

Mid-infrared nanoimaging and nanoscopy of hemoglobin

Eamonn Kennedy, Fiona Yarrow, James H. Rice

Nanophotonics Research Group, School of Physics, University College Dublin, Belfield, Dublin, Ireland e-mail: james.rice@ucd.ie

Abstract Sub diffraction limited infrared absorption imaging of hemoglobin was performed by coupling IR optics with an atomic force microscope. Comparisons between the AFM topography and IR absorption images of micron sized hemoglobin features are presented, along with nanoscale IR spectroscopic analysis of the metalloprotein.

Keywords Infrared imaging, AFM imaging, proteins

Introduction

Unlike UV-Vis spectroscopy which probes the transition between electronic states, IR spectroscopy detects transitions between rotational and vibration energy levels. This allows for chemical characterization based on the detection of absorption bands over the IR range. Relevant infrared absorption bands for metalloproteins like hemoglobin typically reside in the mid-infrared. Examples are amide A, amide B, and amide I, amide II and amide III [1].

Much of our understanding of human physiology has its origin in laboratory studies of hemoglobin, and while IR absorption studies have been well established for proteins, there remain limitations such as the maximum IR image resolution that results from the diffraction limit [2] c.a. $\lambda/2$, which is 2-5 μm for mid-infrared wavelengths. This means that standard IR absorption spectroscopy techniques such as FTIR cannot be applied to study protein features that are on or below the order of microns. This inability to localize IR analysis also increases false readings in the IR spectra if impurities are present alongside the sample on the micron scale, where they cannot be spatially resolved.

However, emergent techniques allow for the utilization of a contact mode AFM to detect thermally induced dilation of a sample surface (e.g., [3, 4, 5]). Light incident on a sample tuned to a suitable absorption band, such as aliphatic C-H stretching for hemoglobin, will be partially absorbed, generating internal heat. The resulting thermal stress produces a small ($>1\text{nm}$) mechanical expansion of the sample surface. If an AFM is in contact with the surface it will be deflected by this surface expansion [5, 6]. In this way the vibration modes of the AFM deflection response can then be linked to the sample absorption via Fourier analysis of the deflection voltage. However, a constant illumination typically results in a spatial equilibrium being reached by the dilated surface. Therefore a pulsed laser excitation is required so that the cantilever displacement, and thus the surface deformation, can be temporally resolved.

The correlation between the AFM deflection mode maximum and the sample absorbance has been documented [3] and is linear for a given wavelength. Other product factors that affect the cantilever response such as the optical contribution and the thermal contribution of the output signal are seen to remain constant for a given cantilever and sample.

Previously we demonstrated single point IR absorption spectra for proteins [4]. In this letter, we demonstrate full IR absorption imaging at sub diffraction resolution of both hemoglobin aggregates and ledges with varying incident excitation radiation. This is performed by raster scanning an area with an AFM tip while continually sampling the AFM tip deflection voltage, producing simultaneous topological and absorption maps of the sample. Additionally, by holding the tip position constant on a hemoglobin region and varying the incident wavelength of light, nanoscopy of hemoglobin can be performed.

Methods

The apparatus consisted of an optical parametric oscillator (OPO) laser coupled with an AFM (fig. 1). The light was generated using an OPO laser based on a periodically poled LiNbO_3 crystal with an output range of 3000 to 3600 nm. The laser was usually focused such that the laser pulse intensity was in the order of $5 \times 10^8 \text{W/m}^2$.

The AFM is operated in contact mode using silicon nitride tips mounted on a V-shaped cantilever with a nominal spring constant of 0.05 N/m. A force setpoint of 2 - 5 nN was applied. A Stanford SR650 (Sunnyvale, CA, USA)

attenuator was used to amplify the signal from the AFM. The signal was routed to an oscilloscope (Agilent Inc., Santa Clara CA, USA). The scope output was digitally sampled using the Agilent connection expert IO libraries suite. The AFM tip deflection was averaged every 4 - 16 laser pulses to increase the signal to noise.

