Infrared Scattering-Type Scanning Near-Field Optical Microscopy in Water

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Infrared (IR) absorption spectroscopy detects state and chemical composition of biomolecules solely by their inherent vibrational fingerprints. Major disadvantages like the lack of spatial resolution and sensitivity were compensated lately by the use of pointed probes as local sensors enabling the detection of quantities as few as hundreds of proteins with nanometer precision. This makes infrared scattering-type scanning near-field optical microscopy a very powerful tool in life science. The strong absorption of infrared radiation of liquid water, however, still prevents to simply access the measured quantity – light scattered at the probing atomic force microscope tip. Here we report on the local IR response of biological membranes immersed in aqueous bulk solution. We make use of a silicon solid immersion lens as substrate and focusing optics to achieve detection efficiencies sufficient to yield IR near-field maps of purple membranes. We scrutinized our experimental findings by applying theoretical models. Finally, we suggest a means to improve the imaging quality by laser scanning assisted scattering-type scanning near-field optical microscopy. We believe that IR scattering-type scanning near-field optical microscopy will resolve biological structures in their native environments at nm resolution without the need for labeling.

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Infrared Scattering-Type Scanning Near-Field Optical Microscopy in Water

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Keywords

Infrared spectroscopy, Scanning near-field optical microscopy, nanoscopy, membrane protein, bacteriorhodopsin, IR spectroscopy, biomembranes

Abstract

Infrared (IR) absorption spectroscopy detects state and chemical composition of biomolecules solely by their inherent vibrational fingerprints. Major disadvantages like the lack of spatial resolution and sensitivity were compensated lately by the use of pointed probes as local sensors enabling the detection of quantities as few as hundreds of proteins with nanometer precision. This makes infrared scattering-type scanning near-field optical microscopy a very powerful tool in life science. The strong absorption of infrared radiation of liquid water, however, still prevents to simply access the measured quantity – light scattered at the probing atomic force microscope tip. Here we report on the local IR response of biological membranes immersed in aqueous bulk solution. We make use of a silicon solid immersion lens as substrate and focusing optics to achieve detection efficiencies sufficient to yield IR near-field maps of purple membranes. We scrutinized our experimental findings by applying theoretical models. Finally, we suggest a means to improve the imaging quality by laser scanning assisted scattering-type scanning near-field optical microscopy. We believe that IR scattering-type scanning near-field optical microscopy will resolve biological structures in their native environments at nm resolution without the need for labeling.

Infrared (IR) spectroscopy is a powerful method in life science as it uses the frequencies of the intrinsic molecular vibrations as a means to record structural information of biological samples (e.g. protein fold) in a label-free manner.^{1, 2} Applications range from assignment of bacterial species,³ detection of light-induced changes in protein conformation, cofactors and protonation state of individual amino acid residues,^{4, 5} aggregation states of proteins related to neurodegenerative diseases like Alzheimer's or Parkinson's disease⁶⁻⁸, and label-free imaging of tissue in cancer diagnosis.⁹⁻¹¹ On top of structural information, the temporal resolution of IR spectroscopy matches the decay times of vibrational modes in the femtosecond range.¹² Despite this broad applicability, the low absorption cross-section of protein (10^{-23} cm²) limits the sensitivity of linear IR absorption spectroscopy to bulk samples. Thus, large amounts of purified samples are required which represents a challenge for proteins that are difficult to express in larger quantities, like e.g. eukaryotic membrane proteins, that are the target of 60% of all drugs on the market.¹³

The observation of spatial heterogeneity is limited by diffraction to the range of a few μ m when IR radiation is used.^{14, 15} This resolution is insufficient for examining e.g. subcellular structures or even single particles. Both challenges can be tackled by using metallic antennae which concentrate the incident radiation in the optical near-field generated at their surfaces. Surface-enhanced infrared absorption spectroscopy (SEIRAS)^{12, 16-18} has been proven to yield spectral information at very high sensitivities down to attomolar concentrations in liquid environments.⁸ Still, diffraction-limited resolution applies and microscopic experiments are not able to resolve structural differences beyond several microns.¹⁹ Complementary to that, scattering-type scanning near-field optical microscopy (sSNOM) utilizes the metallized shaft of an atomic force microscope (AFM) tip as a near-field probe which nanofocuses the light to its apex.²⁰ This allows to increase the resolution of IR microspectroscopy to tens of nm^{21, 22} and in rare cases even beyond.^{23, 24} Single protein complexes, virus particles, biological membranes, whole cells, and tissue can be routinely imaged at specific frequencies limited only by the bandwidth of the illumination source.²⁵⁻²⁸ Lately, it was shown that hyperspectral imaging on biological tissue adopting Fourier-

transform IR (FTIR) methodology which allows for even more sophisticated analysis of heterogeneity and band assignment on the nanoscale.^{29, 30} Yet, all those experiments require dried samples. This circumstance does not allow probing the sample in its native environment and prevents recording dynamic processes by IR nanoscopy.

