our approach has a general scope and is adaptable  
29  
30 to many types of biominerals including calcium phosphate (bone, teeth), calcium oxalates, silica, or any  
31  
32 type of biomineral, resulting from a biologically-controlled or biologically-induced deposition process. In  
33  
34 particular, pathological biomineralizations such as kidney or pancreatic stones may benefit from this  
35  
36 advancement: because they contain a complex cortege of proteins [71, 72], localizing precisely the most  
37  
38 abundant ones may help elucidating the molecular mechanism by which these minerals form, with a  
39  
40 resolution far beyond what is currently done. The technique can also be applied to calcium phosphate-  
41  
42 coated bone / teeth implants where the coating is doped or co-precipitated with one or more bioactive  
43  
44 macromolecules, such as growth factors (BMPs) or peptides/proteins that enhance cellular attachment to  
45  
46 improve osseointegration [73, 74]. We assert that the technique may precisely quantify the density of  
47  
48 protein per square unit, in other words, evaluate the rate of protein incorporation in the coating. In clinical  
49  
50 tests using similar samples in terms of preparation, the acquisition could be automated to allow analyzing  
51  
52 ten samples a day with 3 analyzed areas per sample, on the assumption that the AFM probe is qualified  
53  
54 once. At the end of the series, the first sample should be re-analyzed in order to confirm the successive  
55  
56 analyses. Because of its sensitivity, the technique can also be applied in archeology or paleobiology, on  
57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 fossil or subfossil shell or bone materials that contain only remains of mineralizing matrix [75]. At last, in  
5  
6 addition to proteins, AFM mapping with functionalized tips can localize other types of macromolecules,  
7  
8 such as polysaccharides, the second most abundant components of skeletal matrices. In that case,  
9  
10 antibodies may usefully be replaced by specific lectins. To summarize, we believe that this technique will  
11  
12 bring valuable information in terms of functions of organics in biomineralization process.  
13  
14  
15  
16  
17  
18  
19  
20

## 21 **Acknowledgments**

22  
23  
24 The study has been financially supported by ANR MOBi (ANR-18-CE02-0014-02, 2018-2023, J. Perrin,  
25  
26 coordinator), including the salary of B. Kurshid and most of the consumables and chemicals. Other  
27  
28 financial supports include 1) the Research Project of Excellence BFC "PIA-Récolnat-BFC" supported by  
29  
30 region Burgundy - Franche-Comté and the "Récolnat ANR-11-INBS-0004" program (JT), 2) two SRO  
31  
32 projects, one in 2017, one in 2020 ('Pinocchio', *Pinna NObilis* & sa Calcification Coquillière: une Histoire  
33  
34 Originale) attributed to FM by OSU-Theta (Bourgogne - Franche-Comté), 3) a grant from EMBRC  
35  
36 (European Marine Biological Resource Centre, FM), which provided the logistic support for a field  
37  
38 mission to collect living specimens of *Pinna nobilis* at Villefranche-sur-Mer marine station in spring  
39  
40 2017. All the authors greatly acknowledge the DDTM of Alpes Maritimes (Julien Banus, Serge Castel)  
41  
42 for having issued the authorization (Arrêté Préfectoral n° 2017-459) to sample *Pinna nobilis*.  
43  
44  
45  
46  
47  
48  
49

## 50 **Author contribution**

51  
52  
53 Benazir Khurshid: formal analysis, investigation, methodology, validation, visualization, writing -  
54  
55 original draft preparation, review and editing. Eric Lesniewska: investigation, methodology, resources,  
56  
57 visualization. Luca Polacchi: resources, writing - original draft. Maëva L'Héronde: resources. Daniel J.  
58  
59 Jackson: writing – original draft, review and editing. Sebastien Motreuil: resources. Jérôme Thomas:  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 visualization, funding acquisition. Jean-François Bardeau: conceptualization, formal analysis, writing –  
5  
6 original draft. Stephan E. Wolf: conceptualization, writing – original draft. Daniel Vielzeuf:  
7  
8 conceptualization, funding acquisition. Jonathan Perrin: conceptualization, funding acquisition. Frédéric  
9  
10 Marin: conceptualization, data curation, formal analysis, investigations, methodology, project  
11  
12 administration, resources, supervision, validation, visualization, writing – original draft, review and  
13  
14 editing.  
15  
16

17  
18 The authors have no conflict of interest.  
19  
20

## 21 **References:**

- 22  
23 [1] B. Khurshid, D.J. Jackson, S. Engilberge, S. Motreuil, C. Broussard, J. Thomas, F. Immel, M.J.  
24  
25 Harrington, P.B. Crowley, D. Vielzeuf, J. Perrin, F. Marin, Molecular characterization of accripin11, a  
26  
27 soluble shell protein with an acidic C-terminus, identified in the prismatic layer of the Mediterranean fan  
28  
29 mussel *Pinna nobilis* (Bivalvia, Pteriomorphia), *FEBS Open Bio* 13 (2023) 10-25.  
30  
31 [2] H.A. Lowenstam, S. Weiner, *On biomineralization*, Oxford University Press New York, 1989.  
32  
33 [3] A. Knoll, *Biomineralization and Evolutionary History*, *Reviews in Mineralogy & Geochemistry* 54  
34  
35 (2003) 329-356.  
36  
37 [4] J. Aizenberg, A. Tkachenko, S. Weiner, L. Addadi, G. Hendler, Calcitic microlenses as part of the  
38  
39 photoreceptor system in brittlestars, *Nature* 412 (2001) 819-822.  
40  
41 [5] W. Dong, H. Cheng, Y. Yao, Y. Zhou, G. Tong, D. Yan, Y. Lai, W. Li, Bioinspired synthesis of  
42  
43 calcium carbonate hollow spheres with a nacre-type laminated microstructure, *Langmuir* 27 (2011) 366-  
44  
45 370.  
46  
47 [6] K. Lee, W. Wagermaier, A. Masic, K.P. Kommareddy, M. Bennet, I. Manjubala, S.-W. Lee, S.B.  
48  
49 Park, H. Cölfen, P. Fratzl, Self-assembly of amorphous calcium carbonate microlens arrays, *Nature*  
50  
51 *Communications* 3 (2012) 725.  
52  
53 [7] M. Różycka, I. Coronado, K. Brach, J. Olesiak-Bańska, M. Samoć, M. Zarębski, J. Dobrucki, M. Ptak,  
54  
55 E. Weber, I. Polishchuk, B. Pokroy, J. Stolarski, A. Ozyhar, Lattice shrinkage by incorporation of  
56  
57 recombinant starmaker-like protein within bioinspired calcium carbonate crystals, *Chemistry – A*  
58  
59 *European Journal* 25 (2019) 12740-12750.  
60  
61