The effective spatial resolution is limited by the tip-surface area [7, 8], yielding resolution around 50nm. The excitation IR radiation is directed upward using gold coated mirrors into a lens to direct and focus the laser light. The lens was set on a controllable micrometer z stage so that ambient temperature variations could be accounted for by changing the spot intensity. The sample was produced using lyophilized haemoglobin at <1mg/ml solution in ultrapure water, which was mounted onto a glass substrate to facilitate the optical arrangement. A FTIR spectrometer was used to compare the nanoscopic data with the bulk spectrum.

IR nanoscopy

Nanoscale infrared spectroscopy of samples is performed by detecting the local displacement of the AFM tip caused by thermally induced dilation of the surface topography [9]. As the wavelength of the IR laser source is tuned into resonance with a vibration mode of the sample, absorption of IR radiation is seen to increase. In this way, the response of the cantilever tip can be monitored following the application of the IR radiation at varying frequency to produce an absorption spectrum. AFMIR studies of a deposited layer of hemoglobin on a glass slide were undertaken.

The AFMIR spectrum is shown alongside an FTIR spectrum of hemoglobin in fig 2. The two spectra show peaks at the same wave numbers, with slight variation in relative peak intensities. These changes in the relative amount of different bond excitations by the light arise from small sub-micron changes in the protein conformation [4]. For the region shown, the absorption arises from N-H and C-H vibrations. The amide B band at 3061 cm^{-1} is assigned to an hydrogen-bonded N-H stretching, where as the 2960 cm^{-1} peak is due to aliphatic C-H stretching.

Sub-micron IR imaging

Heme aggregates in the 100 nm height and micron size range in the lateral axial direction were studied using AFMIR. A $10\text{ }\mu\text{m}$ haemoglobin ledge section was irradiated at a resonance wavenumber of 2960 cm^{-1} and raster scanned with an AFM tip. At the same time, the fourier transform of the AFM tip deflection (fig. 3 a) was continually sampled. By monitoring the intensity of the shifted 135 KHz FT peak present in fig. 3, an absorption map was produced. This cantilever mode was not excited above the background when off of the hemoglobin ledge, resulting in a basic oscillation form off sample evident in fig.3 b.

The resulting IR image is shown in fig. 3 alongside the AFM topography. The ledge outline of both images match. The AFM topography shows a two tier structure, with an interior height of 400nm and an outer band of hemoglobin roughly 100nm in height. Interestingly, the thermal imaging shows strong absorption of both sections, albeit at differing amplitudes, indicating the presence of hemoglobin in both with a sharp outer boundary. This demonstrates that the AFMIR image is a chemical specific map, as certain AFM features were not heme, and thus did not absorb, allowing for the clear distinction between heme related and unrelated topography in the AFM image.

AFMIR images of a heme aggregate were recorded for varying excitation wavelengths, shown in Fig 5. The AFM topography image shows the presence of irregular patterns of heme on the substrate. Fig 5 b) shows an AFMIR image of the sample recorded by sampling the N-H stretching band at 2960 cm^{-1} . As with the hemoglobin ledge, the on resonance image shows similar features to the AFM, providing an absorption map of the sample. In comparison, off resonance imaging presented in fig 5 a) showed only residual absorption both on and off sample.

Using this technique, we can now chemically identify hemoglobin as distinct from other materials present on a sub-diffraction limited scale, overcoming one of the significant drawbacks of AFM; topological maps alone do not provide chemical specific information of the sample under study on the nanometer scale. Additionally, high resolution imaging of different absorption band intensities of hemoglobin has been demonstrated.

Acknowledgements

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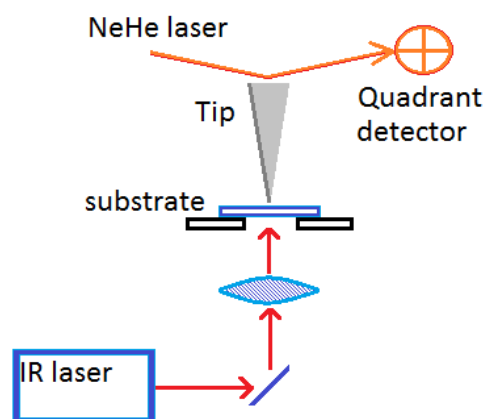