sSNOM has been developed over the past decades to circumvent the limits imposed by diffraction. Particularly, IR near-field spectroscopy shows great potential to address various topics ranging from solid state to life science applications. However, the progress towards application in life science was hampered due to two major reasons: Firstly, water strongly absorbs infrared radiation due to its strong dipole moment. Secondly, optical components for the mid-IR regime like immersion liquids and high numerical aperture (NA) objectives are not readily available. Khatib et al. tried to overcome this challenge by covering the biological sample – tobacco mosaic virus immersed in water – with a graphene monolayer.³¹ Lu et al. succeeded to investigate surface-bound species of water and electrolyte through a graphene layer separating the bulk water and the IR illuminated tip in air.³² Both approaches involve intricate sample preparation and either blur the topographic information or disable its acquisition at all. An alternative route was provided by reading out the IR-induced sample expansion with the AFM tip (AFM IR) which has been demonstrated to allow the spectroscopic investigation of organic and biological samples in liquid water.^{33, 34} Although simple and versatile, AFM IR provides spectral information transduced *via* its thermal expansion which might affect the IR contrast for anisotropic samples.³⁵ Here, we show an alternative illumination and detection scheme which permits IR sSNOM experiments on biological samples, here biomembranes, immersed in bulk aqueous solution. We further prove the imaging capabilities of this geometry and outline strategies for imaging wide fields of view as those typically achieved with atomic force microscopes.

Results and Discussion

Bottom Illumination IR sSNOM. sSNOM typically employs a free-space side illumination and detection scheme which is advantageous in terms of matching the polarization of the

incident light to the tip's long axis and in ease of alignment.³⁶ The coupled dipole-image-dipole of the tip dithering above the sample is effectively excited with p-polarized light.³⁷ Naively switching this scheme to an objective-based illumination from the bottom as common in visible SNOM implementations,³⁸ prevents an efficient excitation of the tip due to the polarization mismatch and the typically available low NA objectives in the IR regime. Here, we address this challenge by introducing a solid immersion lens (SIL) made of silicon (Figure 1 A) as an IR transparent substrate. The Si hemisphere simultaneously enhances the numerical aperture (NA ~ 1.7) and maintains a high polarizability (i.e. high scattering efficiency) of the tip-dipole sample-dipole system by using Si as highly refracting substrate ($n \sim 3.4$).³⁹ Figure 1 B shows approach curves of the homodyne scattered and demodulated light at multiple harmonics ($n = 1,2,3,4$) of the tip oscillation frequency Ω when the tip is vertically moved into the focus at the Si/air interface. The scattered light when demodulated at the fundamental frequency ($n = 1$, blue trace) has significant contributions for tip-sample separations larger than 100 nm because the tip is only partially illuminated by an evanescent field (critical angle Si/air $\theta_c = 17^\circ$, angular range of objective $\theta_o \sim 14^\circ$ - 30°). This is typical for sSNOM approach curves since a significant amount of light is scattered at the tip shaft or the sample which is modulated at the fundamental. The scattered light is, thus, usually demodulated at higher harmonics of the tip ($n = 2$ - 4) to suppress far-field background and extract the near-field contributions only.⁴⁰ The scattered light, demodulated at higher harmonics, clearly shows a drastic increase only for tip-sample separations smaller than 80 nm. We infer that our layout is well-suited to perform IR sSNOM mapping selectively at the Si interface. With higher demodulation orders the sensitivity towards smaller distances further increases,⁴¹ however, at the expense of a lower signal-to-noise ratio.

Figure 1 *Setup and near-field mapping.* **A** Schematic setup of high NA bottom illumination scheme with interferometric pseudo-heterodyne detection of the scattered light. **B** Approach curves in focal spot (homodyne) at higher harmonic demodulation $n = 1 \dots 4$. **C** Map of the near-field distribution in the focal plane (pseudo-heterodyne demodulation) scanning the tip through the focus at the Si/air interface. **D** Calculated distribution of the electric field (E_z) amplitude and phase in the focal plane (Si/air interface).