- 1  
2  
3  
4 [8] A. Jackson, J. Vincent, R.M. Turner, The Mechanical Design of Nacre, Proceedings of The Royal  
5 Society of London 234 (1988) 415-440.  
6  
7  
8 [9] J.G. Carter, Skeletal Biomineralization: Patterns, Processes and Evolutionary Trends, Van Nostrand  
9 Reinhold, New York, 1990.  
10  
11  
12 [10] J.-P. Cuif, Y. Dauphin, A. Denis, D. Gaspard, J. Keller, Etude des caracteristiques de la phase  
13 minerale dans les structures prismatiques du tests de quelques Mollusques, Bulletin du Muséum National  
14 d'Histoire Naturelle de Paris 4e sér. sect. A (1983) 679-717.  
15  
16  
17 [11] W.J. Schmidt, Die Bausteine des Tierkörpers in polarisiertem Lichte, F. Cohen, Bonn, Germany,  
18 1924.  
19  
20  
21 [12] H. Cölfen, M. Antonietti, Mesocrystals: inorganic superstructures made by highly parallel  
22 crystallization and controlled alignment, Angewandte Chemie International Edition 44 (2005) 5576-5591.  
23  
24  
25 [13] H. Cölfen, M. Antonietti, Mesocrystals and Nonclassical Crystallization, John Wiley & Sons, Ltd.,  
26 Chichester, 2008.  
27  
28  
29 [14] J.J. De Yoreo, P.U.P.A. Gilbert, N.A.J.M. Sommerdijk, R.L. Penn, S. Whitlam, D. Joester, H.  
30 Zhang, J.D. Rimer, A. Navrotsky, J.F. Banfield, A.F. Wallace, F.M. Michel, F.C. Meldrum, H. Cölfen,  
31 P.M. Dove, Crystallization by particle attachment in synthetic, biogenic, and geologic environments,  
32 Science 349 (2015) aaa6760.  
33  
34  
35 [15] S.E. Wolf, C.F. Böhm, J. Harris, B. Demmert, D.E. Jacob, M. Mondeshki, E. Ruiz-Agudo, C.  
36 Rodríguez-Navarro, Nonclassical crystallization in vivo et in vitro (I): Process-structure-property  
37 relationships of nanogranular biominerals, Journal of Structural Biology 196 (2016) 244-259.  
38  
39  
40 [16] C. Rodríguez-Navarro, E. Ruiz-Agudo, J. Harris, S.E. Wolf, Nonclassical crystallization in vivo et in  
41 vitro (II): Nanogranular features in biomimetic minerals disclose a general colloid-mediated crystal  
42 growth mechanism, Journal of Structural Biology 196 (2016) 260-287.  
43  
44  
45 [17] F. Marin, N. Le Roy, B. Marie, The formation and mineralization of mollusk shell, Frontiers in  
46 Bioscience (Scholar edition) 4 (2012) 1099-1125.  
47  
48  
49 [18] I. Weiss, V. Schönitzer, N. Eichner, M. Sumper, The chitin synthase involved in marine bivalve  
50 mollusk shell formation contains a myosin domain, FEBS Letters 580 (2006) 1846-1852.  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

- 1  
2  
3  
4 [19] I. Herlitzke, B. Marie, F. Marin, D.J. Jackson, Molecular modularity and asymmetry of the molluscan  
5 mantle revealed by a gene expression atlas, *GigaScience* 7 (2018) giy056.  
6  
7  
8 [20] F. Marin, B. Marie, S. Hamada, P. Ramos-Silva, N. Le Roy, N. Guichard, S. Wolf, C. Montagnani,  
9 C. Joubert, D. Piquemal, D. Saulnier, Y. Gueguen, 'Shellome': Proteins Involved in Mollusc Shell  
10 Biomineralization —Diversity, Functions., in: S. Watabe, K. Maeyama, H. Nagasawa (Eds.), *Recent*  
11 *Advances in Pearl Research - Proceedings of the International Symposium on Pearl Research 2011*,  
12 Terrapub, Tokyo, 2013, pp. 149-166.  
13  
14  
15 [21] F. Marin, Mollusc shellomes: Past, present and future, *Journal of Structural Biology* 212 (2020)  
16 107583.  
17  
18 [22] T. Yarra, M. Blaxter, M.S. Clark, A Bivalve Biomineralization Toolbox, *Molecular Biology and*  
19 *Evolution* 38 (2021) 4043-4055.  
20  
21  
22 [23] S. Cohen, E. Kalfon-Cohen, Dynamic nanoindentation by instrumented nanoindentation and force  
23 microscopy: A comparative review, *Beilstein Journal of Nanotechnology* 4 (2013) 815-833.  
24  
25  
26 [24] U. Dammer, M. Hegner, D. Anselmetti, P. Wagner, M. Dreier, W. Huber, H.J. Güntherodt, Specific  
27 antigen/antibody interactions measured by force microscopy, *Biophysical Journal* 70 (1996) 2437-2441.  
28  
29  
30 [25] P.K. Hansma, J.P. Cleveland, M. Radmacher, D.A. Walters, P.E. Hillner, M. Bezanilla, M. Fritz, D.  
31 Vie, H.G. Hansma, C.B. Prater, J. Massie, L. Fukunaga, J. Gurley, V. Elings, Tapping mode atomic force  
32 microscopy in liquids, *Applied Physics Letters* 64 (1994) 1738-1740.  
33  
34  
35 [26] M. Pfreundschuh, D. Alsteens, M. Hilbert, M.O. Steinmetz, D.J. Müller, Localizing Chemical  
36 Groups while Imaging Single Native Proteins by High-Resolution Atomic Force Microscopy, *Nano*  
37 *Letters* 14 (2014) 2957-2964.  
38  
39  
40 [27] M. Rief, M. Gautel, F. Oesterhelt, J.M. Fernandez, H.E. Gaub, Reversible unfolding of individual  
41 titin immunoglobulin domains by AFM, *Science* 276 (1997) 1109-1112.  
42  
43  
44 [28] C.S. Sikes, A. Wierzbicki, V.J. Fabry, From atomic to global scales in biomineralization, *Bulletin de*  
45 *l'Institut Océanographique* 14 (1994) 1-47.  
46  
47  
48 [29] S. Manne, C.M. Zaremba, R. Giles, L. Huggins, D.A. Walters, A. Belcher, D.E. Morse, G.D. Stucky,  
49 J.M. Didymus, S. Mann, P.K. Hansma, Atomic force microscopy of the nacreous layer in mollusc shells,  
50 *Proceedings of the Royal Society of London. Series B: Biological Sciences* 256 (1994) 17-23.  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

- 1  
2  
3  
4 [30] A.G. Checa, C.M. Pina, A.J. Osuna-Mascaró, A.B. Rodríguez-Navarro, E.M. Harper, Crystalline  
5 organization of the fibrous prismatic calcitic layer of the Mediterranean mussel *Mytilus galloprovincialis*,  
6 European Journal of Mineralogy 26 (2014) 495-505.  
7  
8  
9  
10 [31] S. Senapati, S. Lindsay, Recent progress in molecular recognition imaging using Atomic Force  
11 Microscopy, Accounts of Chemical Research 49 (2016) 503-510.  
12  
13  
14 [32] H. Clausen-Schaumann, M. Seitz, R. Krautbauer, H.E. Gaub, Force spectroscopy with single bio-  
15 molecules, Current Opinion in Chemical Biology 4 (2000) 524-530.  
16  
17  
18 [33] T.E. Fisher, P.E. Marszalek, J.M. Fernandez, Stretching single molecules into novel conformations  
19 using the atomic force microscope, Nature Structural Biology 7 (2000) 719-724.  
20  
21  
22 [34] C.D. Frisbie, L.F. Rozsnyai, A. Noy, M.S. Wrighton, C.M. Lieber, Functional Group Imaging by  
23 Chemical Force Microscopy, Science 265 (1994) 2071-2074.  
24  
25  
26 [35] S. Allen, J. Davies, M.C. Davies, A.C. Dawkes, C.J. Roberts, S.J.B. Tendler, P.M. Williams, The  
27 influence of epitope availability on atomic-force microscope studies of antigen–antibody interactions,  
28 Biochemical Journal 341 (1999) 173-178.  
29  
30  
31  
32 [36] P. Hinterdorfer, W. Baumgartner, H. Gruber, K. Schilcher, H. Schindler, Detection and localization  
33 of individual antibody-antigen recognition events by Atomic Force Microscopy, Proceedings of the  
34 National Academy of Sciences of the United States of America 93 (1996) 3477-3481.  
35  
36  
37 [37] Y. Jiang, C. Zhu, L. Ling, L. Wan, X. Fang, C. Bai, Specific Aptamer–Protein Interaction Studied by  
38 Atomic Force Microscopy, Analytical Chemistry 75 (2003) 2112-2116.  
39  
40  
41 [38] J.S. Kim, S. Jang, U. Kim, K. Cho, AFM Studies of Inhibition Effect in Binding of Antimicrobial  
42 Peptide and Immune Proteins, Langmuir 23 (2007) 10438-10440.  
43  
44  
45 [39] H. Wang, Y. Dalal, S. Henikoff, S. Lindsay, Single-epitope recognition imaging of native chromatin,  
46 Epigenetics & Chromatin 1 (2008) 1-9.  
47  
48  
49 [40] P.-H. Puech, K. Poole, D. Knebel, D.J. Muller, A new technical approach to quantify cell–cell  
50 adhesion forces by AFM, Ultramicroscopy 106 (2006) 637-644.  
51  
52  
53 [41] A.V. Taubenberger, D.W. Hutmacher, D.J. Muller, Single-Cell Force Spectroscopy, an Emerging  
54 Tool to Quantify Cell Adhesion to Biomaterials, Tissue Engineering Part B: Reviews 20 (2013) 40-55.  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

