Effects of Ethanol, Formaldehyde, and Gentle Heat Fixation in Confocal Resonance Raman Microscopy of Purple NonSulfur Bacteria

ANN-KATHRIN KNIGGENDORF,* TOBIAS WILLIAM GAUL, AND MERVE MEINHARDT-WOLLWEBER
Institute of Biophysics, Gottfried Wilhelm Leibniz University of Hannover, Herrenhaeuser Str. 2, 30419 Hannover, Germany

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ABSTRACT Resonance Raman microscopy is well suited to examine living bacterial samples without further preparation. Therefore, comparatively little thought has been given to its compatibility with common fixation methods. However, fixation of cell samples is a very important tool in the microbiological sciences, allowing the preservation of samples in a specific condition for further examination, future measurements, transport, or later reference. We examined the effects of three common fixatives—ethanol, formaldehyde solution, and gentle heat—on the resonant Raman spectrum of three generic bacteria species, *Rhodobacter sphaeroides* DSM 158^T, *Rhodopseudomonas palustris* DSM 123^T, and *Rhodospirillum rubrum* DSM 467^T, holding carotenoid- and heme-chromophores in confocal Raman microscopy. In addition, we analyzed the effect of poly-L-lysine coating of microscope slides, widely used for mounting biological and medical samples, on subsequent confocal Raman measurements of native and fixed samples. The results indicate that ethanol is preferable to formaldehyde as fixative if applied for less than 24 h, whereas heat fixation has a strong, detrimental effect on the resonant Raman spectrum of bacteria. Formaldehyde fixation excels at fixation times above 24 h, but causes an overall reduction in signal intensity. Poly-L-lysine coating has no discernable effect on the Raman spectra of samples fixed with ethanol or heat, but it further decreases the signal intensity, especially at higher wavenumbers, in the spectra of samples fixed with formaldehyde. *Microsc. Res. Tech. 74:177–183, 2011.*

INTRODUCTION

Confocal resonance Raman micro-spectroscopy is a promising upcoming tool in many scientific fields from biology to solid state physics. Non-destructive measurements based on ubiquitous chromophores give it an extremely broad range of accessible samples. For example, hemes and carotenoids—abundant in most organisms throughout the biological kingdoms—are chromophores especially useful in biological and medical research.

Confocal resonance Raman microscopy combines the specificity of Raman fingerprints and a highly increased sensitivity under resonant excitation conditions with the spatial resolution of confocal laser microscopy. Its ability to analyze and identify microorganisms, if necessary even based on a single cell, without further preparation or previous cultivation is of great interest in a wide field of applications requiring fast and reliable methods of identification of microbial organisms (Harz et al., 2009; Kudelski, 2008).

As resonance Raman microscopy is well suited for analyzing complex bacterial samples in vivo, comparatively little thought has been given to its compatibility with common fixation methods and cell adhesive coatings widely used in microbiological research.

Fixation in its broadest sense is an important tool in the biological, medical, and pathological sciences. It allows biological aggregates such as tissues or bacterial communities—no matter if obtained from natural habitats or cultured from clinical samples—to be stored over extended periods of time while remaining unaltered regarding the respective properties under study. Many analytical methods regularly applied to biological samples require fixation. Considering the vast number of analytical methods available, ranging from the extraction of specific molecules or organelles from single cells to the preservation of the spatial structure of complex cell aggregates, it is unsurprising that a multitude of fixation procedures has been developed.

Recent developments combine Raman microscopy with other methods of microbial analysis requiring fixation. For example, Huang et al. (2007) reported to have successfully combined Raman microscopy with fluorescent in situ hybridization (FISH) on cells incubated with ^{13}C-labeled compounds. Fixation for FISH requires the spatial allocation of cells while preserving the cell shape and increasing the permeability of cell walls and membranes in order to achieve large-scale hybridization of the binding sites (ribosomes) with specific oligonucleotide probes.