Figure 1 C shows the complex near-field distribution (left to right: magnitude and phase) when the tip is scanned across the clean Si hemisphere and the scattered light ($\lambda = 6.02 \mu\text{m}$) is recorded interferometrically with a pseudo-heterodyne detection method as previously reported.⁴² Near-field maps of all harmonics can be found in supporting figure 1. The amplitude map shows two central maxima aligned along the incident polarization with side lobes decaying when the tip moves away from the focus. In line with previous work we, anticipate the detected light to report on the z-component of the local electric field at the Si-air interface exclusively.⁴³ We calculated the z-component of the electric field in the focal plane assuming a NA of 1.7, Si ($n = 3.4$) as material between the lens and the focal plane, a dielectric constant of 1 in the focal plane at $\lambda = 6 \mu\text{m}$.⁴⁴ The experimental near-field amplitude

and phase maps excellently match the calculated ones verifying selectivity for the near-field z-component. Scanning the SIL underneath the tip instead of tip scanning leads to near-field distributions with the same shape but larger lateral spread (Supporting figure 2). The focal spot is moved proportionally to the displacement of the SIL in the focal plane of the objective due to the movement of the hemisphere's curved interface. Although the sample scanning misaligns the focus with regard to the tip, the amplitude and phase stays relatively constant within a $1 \times 1 \mu\text{m}^2$ area in the central region of the focus. For sSNOM experiments which opt for nanoscale analysis and only require a region of interest smaller than $1 \times 1 \mu\text{m}^2$, the outlined bottom illumination approach is a viable solution. Tilting the incident beam entering the objective allows to match the region of interest on the silicon interface with the focal region as usually done in laser scanning microscopy.

IR sSNOM in Water. As a proof of concept for IR nanoimaging of biological samples in aqueous solution, we used patches of membrane fragments of the archaeon *H. salinarum*, termed purple membrane (PM), and deposited these on the silicon surface. PM is densely packed with the seven transmembrane α -helical protein bacteriorhodopsin (bR).⁴⁵ Figure 2 shows the near-field amplitude (A), near-field phase (B) and topography (C) of the focal region under ambient condition (air humidity). The PM patches as identified from the topography (Fig. 2C) can be clearly correlated with the patches in the amplitude map (Fig. 2A). Due to their lower index of refraction than silicon, the patches appear as dark structures with lower amplitude on top of the bright background. In the phase map, which resembles well the absorptive information,^{22, 25} barely any feature is visible due to the strong variation of the phase between $\pm\pi$. However, in the central region of the focus the phase changes are less extreme. Zooming into one patch indeed reveals a correlation of the phase with topography when imaged at 1659 cm^{-1} , matching the amide I absorption band of bR, in good agreement with other reports (Figure 2D).^{25, 26} Imaging the same patch at an IR frequency devoid of any absorptive feature ($\nu = 1710 \text{ cm}^{-1}$, Figure 2E), does not yield any significant contrast in the near-field phase. Upon repetition of the image recording with the cantilever, tip, and sample immersed in 200-500 μl buffer solution (Figure 2 G-I), we clearly identified

the local IR absorption in the map recorded when the illuminating laser is tuned to the amide I absorption ($\nu = 1659 \text{ cm}^{-1}$, Figure 2G). Only a slight negative phase contrast was resolved in the range of optical transparency (e.g. $\nu = 1710 \text{ cm}^{-1}$, Figure 2 H). The phase contrast appears noisier in both maps although the amplitude map showed the same magnitude. This might have been caused by the use of Pt-coated AFM tips which are optimized for operation in air. The lock-in amplifier is not able to track the tips oscillation frequency accurately at all times due to the significantly lowered Q-factor in liquid resulting in increased noise in the demodulated signal. Extracting line profiles from the phase maps across an edge of a PM patch (Supporting figure 3) reveals an increase in lateral resolution from 26 nm to 81 nm. This increase is most likely related to the non-optimal operation of the cantilever which might have damaged the tip while imaging. As a consequence, the tip diameter is effectively increased and, correspondingly, the spatial resolution is less. This agrees with the observation that the topography is slightly more blurred in liquid as in air. We infer that the use of cantilevers optimized for tapping mode operation in liquid will significantly improve the signal-to-noise ratio and the spatial resolution.