- 1  
2  
3  
4 [42] V.T. Moy, E.L. Florin, H.E. Gaub, Intermolecular forces and energies between ligands and receptors,  
5 Science 266 (1994) 257-259.  
6  
7  
8 [43] B. Marie, G. Luquet, J.-P. Pais De Barros, N. Guichard, S. Morel, G. Alcaraz, L. Bollache, F. Marin,  
9 The shell matrix of the freshwater mussel *Unio pictorum* (Paleoheterodonta, Unionoida). Involvement of  
10 acidic polysaccharides from glycoproteins in nacre mineralization., The FEBS Journal 274 (2007) 2933-  
11 2945.  
12  
13  
14  
15 [44] J.H. Morrissey, Silver stain for proteins in polyacrylamide gels: A modified procedure with enhanced  
16 uniform sensitivity, Analytical Biochemistry 117 (1981) 307-310.  
17  
18  
19  
20 [45] P. Matsudaira, Sequence from picomole quantities of proteins electroblotted onto polyvinylidene  
21 difluoride membranes, Journal of Biological Chemistry 262 (1987) 10035-10038.  
22  
23  
24 [46] S. Allen, X. Chen, J. Davies, M.C. Davies, A.C. Dawkes, J.C. Edwards, C.J. Roberts, J. Sefton,  
25 S.J.B. Tendler, P.M. Williams, Detection of antigen-antibody binding events with the Atomic Force  
26 Microscope, Biochemistry 36 (1997) 7457-7463.  
27  
28  
29  
30 [47] A. Vinckier, I. Heyvaert, A. D'Hoore, T. McKittrick, C. Van Haesendonck, Y. Engelborghs, L.  
31 Hellemans, Immobilizing and imaging microtubules by atomic force microscopy, Ultramicroscopy 57  
32 (1995) 337-343.  
33  
34  
35  
36 [48] A. Yersin, H. Hirling, P. Steiner, S. Magnin, R. Regazzi, B. Hüni, P. Huguenot, P. De Los Rios, G.  
37 Dietler, S. Catsicas, S. Kasas, Interactions between synaptic vesicle fusion proteins explored by atomic  
38 force microscopy, Proceedings of the National Academy of Sciences of the United States of America 100  
39 (2003) 8736-8741.  
40  
41  
42  
43 [49] G. Leclercq, I. Laïos, C. Elie-Caille, D. Leiber, G. Laurent, E. Lesniewska, Z. Tanfin, Y. Jacquot,  
44 ER $\alpha$  dimerization: a key factor for the weak estrogenic activity of an ER $\alpha$  modulator unable to compete  
45 with estradiol in binding assays, Journal of Receptors and Signal Transduction 37 (2017) 149-166.  
46  
47  
48  
49 [50] B. Alexandre, O. Ben, Common approaches to tip functionalization for AFM-based molecular  
50 recognition measurements, Bruker guide, Application Note #130 (2010).  
51  
52  
53  
54 [51] P. Silberzan, L. Leger, D. Ausserre, J.J. Benattar, Silanation of silica surfaces. A new method of  
55 constructing pure or mixed monolayers, Langmuir 7 (1991) 1647-1651.  
56  
57  
58 [52] G. Lykotrafitis, AFM tip functionalization with glutaraldehyde, protocols.io (2019).  
59  
60  
61  
62  
63  
64  
65

- 1  
2  
3  
4 [53] Y. Jacquot, D. Spaggiari, J. Schappler, E. Lesniewska, S. Rudaz, and G. Leclercq, ERE-dependent  
5 transcription and cell proliferation: Independency of these two processes mediated by the introduction of  
6 a sulfone function into the weak estrogen estrothiazine, *European Journal of Pharmaceutical Sciences* 109  
7 (2017) 169-181.  
8  
9  
10  
11 [54] V. Filipe, R. Poole, O. Oladunjoye, K. Braeckmans, W. Jiskoot, Detection and Characterization of  
12 Subvisible Aggregates of Monoclonal IgG in Serum, *Pharmaceutical Research* 29 (2012) 2202-2212.  
13  
14  
15 [55] G. Muyzer, P. Westbroek, An immunohistochemical technique for the localization of preserved  
16 biopolymeric remains in fossils, *Geochimica et Cosmochimica Acta* 53 (1989) 1699-1702.  
17  
18  
19 [56] F. Marin, P. Narayanappa, S. Motreuil, Acidic shell proteins of the Mediterranean fan mussel *Pinna*  
20 *nobilis*, in: W.E.G. Müller (Ed.), *Molecular Biomineralization: Aquatic Organisms Forming*  
21 *Extraordinary Materials*, Springer Berlin Heidelberg, Berlin, Heidelberg, 2011, pp. 353-395.  
22  
23  
24 [57] F. Marin, B. Pokroy, G. Luquet, P. Layrolle, K. De Groot, Protein mapping of calcium carbonate  
25 biominerals by immunogold, *Biomaterials* 28 (2007) 2368-2377.  
26  
27  
28  
29 [58] A.J. Osuna-Mascaró, T. Cruz-Bustos, F. Marin, A.G. Checa, Ultrastructure of the interlamellar  
30 membranes of the nacre of the bivalve *Pteria hirundo*, determined by immunolabelling, *PLOS ONE* 10  
31 (2015) e0122934.  
32  
33  
34 [59] A. Osuna-Mascaró, T. Cruz Bustos, B. Marie, A. Checa, F. Marin, Heavy Metals in Mollusc Shells:  
35 A Quick Method for their Detection, *Key Engineering Materials* 672 (2015) 340-346.  
36  
37  
38 [60] F. Nudelman, B. Gotliv, L. Addadi, S. Weiner, Mollusk shell formation: Mapping the distribution of  
39 organic matrix components underlying a single aragonitic tablet in nacre, *Journal of Structural biology*  
40 153 (2006) 176-187.  
41  
42  
43 [61] L.M. Gordon, D. Joester, Nanoscale chemical tomography of buried organic–inorganic interfaces in  
44 the chiton tooth, *Nature* 469 (2011) 194-197.  
45  
46  
47 [62] S. Amarie, P. Zaslansky, Y. Kajihara, G. Erika, W. Schmahl, F. Keilmann, Nano-FTIR chemical  
48 mapping of minerals in biological materials, *Beilstein Journal of Nanotechnology* 3 (2012) 312-323.  
49  
50  
51 [63] N. Trinkler, M. Labonne, F. Marin, A. Jolivet, M. Bohn, C. Poulain, J.F. Bardeau, C. Paillard, Clam  
52 shell repair from the brown ring disease: A study of the organic matrix using Confocal Raman micro-  
53 spectrometry and WDS microprobe, *Analytical and Bioanalytical Chemistry* 396 (2010) 555-567.  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