By far the most frequently and routinely applied fixation protocols in this context are those using parafor-

*Correspondence to: Ann-Kathrin Kniggendorf, Institute of Biophysics, Gottfried Wilhelm Leibniz University of Hannover, Herrenhaeuser Str. 2, 30419 Hannover, Germany. E-mail: kniggendorf@biophysik.uni-hannover.de

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Fig. 1. Phase contrast micrographs of growing PNS type strain cultures showing different cell shape and size: *Rhodobacter sphaeroides* 158 (**a**), *Rhodopseudomonas palustris* 123 (**b**), and *Rhodospirillum rubrum* 467 (**c**). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

maldehyde (PFA) for gram-negative bacteria, generally equipped with a double-layered cell membrane, and ethanol (EtOH) for gram-positive bacteria with just a single membrane but a rigid outer cell wall.

However, given the long history of EtOH and formaldehyde solution being used as fixatives for bacterial cells, the number of published studies detailing their effects on the molecular and cellular level is—to the best of our knowledge—surprisingly small.

Formaldehyde solution, which has been used as a fixative for cells and tissues in the biological, medical, and pathological sciences for more than a century (Fox et al., 1985), causes substantial chemical changes in peptides, most notably stable methylene bridges linking primary amino (including lysine) and thiol groups irreversibly with several other amino acid residues (Metz et al., 2004).

EtOH is assumed to displace intracellular water, thus reducing the water activity (a_w) of the cells and ultimately causing their proteins to coagulate (Denyer 1995, 1998; Rutala, 1989). Since the coagulation effect is pronounced more strongly with some residual water present, EtOH is generally considered to be most effective as bactericidal agent when used in a final concentration of 60–70% vol/vol (Larson and Morton, 1991). Another effect associated with EtOH exposure at lower concentration is the solubilisation of the phospholipids as integral elements of the bacterial membrane (Ballesteros et al., 1993).

Although moist heat under overpressure (autoclavation; at least 121°C in hot steam) is known to effectively kill vegetative bacteria as well as their even more resistant spores for most hygienic applications, the mechanisms of action at temperatures well below 100°C still lack full understanding. Heating suspended bacterial specimen to temperatures in the range of 60 to 90°C generally results in an inactivation, i.e., loss of capability to reproduce. Other specific cell properties, such as the antigenic identity, may remain intact. Among the reported heat-related effects are protein coagulation, breakdown of nucleic acids, and leakage of molecules of low-molecular weight through the cell membrane. There are, however, effective mechanisms known how several species can cope with short-time heat exposure (Russel, 1991).

Cell adhesive coatings are an important aid for preparing samples of cells with little surface affinity of their own. For example, polylysine has been used as a highly adhesive coating for microscope slides and other laboratory equipment for more than thirty years. Its polycationic molecules adsorb strongly to various carrier materials such as glass, quartz, or various plastics, providing cationic surface sites to combine with the anionic sites on outer cell membranes. Cells attached to a polylysine coating spread and flatten along the surface. The adhesion is strong enough to withstand most fixation baths, though an ethanol acetate solution of three to one has been reported to cause partial cell detachment (Mazia et al., 1975). A poly-L-lysine coating is a standard feature of many readily available high-quality microscope slides these days.

Purple non-sulfur bacteria (PNS) were chosen as sample organisms, because they are comparatively well understood and of widespread use in many fields of research, especially anoxic bacteriophotosynthesis (Cogdell et al., 2006; Mackenzie et al., 2007). Phylogenetically, they belong to the Alphaproteobacteria (with few exceptions grouped into the Betaproteobacteria) and generally show a wide range of physiological flexibility. Under anaerobic conditions, PNS-bacteria are able to grow photoheterotrophically, expressing bacteriochlorophyll and strain-specific carotenoids, thus providing useful chromophores for resonant Raman excitation (Imhoff, 2006).