To scrutinize our results by quasi-electrostatic theory, we calculated the modulus and argument of the tips polarizability when placed atop a generic protein (permittivity composed of an amide I and amide II vibrational mode as described in ref. ⁴⁶) or a pure Si surface immersed either in air or in water with the tip approximated as a polarizable sphere (Supporting Figure 4A&B).⁴⁷ Although the spectral response includes as well the vibrational modes of bulk water, a normalization procedure as commonly done in sSNOM⁴⁸ and nanoFTIR⁴⁹ reproduces well the characteristic shape of both the amplitude and phase information (Supporting Figure 4C&D). Interestingly, the expected phase contrast seems to be enhanced due to the larger polarizability of the aqueous environment rendering the use of a highly polarizable medium even more advantageous.

Figure 2 Near-field mapping of PM in dry and aqueous environment. **A-C** Near-field amplitude (**A**), near-field phase (**B**) and topography (**C**) of an 10x10 μm overview of region of focal region in dry condition. **D-F** Near-field map of a PM patch in dry recorded at 1659 cm^{-1} (**D**) and 1711 cm^{-1} (**E**) and corresponding topography (**F**). **G-I** Near-field map of a PM patch immersed in buffer recorded at 1659 cm^{-1} (**G**) and 1711 cm^{-1} (**H**) and corresponding topography (**I**).

Laser-scanning assisted sSNOM. Although other strategies were proposed and tested for recording the scattered light of samples located in liquid water^{31, 32} the here proposed method appeals with simplicity, the versatility to investigate, in principle, any sample geometry (as long as the sample is sufficiently thin for the IR radiation to be transmitted) and simultaneously map the samples topography. Yet, the strong variation of amplitude and phase limits the region of interest to an area of $1 \times 1 \mu\text{m}^2$. In the following we will address this issue.

Any displacement of the Si-SIL leads to a displacement of the focal spot relative to the steady tip due to the curved interface of the hemisphere. This misalignment of the tip and the focus can be compensated by tracking the tip's position by scanning the beam system much like in laser scanning confocal microscopy.⁵⁰ Although tracking the tip with a scanned laser is not genuine,⁵¹ it comes at full potential here. We modified our home-built pseudo-heterodyne sSNOM system such that the illumination and detection paths of the near-field scattered light pass a pair of galvanometric scan mirrors relayed by a pair of telecentric lenses imaging the beam into the entrance pupil of the Schwarzschild objective (Figure 3A). With this geometry,

the focus is precisely steered in the focal plane such that it is aligned with the tip of the AFM while scanning the sample relative to the tip. As a result, the field of view ($30 \times 30 \mu\text{m}^2$) is illuminated homogeneously (Figure 3B&C). The amplitude (Figure 3B) and phase (Figure 3C) are constant except for the very central region. This is most likely due to interference of light reflected at the lower curved faces of the hemisphere yielding interference patterns. The latter will be significantly reduced by applying an anti-reflection coating to the curved face if necessary. This modification allows imaging the chemical composition of areas larger by more than an order of magnitude compared to the unmodified system.

Figure 3 *Scanning focus enables wide field of view.* **A** Bottom-illumination setup modified with a laser-scanning system using a pair of galvanometric scan mirrors relayed by two telecentric lenses in 4f configuration. **B & C** Near-field amplitude (**B**) and phase (**C**) while scanning a range of $30 \times 30 \mu\text{m}^2$ (sample scanning).

Conclusion

Chasing a viable solution for sSNOM in liquid environments, we presented a modification of conventional free-space illumination sSNOM. Here, side illumination is replaced by an illumination scheme from the bottom consisting of a Schwarzschild objective focusing the incident light through a Si-SiL. The enhanced NA allows to access the scattered light even if the tip is immersed in (any) liquid. We demonstrate that this mode of operation works as well when applied to biological samples. We provide evidence for increasing the field of view essentially by tracking the tip with the focus *via* laser scanning. The presented experimental

design can be extended to near-field spectroscopy by means of nanoFTIR with minor modifications. In addition, biological samples can be investigated in their native environment which allows to capture dynamic processes like association/dissociation reactions, structural changes upon ligand binding or light activation, polymerization reactions, and many more. We believe that this work can transform sSNOM into an even more valuable tool for nano-science, particularly at the interface of life science, and push its range of applications significantly.