- 1  
2  
3  
4 [64] C. Hedegaard, J.F. Bardeau, D. Chateigner, Molluscan shell pigments: an *in situ* Resonance Raman  
5 Study, *Journal of Molluscan Studies* 72 (2006) 157–162.  
6  
7  
8 [65] R. Hovden, S.E. Wolf, M.E. Holtz, F. Marin, D.A. Muller, L.A. Estroff, Nanoscale assembly  
9 processes revealed in the nacreprismatic transition zone of *Pinna nobilis* mollusc shells, *Nature*  
10 *Communications* 6 (2015) 10097.  
11  
12  
13 [66] D. L. Masica and J. J. Gray, Solution-and Adsorbed-State Structural Ensembles Predicted for the  
14 Statherin-Hydroxyapatite System, *Biophysical Journal* 96 (2009) 3082–3091.  
15  
16  
17 [67] L. Faye, M.J. Chrispeels, Common antigenic determinants in the glycoproteins of plants, molluscs  
18 and insects, *Glycoconjugate Journal* 5 (1988) 245-256.  
19  
20  
21 [68] V. Mouchi, F. Lartaud, N. Guichard, F. Immel, M. de Rafélis, C. Broussard, Q.G. Crowley, F.  
22 Marin, Chalky versus foliated: a discriminant immunogold labelling of shell microstructures in the edible  
23 oyster *Crassostrea gigas*, *Marine Biology* 163 (2016) 1-5.  
24  
25  
26 [69] J.R. Rao, S.C. Yadav, G.C. Ram, P.R.S. Raghav, R.B. Lal, Common antigenic determinants of  
27 *Fasciola gigantica* as defined by monoclonal antibodies to phosphocholine, *Journal of Applied Animal*  
28 *Research* 9 (1996) 95-104.  
29  
30  
31 [70] J.K. Stuart, V. Hlady, Effects of Discrete Protein-Surface Interactions in Scanning Force Microscopy  
32 Adhesion Force Measurements, *Langmuir* 11 (1995) 1368-1374.  
33  
34  
35 [71] D.C. Zhang, J.B. Xiang, L.Y. Wang, Z.B. Xu, L.D. Sun, F. Zhou, X.L. Zha, D. Cai, Comparative  
36 Proteomic Analysis of Gallbladder Bile Proteins Related to Cholesterol Gallstones, *PLOS ONE* 8 (2013)  
37 art n° e54489.  
38  
39  
40 [72] P. Peerapen, V. Thongboonkerd, Kidney stone proteomics: an update and perspectives, *Expert*  
41 *Review of Proteomics* 18 (2021) 557-569.  
42  
43  
44 [73] Y. Liu, C. Schouten, O. Boerman, G. Wu, J.A. Jansen, E.B. Hunziker, The kinetics and mechanism  
45 of bone morphogenetic protein 2 release from calcium phosphate-based implant-coatings, *Journal of*  
46 *Biomedical Materials Research Part A* 106 (2018) 2363-2371.  
47  
48  
49 [74] C.D. Reyes, T.A. Petrie, K.L. Burns, Z. Schwartz, A.J. García, Biomolecular surface coating to  
50 enhance orthopaedic tissue healing and integration, *Biomaterials* 28 (2007) 3228-3235.  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

[75] J. Sakalauskaite, F. Marin, B. Pergolizzi, B. Demarchi, Shell palaeoproteomics: first application of peptide mass fingerprinting for the rapid identification of mollusc shell in archaeology, *Journal of Proteomics* 227 (2020), 103920.

## Figures captions

Figure 1. The shell of *Pinna nobilis*. a) Inner view of the right valve of an adult specimen. The internal nacreous layer (NL, whitish, delineated by a black dashed line) covers less than one half of the shell, contrarily to the prismatic outer layer (PL, brown-red). b) Inner SEM view of the prismatic layer, that consists of needles of polygonal section that are densely packed together and glued by thin interprismatic sheaths (dark line). The blue arrow symbolizes the growth axis of the prisms. c) An isolated single prism observed by SEM. Note that the prism is not homogeneous but constituted of a stack of thin mineralized layers perpendicular to its elongation axis.

Figure 2. (a) Amino acid sequence of accripin11, the protein of the prismatic layer, localized in this study by AFM with functionalized tip. The signal peptide is indicated in red. The two domains underlined in yellow and grey correspond to the two synthetic peptides used for generating polyclonal antibodies in white rabbits. (b) 3D-structure of accripin11, deduced from AlphaFold2 simulation. The two alpha helices are stabilized by four disulfide bonds while the C-terminus is disordered.

Figure 3. Principle of AFM surface scanning with functionalized tip. a) General scheme of molecular recognition imaging measurements in which ligand molecules (A) are attached to the AFM tip, whereas receptor molecules (B) are present on the sample surface. The tip is symbolized in grey. b) The amine group was introduced by silanization reaction between silicon surface of the tip and a trichlorosilane group of APTES, the silane reagent. After silanization, glutaraldehyde was used as a binding agent to attach antibodies (IgG) covalently to the tip. c) Typical Force-Distance curves recorded upon approach (blue curve) and retraction (red curve). The black triangles symbolize the AFM tip in different positions. From position 1–2, the tip is approaching the biomineral surface. Then, the cantilever bends until the force reaches the maximum limit as shown in position 3. The tip starts withdrawing at position 4 that further detaches from the surface and returns to its resting position 5. d)

more detailed theoretical view of the interaction between the antibody-grafted tip and the surface of the sample with the target protein.

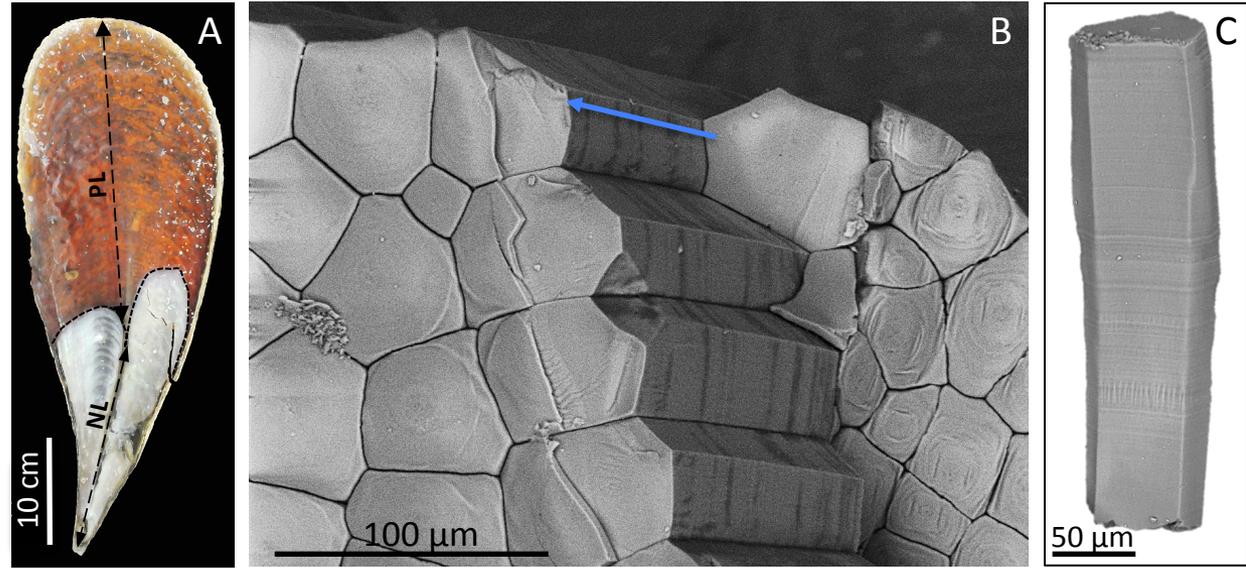
Figure 4. Specificity of the anti-accripin11 antibody preparation. a) Test of the unpurified antibody against the soluble prism matrix. The soluble prism matrix, which contains several discrete bands, was checked on a monodimensional SDS-PAGE gel stained with silver (lane 2) and tested in parallel against the unpurified anti-accripin11 antibody (lane 4). Note the specificity of the antibody. Lanes 1 and 3: prestained molecular markers. b) Purification of the anti-accripin11 immunoglobulins after their elution from the affinity chromatography column. Lanes 2 to 6 correspond to eluates 1 to 5. Note that most of the immunoglobulins are concentrated in eluates 3 and 4 (lanes 4 and 5). Lane 1: molecular weight markers. c) Same gel as Fig. 4b electroblotted on a nitrocellulose membrane and directly tested against the secondary antibody (Goat anti-Rabbit, Alkaline Phosphatase conjugate). The three bands correspond, from top to bottom, to the full IgG, to the heavy chain and to the light chain, respectively.