Fig 1. The key experimental elements. The quadrant detector output is then attenuated, locked and triggered via the laser pulse on the oscilloscope

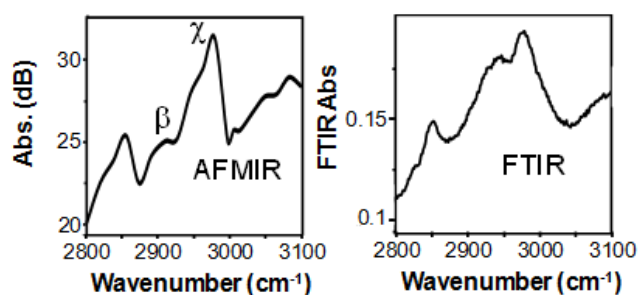


Fig 2. IR absorption spectra for haemoglobin, left) is the AFMIR spectrum and right) is the FTIR spectrum taken using a commercial FTIR instrument recording the bulk sample IR spectra.

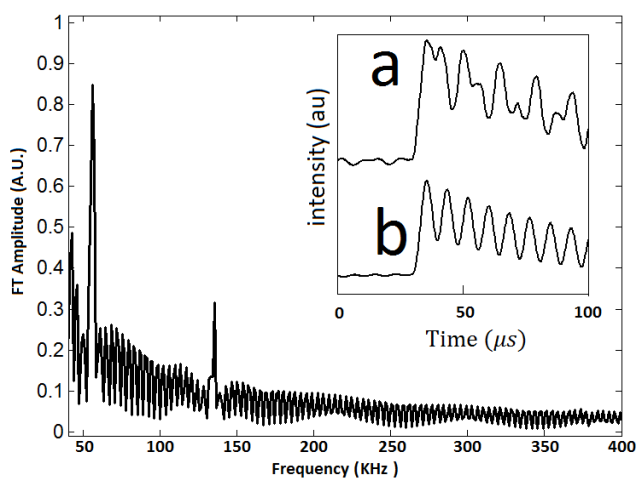


Fig 3. Fourier transform of the on-resonance oscillations with a 20MHz sampling rate. a) a detail of the Cantilever oscillations for the N-H region on the hemoglobin ledge and b) off of the Hemoglobin ledge.

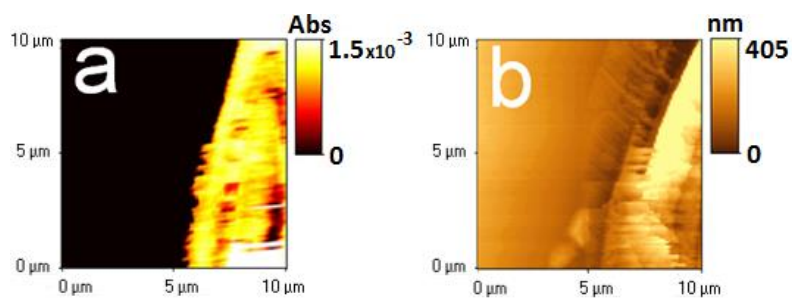


Fig 4. a) AFMIR image demonstrating near zero absorption off of the heme ledge, b) AFM topography image of the sample, recorded simultaneously, showing a two tier structure near the ledge, matching the perimeter detailed in the IR absorption map.

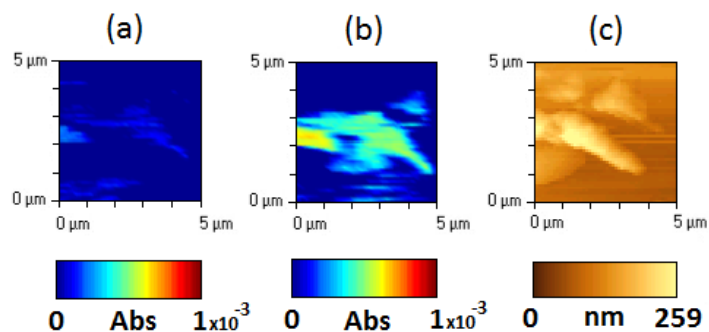


Fig 5. a) AFMIR image of heme recorded off resonance, b) AFMIR image recorded at resonance, c) the AFM topography image of the sample, recorded simultaneously.