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COMMUNICATION

Characterizing cytochrome c states – TERS studies of whole mitochondria†

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Protein structures (cytochrome c) were visualized by TERS measurements on whole mitochondria referring to specific spectral features describing the electronic state of the heme moiety.

Detecting single molecules is one of the most current issues in biosciences, since it provides individual information, inaccessible by ensemble averaged measurements. Hence, various detection methods are extended towards the molecular limit. Using reporter molecules for fluorescent based statistical approaches is well known to visualize several biomolecular species within cellular systems on a nanometre scale.¹ However, sample pre-treatment is required which may influence its behavior after labeling.

Further single molecule results have been reported by using surface-enhanced Raman spectroscopy (SERS) using all kinds of enhancing substrates.² The challenge is here the correlation of position and structural information. Confining the enhancement region to a single metallic feature results in so-called tip-enhanced Raman spectroscopy (TERS).³ The precise positioning of the enhancing particle using an AFM allows the correlation of sample position and structural information down to a nanometre or even less. The lateral resolution of the enhancement can be estimated by a dipole – mirror dipole approximation. This reveals that for a single 20 nm diameter Au-sphere the Raman enhancement decays to 10% of the maximum value within the very first 3.5 nm. Hence, a conservative estimation for the resolution of such a probe would be 7–10 nm. The simultaneous recording of the AFM topography as well as the spectral information provides highly resolved structure information in principle suited for single molecule detection. This general concept has been used for various applications, which require this outstanding spatial resolution.^{3–5}

In this contribution, we present TERS investigations of isolated mitochondria, extracted from yeast cells. Using an

excitation wavelength of 532 nm, the cytochrome protein structures embedded in the mitochondrial membrane system become resonantly excited. Hence, conventional Raman spectra recorded at this excitation wavelength are dominated by cytochrome signals, attributed to the central iron containing heme moiety.⁶ Consequently this resonance Raman effect must be considered in the following experiments.

Cytochrome proteins are known to play a crucial role in several steps of the respiratory chain of mitochondria, which takes place in their membrane system.⁷ Although most of the processes are located at the inner membrane of the mitochondrion, a key step involving the electron transfer is performed by cytochrome c, an electron carrier located in the intermembrane space. This is indicated by a change of the oxidative state of the central iron between Fe II and Fe III, which is schematically illustrated in Fig. 1. Since cytochrome c is in closer proximity to the TERS probe compared to the other cytochrome species in the mitochondrion, spectral contributions typical for a heme moiety are assigned to cytochrome c. With respect to the sample treatment (drying) we suggest a squeezed double membrane model. It is well known from the literature that the

Fig. 1 Schematic TERS-probe sample interaction during a measurement on a mitochondrial membrane. Initially, the evanescent field interacts with the outer membrane (OM). Cytochrome c (cyt c) is mostly located in the intermembrane space (IMS), acting as an electron carrier within the mitochondrial respiratory chain between complex III (cytochrome c reductase) and complex IV (cytochrome c oxidase), which are located in the inner membrane (IM) (sizes not to scale). The gap between OM and IM probably shrinks during the sample preparation, which slightly facilitates TERS of cyt species.

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Table 1 Distinct mode assignment (cm^{-1}) of oxidation and spin marker bands of cytochrome c (cyt c) adapted from ref. 6

Oxidation state	cyt c (III)		cyt c (II)	
ν_4	1373		1362	
Spin state	LS	HS	LS	HS
ν_3	1507	(1492)	1497	(1473)
ν_{11}			1543	
$\nu_{2/19}$	1584	1568	1590	1565
ν_{10}	1636	1625	1622	1607

distinct oxidation states can be distinguished by Raman bands occurring around 1360 cm^{-1} typical for Fe II and around 1373 cm^{-1} characteristic for Fe III, respectively.⁶ Furthermore, also the spin state of the iron can be deduced when considering specific combinations of Raman modes appearing from approx. 1480 cm^{-1} to 1640 cm^{-1} . A detailed diagram of the respective Raman modes using the currently accepted assignment is given in Table 1.⁶ We present a novel TERS approach towards single molecule spectroscopy based on discrete variations of the oxidation or spin state. Accordingly, localized changes in the oxidation state of cytochrome c are shown that are caused by only a few individual protein structures.