Figure 5. Interaction force (pN) measured on *Pinna nobilis* periprismatic membranes for various loading rates from 10 to 1740 nm/s, expressed on a logarithmic scale. For all further experiments, a loading rate of 500 nm/s was selected.

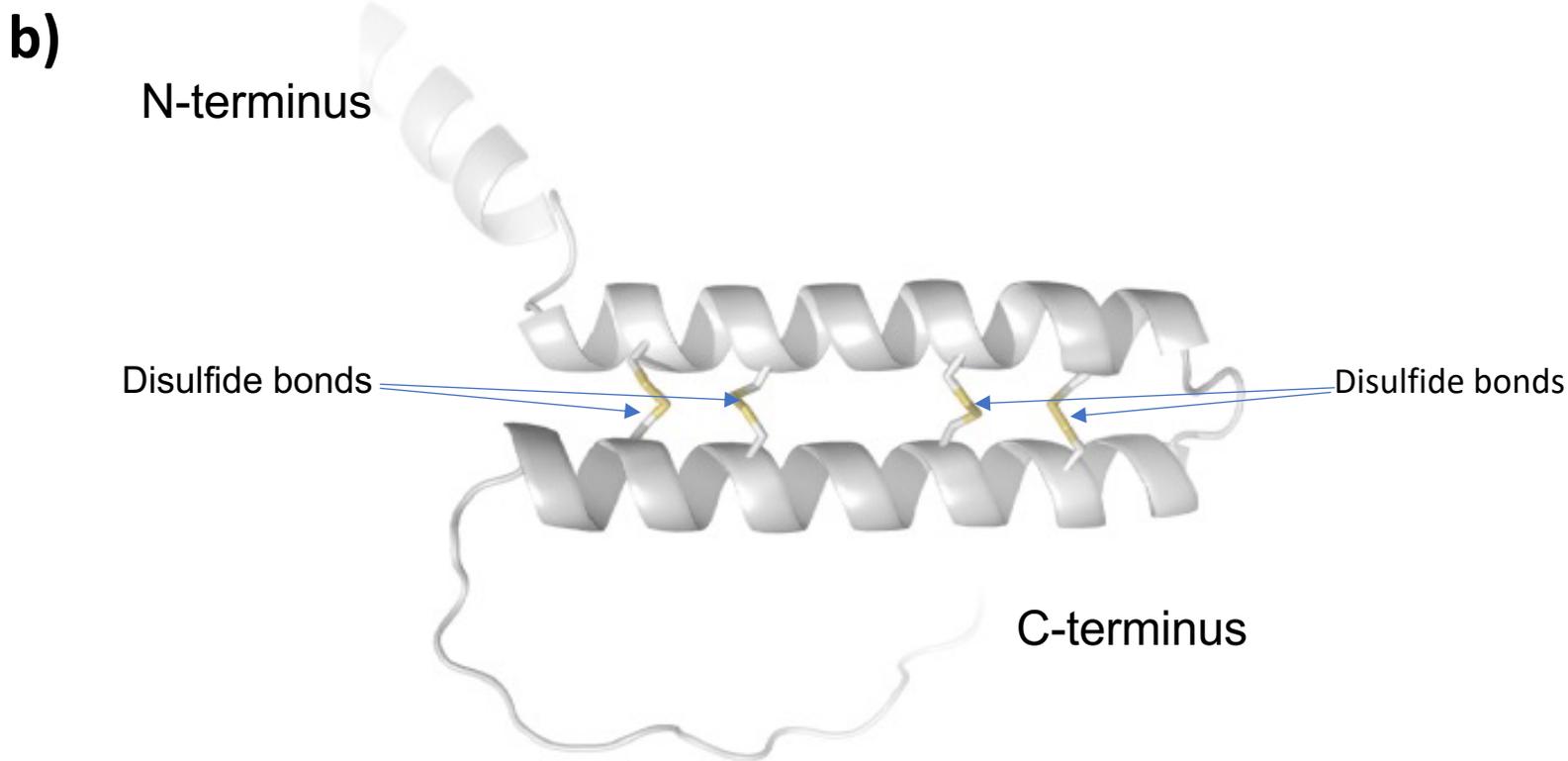
Figure 6. AFM on the calcitic prisms of *Pinna nobilis*. Topographic images in transverse (a, b, c) and in longitudinal (d, e, f) sections, prepared by microtomy: a and d correspond to a 3D block of the surface topography of a prism, b and e, to a 2D image and c and f, to the topographic curve across the periprismatic sheath. In b and e, the white bar symbolizes the sections illustrated by c and f. Note that the difference of height is, at maximum (see c) 140 nm between the top of the periprismatic sheath and the mineral surface, and in general less than 100 nm (f). The AFM tip can accommodate such a difference.

Figure 7. AFM scanning of a prism surface, in transverse (a, b) and longitudinal (c, d) sections, adhesion maps. The surface was prepared by microtomy, without polishing. Figures a and c represent negative controls obtained with non-functionalized tips, while figures b and d are positive controls obtained with accripin11-functionalized tips. The numerous white dots correspond to locations where the adhesion between the functionalized tip and the surface is the highest, *i.e.*, where accripin11 is present. Accripin11 seems to be homogeneously distributed in the prism, but presents also high concentrations along the periprismatic sheaths in d, and at the triple junctions in b. For each of the four maps, the scales are relative.

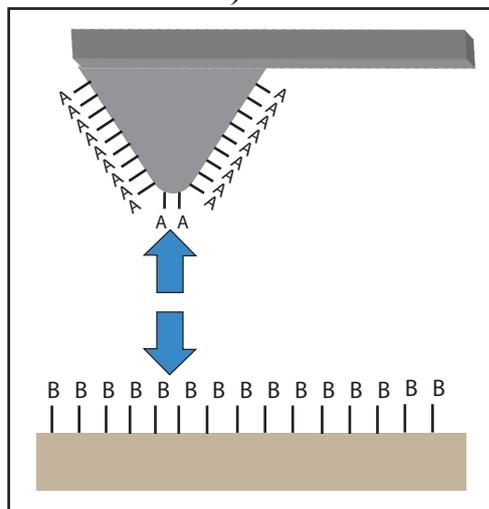
Figure 8. Interaction force measured on *Pinna nobilis* transversal and longitudinal sections, for a loading rate of 500 nm/s. The two negative control histograms (A and C) correspond to zones devoid of accripin11. Note the difference in adhesion in transversal (B) and longitudinal (D) sections.