Methods

Bottom illumination scheme. The IR sSNOM setup has been described elsewhere.⁵² The presented bottom illumination scheme sSNOM is based on a NanoWizard II (JPK Instruments AG, Germany) equipped with a x-y sample scanner stage (TAO). A silicon hemisphere ($\varnothing = 10$ mm, AMS Technologies AG, Germany) is fit centrally into a home-built aluminum plate and fixed by a PEEK gasket. One part of the collimated light from an attenuated external cavity quantum cascade laser (QCL, Daylight Solutions, USA) is guided through a beamsplitter (1" ZnSe window, Eksma Optics UAB, Lithuania) into the entrance pupil of a 40x Schwarzschild objective (LMM-40x-P01, 40x reflective objective, 0.5 NA, infinity corrected back focal plane, Thorlabs GmbH, Germany) and focused through the Si hemisphere onto the Pt-coated AFM tip (Arrow NCPT, NanoAndMore GmbH, Germany) oscillating at $\Omega \sim 280$ kHz in air or $\Omega \sim 130$ kHz in H₂O. The scattered light is guided back to the ZnSe window and is directed onto a mercury-cadmium-telluride photodetector (KLD-0.1-J1/DC/11, Kolmar Technologies Inc., USA) where the light is interferometrically detected and fed into a lock-in amplifier (HF2LI, Zurich Instruments, Switzerland). The signal is then analyzed using a pseudo-heterodyne detection scheme as previously described.⁴⁸ All near-field maps are postprocessed to retrieve the full $-\pi$ to π phase information.⁵³ Phase maps were then corrected by subtracting the median of the phase line-by-line. Phase maps in Figure 2 D, E, G, & H are subject to a more elaborate line-y-line phase correction algorithm (see Supporting Information).

Laser scanning assisted sSNOM. A pair of 5 mm galvanometric scan mirrors (GVS102, 2D Galvo System, gold coated, Thorlabs GmbH, Germany) was installed in between the ZnSe window and the objective to track the scanning tip with the IR focus. The scanned beam was relayed by a pair of telecentric lenses (LA5040, ½" uncoated CaF₂ lens, f = 40 mm, Thorlabs GmbH, Germany) and imaged into the entrance pupil of the objective. The auxiliary outputs of the x-y scanner are scaled and offset by a homebuilt circuit and fed into the controller of the 2D galvanometric scan mirror to accurately match the beam-scanning with the tip/sample scanning.

Sample preparation. Purple membrane was kindly provided by the group of Dr. Ramona Schlesinger (Freie Universität Berlin, Germany). Before each experiment the Si hemisphere was cleansed by stepwise sonication in acetone, isopropanol and pure water for at least 15 min. Purple membrane patches were diluted to 10 µg/ml in a buffered solution (300 mM KCl, 10 mM Tris, pH 7.8, filtered through 0.2 µm filter) according to ref. ⁵⁴. 50 µl of this solution was incubated for 30 min on the flat face of the hemisphere to allow the patches to adsorb. The hemisphere was then gently rinsed with pure water subsequently and dried. For experiments in an aqueous environment, 200-500 µl of (150 mM KCl, 10 mM Tris, pH 7.8, filtered through 0.2 µm filter) were added to the surface.

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Notes

The authors declare no competing financial interest.

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Supporting Information

Supporting Information Available:

Supporting information contains extended data on focal intensity distributions, analytical modeling of the near-field contrast and line profiles extracted from near-field phase maps.

This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Supporting Information to

Infrared Scattering-Type Scanning Near-Field Optical Microscopy in Water

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Supporting text contains details about computations shown in supporting figure 4 and phase correction in Figure 2 C, D, G, & H. Supporting figure 1 and 2 contain near-field maps for demodulation orders $n=1, 2$, & 3 for tip or sample scanning. Supporting figure 3 shows line profiles extracted from the near-field maps of purple membrane patches when the QCL is tuned to the amide I absorption band. Supporting figure 4 shows the results of the point dipole model applied to the experimental scheme described in the main text.