TERS measurements on isolated dried mitochondria were performed using a combination of an inverted Raman microscope (S & I GmbH, Germany) and an AFM set-up (Nano Wizard II, JPK Instrument AG, Germany) similar to previously described systems.³ For all TERS spectra the excitation wavelength was 532 nm and the laser was focused *via* an oil immersion microscope objective ($60\times$, NA 1.45, Olympus) onto the silver coated AFM tip. The Raman signal is collected by the same microscope objective. The non-contact AFM tips (NSG10, resonant frequency $190\text{--}325 \text{ Hz}$, NT-MDT) were coated with 20 nm silver (99.99% pure, Balzers Materials).

Fig. 2a shows an AFM image of an isolated and dried mitochondrion, recorded before the actual TERS experiment, but already using a silver coated probe to get an idea of the actual sample surface. No obvious damage can be observed at the surface and also the size represents an individual mitochondrion (150 nm). For the subsequent TERS measurements, the tip was positioned along a line as indicated in Fig. 2a. The spectra were then obtained using a step size of 8 nm . Although larger than the expected size of single cytochrome c structures ($\sim 2 \text{ nm}$),⁷ this step-size has been chosen due to the distribution of the proteins within the mitochondria (for details see ref. 7). The TERS recording sites in Fig. 2a are color coded according to the oxidation state (Fe III – red; Fe II – blue) of the heme moiety, which is shown in the two diagrams in part b and c of Fig. 2. For the assignment, the band positions were determined by a standard non-linear curve fitting (IGOR Pro 6.12, Wavemetrics, USA) Fig. 2b illustrates the change of the oxidation state using ν_4 as a marker band with respect to the position of the TERS probe. The shift from 1373 cm^{-1} (Fe III) to 1360 cm^{-1} (Fe II) clearly indicates a transition from Fe III to Fe II. Although mostly dominated by the oxidized species, only position 3 (16 nm) refers to a mixed state. Here, a mixed state most likely implies a tip position between a Fe III and Fe II state. In addition to the oxidation state, Raman modes are

Fig. 2 (a) AFM image of the isolated mitochondrion selected for the TERS experiments. The dots indicate the positions of the TERS experiment. Oxidized (Fe III – red) or reduced (Fe II – blue) species of cytochrome c are indicated by the colour coding. (b) The diagram shows the shift of the oxidation marker band ν_4 with respect to the TERS probe position. An effective shift from oxidized to reduced cytochrome c species can be observed and is displayed using the same colour coding as in (a). The slight shift towards lower energies at point 3 (16 nm) is likely to result from a mix of oxidation states, with Fe III as a major contribution. (c) The distribution of several Raman modes characteristic for the spin marker region vs. the TERS probe position is shown. The spin classification follows the assignment, which is shown in Table 1. Considering the respective intensities, weak bands are colored brown. The individual peak positions and error bars in part b and c are provided by a standard curve fitting procedure.