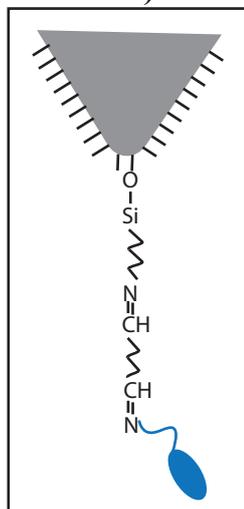
a) MRLLIVFILF VTLAQVFAKP ASNRRNRYMN MVGSVLDNCR RKCIFDNFSC NIPCRLFYTT  
70 80 90 100 110 120  
QRTYKDCAQQ CTRDRETCFG ECTANHGPKK ATPAPTTAAP KPAKEPSSAD DDDDESDESF D



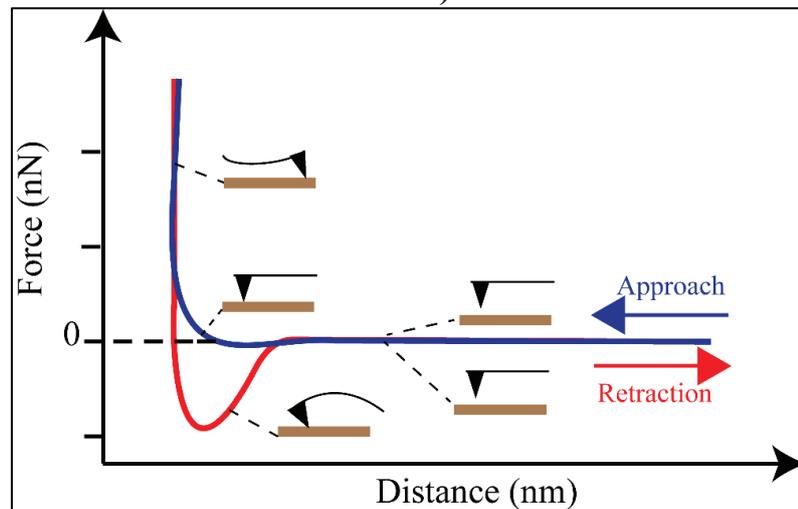
a)



b)



c)