Supporting Text

Point-Dipole-Model in Water. We used the formalism described by Knoll et al.^{1, 2} This model simplifies the tip apex as a polarizable sphere with polarizability

$$\alpha = 4\pi a^3 \frac{(\epsilon_t - 1)}{(\epsilon_t + 2)}, \quad (1)$$

where ϵ_t is the dielectric function of the sphere with radius a resting in distance z above the lower half space with dielectric function ϵ_s . The samples polarizability can be then described by an image dipole with polarizability

$$\alpha\beta = \alpha \frac{(\varepsilon_s - 1)}{(\varepsilon_s + 1)}. \quad (2)$$

The combined polarizability is written as

$$\alpha_{eff} = \frac{\alpha(1 + \beta)}{1 - \frac{\alpha\beta}{16\pi(a + z)^3}}. \quad (3)$$

This effective polarizability is directly proportional to the scattered field detectable in the far-field. For this model, however, a vacuum polarizability of the upper half space was assumed. We now generalize equation (1) and (2) which permits assigning an arbitrary dielectric function ε_u in the upper and ε_s in the lower half space:

$$\alpha = 4\pi a^3 \frac{(\varepsilon_t - \varepsilon_u)}{(\varepsilon_t + 2\varepsilon_t)} \quad (4)$$

and

$$\alpha\beta = \alpha \frac{(\varepsilon_s - \varepsilon_u)}{(\varepsilon_s + \varepsilon_u)}. \quad (5)$$

Inserting equations (4) and (5) into equation (1) now allows to calculate the polarizability of the coupled dipole for the tip resting in arbitrary environments. Supporting figure 4 shows the magnitude (Supporting Figure 4A) and argument (Supporting Figure 4B) of the polarizability of a Pt sphere (permittivity modeled by the 4-term Lorentz-Drude model according to ref. ³) of radius 20 nm resting in 10 nm distance of the lower half space with refractive index of either silicon ($n = 3.4$, black traces) or a generic protein (permittivity modeled as Lorentz oscillator from ref. ⁴, red traces). The dielectric function of the upper half space is varied between vacuum ($n = 1$, dashed lines) or water (complex refractive index from ref. ⁵, interpolated by cubic splines, solid lines). A simple subtraction of the spectral response above Si from the one above the protein recovers the proteins spectral features (Supporting Figure 4C&D).

Line-by-line Phase Correction. All near-field phase maps recorded with this technique can, in principle, suffer from offsets in the phase by drift of the optical path lengths of both the tip arm and the reference arm. To compensate that all maps, unless noted, are corrected by subtracting the median of the phase of each line. In Figure 2 D,E,G, & H the phase is corrected line-by-line as follows: for each line i the pixels j with a near-field amplitude $s_i(j)$ larger than a threshold $s_i(j) > \max_j \{s_i(j)\}$ are selected. The phase $\varphi_i(j)$ of the corresponding pixels j is then fitted either by a constant or linear function and subtracted from the all phase values in the line i .

Supporting Figure 1 *Near-field distributions for all harmonics (tip scanning).* Near-field amplitude, phase and topography (left to right) for harmonics $n=1$ (**A**), $n=2$ (**B**), and $n=3$ (**C**). The white arrow indicates the incident polarization.

Supporting Figure 2 *Near-field distributions for all harmonics (sample scanning).* Near-field amplitude, phase and topography (left to right) for harmonics $n=1$ (**A**), $n=2$ (**B**), and $n=3$ (**C**). The white arrow indicates the incident polarization. Please note, that the scale bar is the same as in supporting figure 1.

Supporting Figure 3 *Line profiles extracted from near-field phase maps. A-B* Near-field phase maps of a PM patch (identical to Figure 2 in the main text) in air (**A**) and in H₂O (**B**) when the QCL is tuned to 1659 cm⁻¹. The red line indicates the line along which the line profile was extracted. **C** Extracted line profiles (average of three adjacent lines) as indicated in **A** and **B** for maps recorded in air (black trace) and H₂O (red trace). The solid line indicates a sigmoidal fit to the extracted traces. The distance for the phase to drop from 90% to 10% of the sigmoidal fit is $\Delta x_{\text{air}} = 26 \pm 12$ nm and $\Delta x_{\text{H}_2\text{O}} = 81 \pm 46$ nm, respectively.

Supporting Figure 4 Point-dipole model for the tip above PM resting on Si in air and H_2O .

A-B Magnitude (**A**) and argument (**B**) of the polarizability when a 20 nm sphere of Pt resides 10 nm above Si (black traces) or a generic protein (red traces). The tip is placed in H_2O (solid trace) or air (dashed trace). **C** Magnitude of the polarizability above a generic protein normalized to the magnitude above Si in air (black trace) and H_2O (red trace). **D** Argument of the polarizability above a generic protein corrected (ordinary subtraction) for the its argument above Si in air (black trace) and H_2O (red trace). The refractive index of H_2O is taken from ref. ⁵ and interpolated by a spline to 1 cm^{-1} spectral resolution. The refractive index of the generic protein is taken from ref. ⁴.

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