also sensitive for the spin state.⁶ This spectral dependence is shown in Fig. 2c. With the exception of positions 1 and 2 (low spin – LS), every spectrum shows features typical for a low and a high spin (HS) configuration. However, the HS dominates the spectral appearance on points 3–6. Since several specific spin marker modes are directly related with a specific oxidation state (for instance ν_{11} of the reduced species), this additionally supports the assignment to cytochrome c. The individual classification for LS and HS of the oxidized (III) and reduced (II) form is accomplished using Table 1, which is illustrated in the following section. In Fig. 3 the comparison of spectra from two adjacent positions (3 (16 nm) and 4 (24 nm)) further strengthens the previous assumptions. The effective band shift can be clearly seen when zooming into the region presented in Fig. 3b. Vertical lines guide the eye towards the important changes in the oxidation and spin marker regions when shifting from a mixed, however, Fe III dominated region (point 3 at 16 nm), to the reduced (point 4 at 24 nm) species. The simultaneous switching of the C=C stretching mode (1626 cm^{-1} to 1618 cm^{-1}) and the ν_4 oxidation marker band supports the observation of different electronic states rather than shifts due to nanoscale effects as has been also shown.³ Furthermore, the ν_{11} band at 1543 cm^{-1} , indicating low spin Fe II, only occurs in spectrum 4, again supporting the electronic change hypothesis. A high spin dominated configuration is detected in spectra 3 and 4. In spectrum 3 this is indicated by Raman modes at 1566 cm^{-1} and 1626 cm^{-1} .

Fig. 3 (a) TERS spectra of the mitochondrial surface showing the oxidation shift, recorded at positions 3 (16 nm) and 4 (24 nm) as indicated in Fig. 2 (excitation at 532 nm, incident laser power 0.5 mW, sampling time 5 s). Strong lipid signals are marked by an L. (b) Enlarged region displaying the discussed electronic marker bands.

A low spin contribution that is suggested by the shoulder at 1588 cm^{-1} can be disregarded based on intensity arguments. In spectrum 4, again the low spin indicators at 1505 cm^{-1} and 1542 cm^{-1} are weak and the high spin markers at 1568 cm^{-1} and 1618 cm^{-1} are more intense. The appearance of such distinctive changes clearly points towards only a few molecules, if not even single molecules, contributing to the electronic changes on a length scale of less than 10 nm.

An important issue is a possible interaction of the metal coated tip with the redox active protein. But, as the cytochrome c structures are located in the intermembrane space on the inner membrane,⁷ at least 8 nm below the mitochondrial surface, as indicated in Fig. 1, no electronic interaction with the tip is possible without destruction of the whole cell organelle. Such a destructive process can be safely excluded by the spectral information, given in Fig. 3a as well as the topography information in Fig. 2a. Interestingly all the spectra show a strong contribution of the lipid matrix. The corresponding bands are marked ("L"). Raman modes typical for the phosphate residue of lipid molecules occur around $790\text{--}800\text{ cm}^{-1}$ and 1080 cm^{-1} and are usually very intensive features. Furthermore, specific C–H deformation bands

typical for lipids can be found at approx. 1310 cm^{-1} and 1440 cm^{-1} .⁸ Such a strong lipid contribution to the Raman spectrum is unexpected considering the excitation wavelength, which is within the electronic resonance of the cytochromes. The reason for this effect is intrinsic to the TERS technique and illustrated in Fig. 1. The evanescent field decays very rapidly, mostly within the first 10–15 nm.⁹ Hence, most of the enhancing electromagnetic field interacts with the lipid environment of the outer membrane. Due to the comparatively large distance of the cytochrome c from the tip, the resonance Raman effect plays a much lesser role for the appearance of the spectra compared to conventional resonance Raman, where the whole spectrum is dominated by the heme group scattering. Furthermore, TERS spectra (data not shown) were recorded on the same mitochondrial surface, which are free of cyt c. From these observations it can be concluded that the redox active proteins are separated from the TERS probe by the lipid environment of the outer membrane, further ruling out any direct tip – cytochrome c interaction. Other contaminations of the tip were excluded by retracting the tip and refocusing to the initial silicon signal.¹⁰

In conclusion, we report the use of TERS towards the detection of very few, potentially even single, cytochrome c structures, due to their individual distribution within the mitochondrial membrane system.⁷

Future experiments aim to explain potential effects of sample preparation by investigating the specimen under controlled oxidation conditions as a further step towards nanoscale investigations under physiological relevant conditions.

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