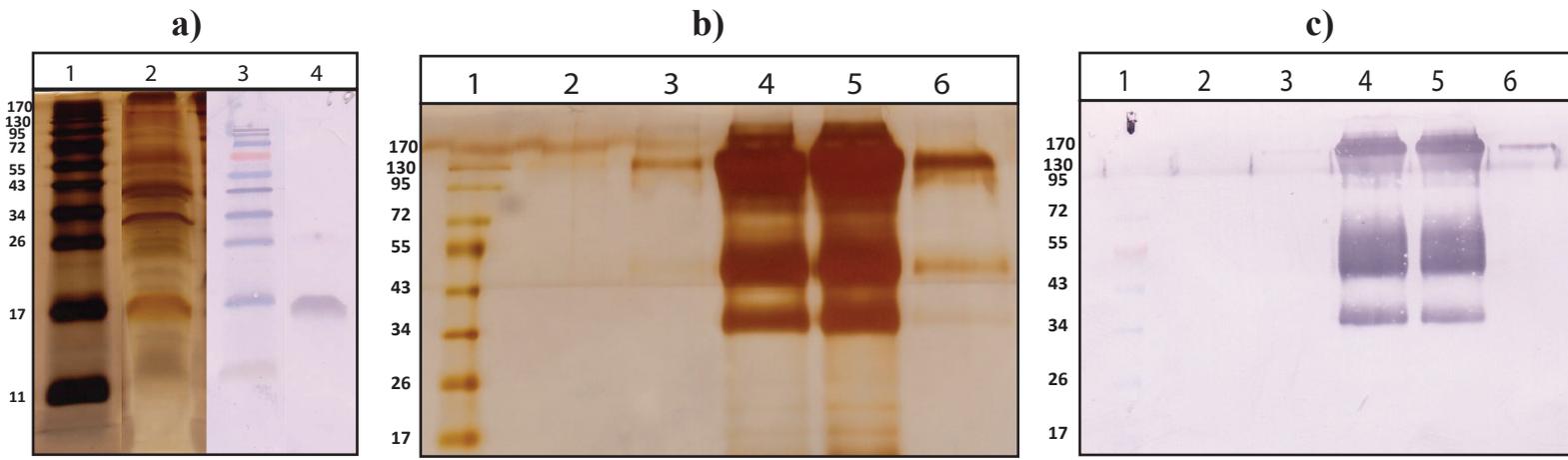
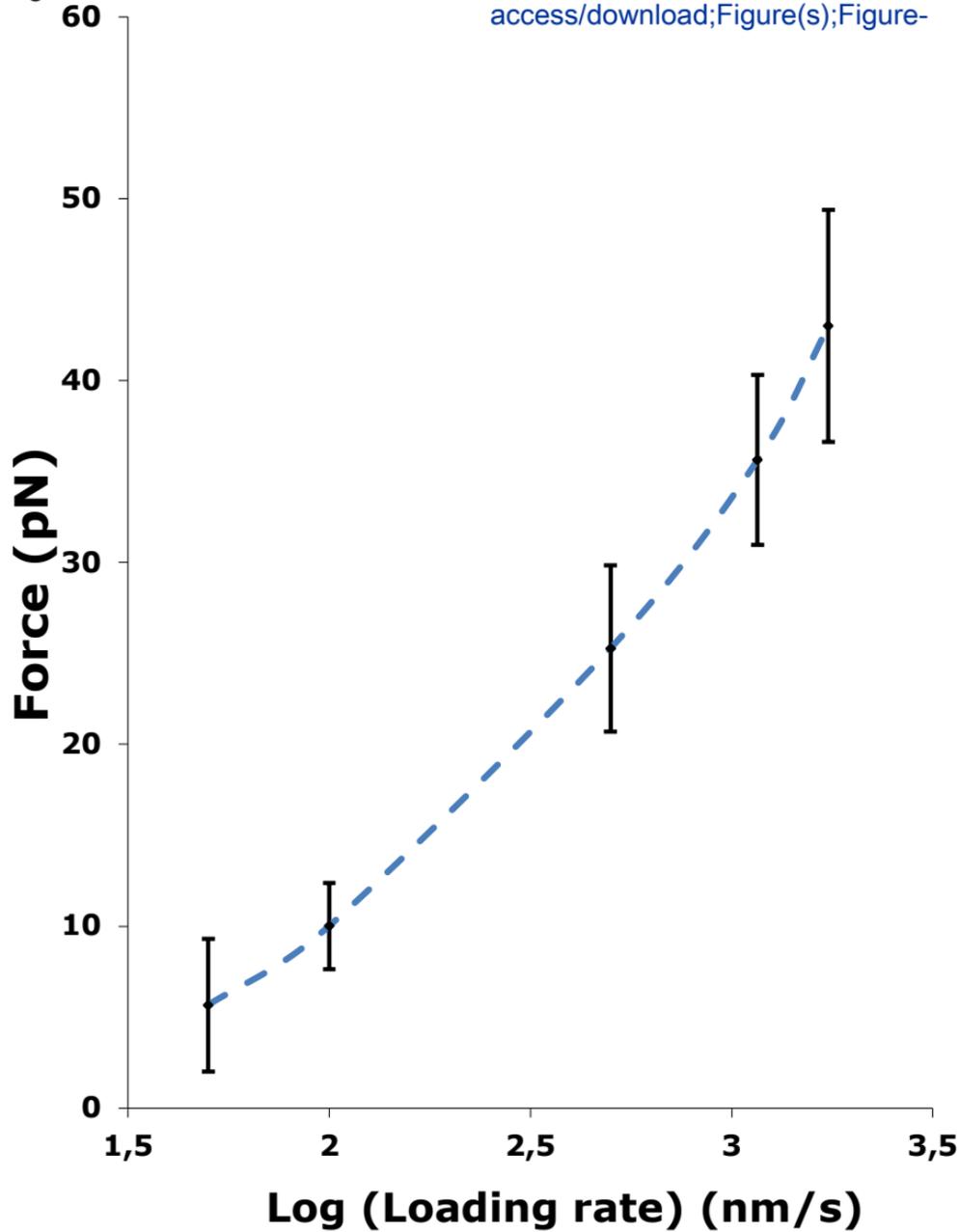
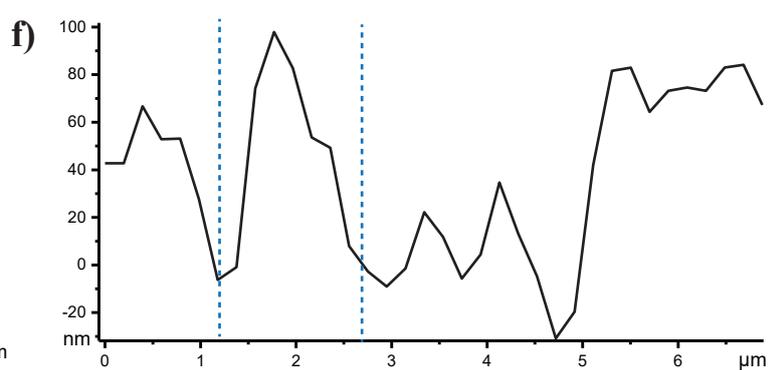
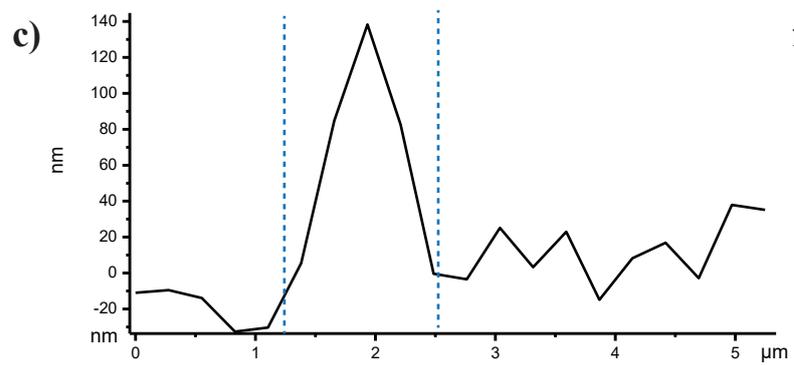
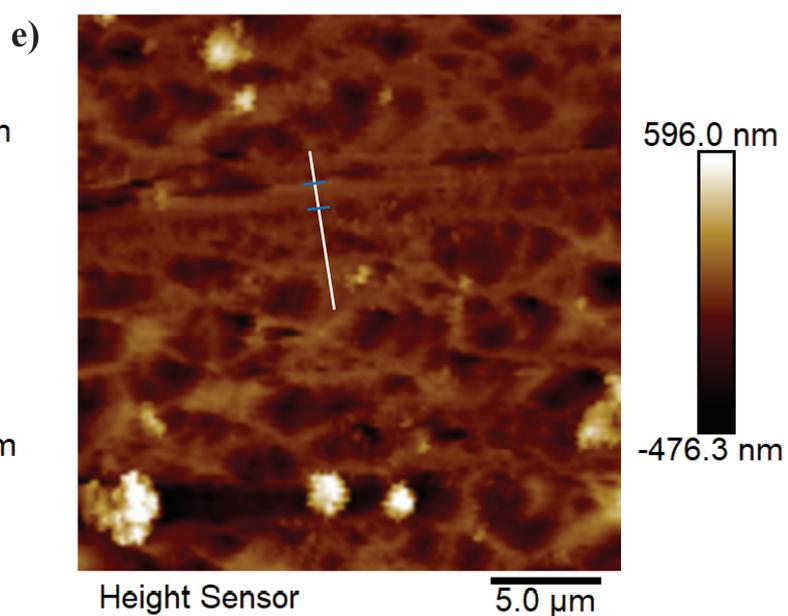
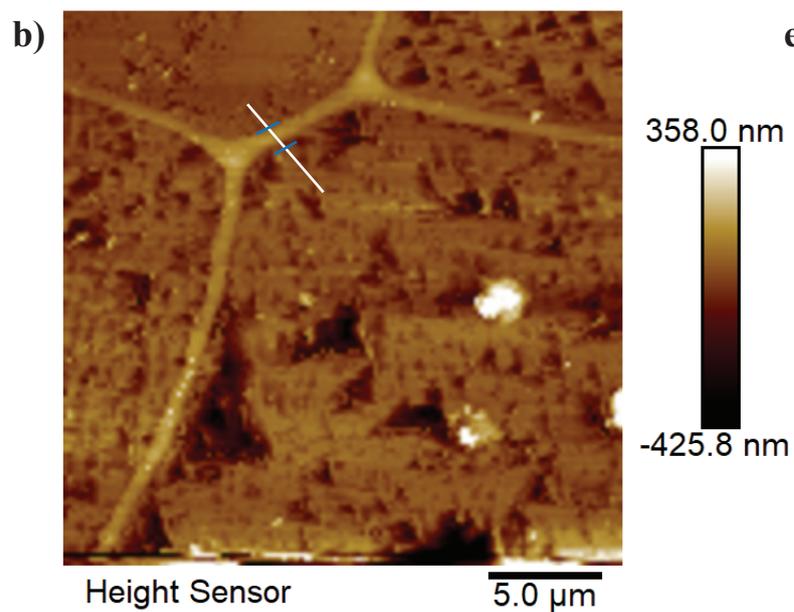
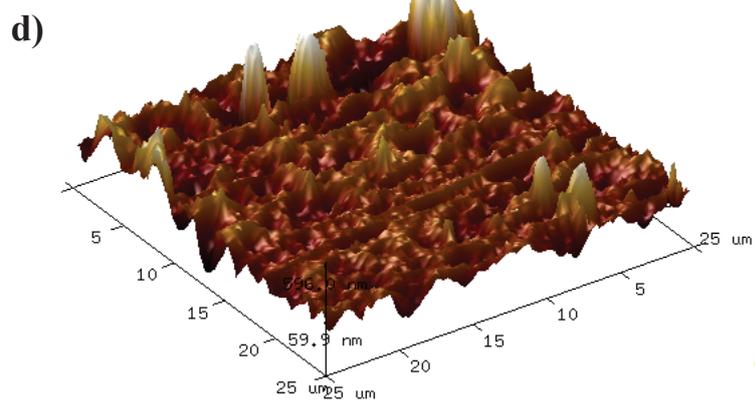
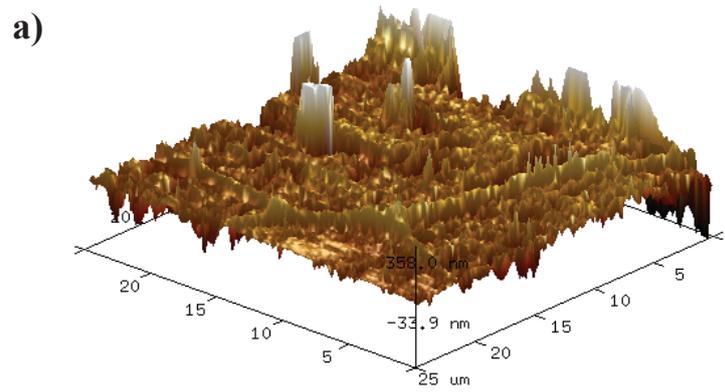


Figure 5

[Click here to access/download;Figure\(s\);Figure-](#)





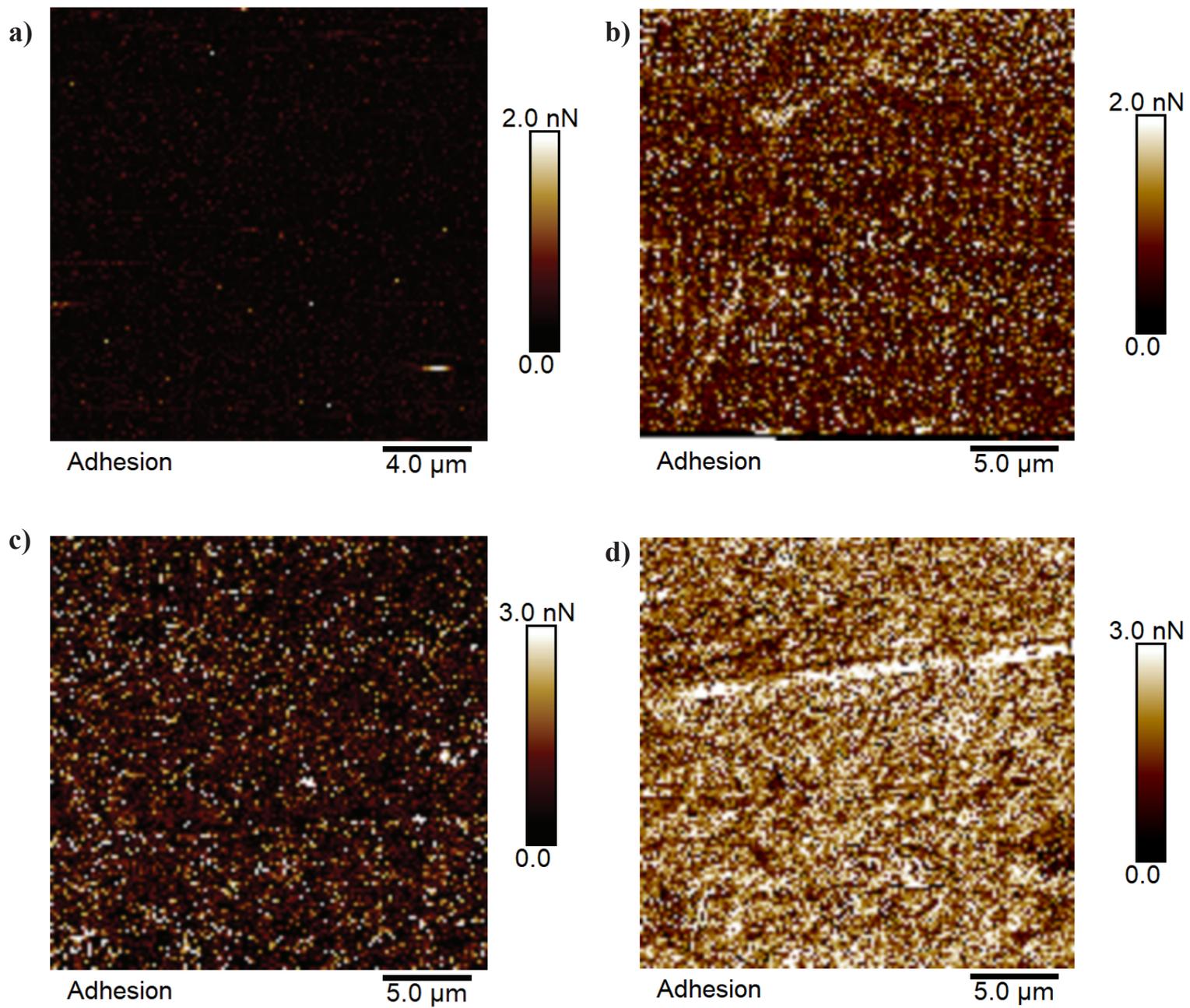
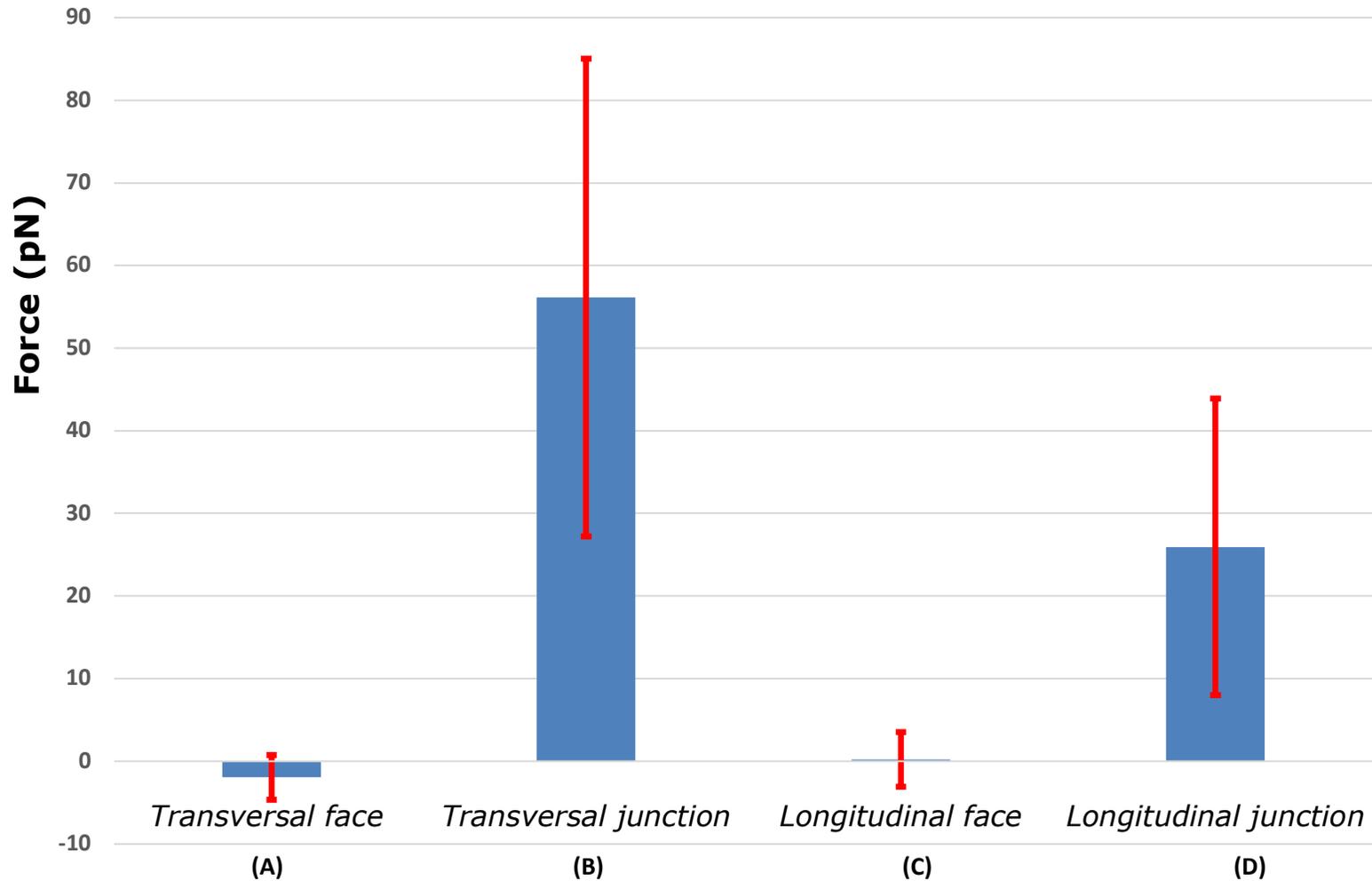


Figure 8

# Adhesion Force

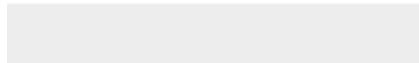
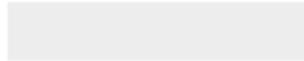




Click here to access/download

**Supplementary Material**

4-Supplementary figure + figure caption.docx



### **Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.