Figure 1 shows phase-contrast micrographs of the three type strains of PNS-bacteria within the Alphaproteobacteria chosen for this study: *Rhodobacter sphaeroides*, *Rhodopseudomonas palustris*, and *Rhodospirillum rubrum* according to Imhoff et al. (1984).

The resonant Raman spectrum at 532 nm excitation of native *Rhodobacter sphaeroides* 158, as well as the spectra of *Rhodopseudomonas palustris* and *Rhodospirillum rubrum*, is dominated by the strong Raman lines of the carotenoids associated with their photoreaction centers. The carotenoids typically found in *Rhodobacter sphaeroides* are spheroidene with its strongest line at 1,519 cm^{-1} or neurosporene with its strongest line at 1,528 cm^{-1}, whereas *Rhodospirillum rubrum* holds a spirilloxanthin with the strongest line at 1,509 cm^{-1} (Koyama, 1991, 1995). Additional Raman chromophores resonant to 532 nm excitation in *Rhodobacter* are cytochromes c and bacteriopheophytins (Eads et al., 2000; Imhoff et al., 1984; Varotsis et al., 1995).

In this work, we examine the compatibility of several, widely used fixation methods for bacterial samples with subsequent confocal resonance Raman microscopy. We also analyzed the effect of the poly-L-lysine coating of microscope slides in this context. The used types of sample bacteria were chosen with respect to their widespread use in research and type of dominant chromophore for comparability.

MATERIALS AND METHODS

Bacterial Cultures

Three bacterial type strains of photosynthetic Alphaproteobacteria—*Rhodobacter sphaeroides* DSM 158^T, *Rhodopseudomonas palustris* DSM 123^T and *Rhodospirillum rubrum* DSM 467^T—were obtained as freeze-dried cultures from German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ]), Braunschweig, Germany. The freeze-dried pellets were activated as recommended by DSMZ and cultured anaerobically in medium DSM 27 modified for oxygen sensitive species.

All cultures were incubated at 25°C in an illuminated climate chamber on a rotary shaker at 200 rpm. Growing cultures were regularly inoculated in fresh medium as soon as the photometrically determined optical density at 578 nm (OD_{578}) exceeded 2.0 compared with sterile medium as control (spectrophotometer Cadas 200 by Hach-Lange GmbH, Berlin, Germany; standard single-use cuvettes).

Before each sampling for subsequent resonance Raman analysis, the purity of cultures was checked microscopically (phase contrast, 100-fold magnification, Zeiss GmbH, Oberkochen, Germany).

The sampling volume was set to 1 ml of cell suspension from the respective, actively growing culture at a cell density of 10^6 to 2×10^6 cells per ml (as estimated from OD), centrifuged for 5 min at 14,500*g* at 4°C, washed with Phosphate Buffered Saline (PBS; pH 7.2) and pelletized again. Subsequently, cell pellets were either subjected to fixation or measured directly as native reference.

Fixation Methods

Three methods of fixation were used: EtOH, formaldehyde, and gentle heat. Harvested, PBS-washed, and pelletized cells were further processed as follows:

Ethanol (EtOH): Cells were resuspended in 300 μl of sterile PBS before adding 700 μl of pure ethanol (analytical grade) to a final volumetric concentration of ethanol of ~70% and stored at room temperature.

Formaldehyde (PFA): Cells were resuspended in 1 mL of 4% PFA solution (4% wt/vol in PBS, pH 7.2) and stored at room temperature.

Heat: The harvested pellet was resuspended in 1 mL sterile PBS and heated to 70°C in a thermostatted shaker (Thermomixer 5436, Eppendorf AG, Hamburg, Germany).

After the respective fixation time (15 min–48 h, see results section), fixatives were thoroughly removed by centrifuging and washing with PBS, as described above. This step was applied twice. The heat fixed samples were treated identically.

Efficiency of fixation (i.e., cultured bacteria were no longer viable, but cells morphologically intact) was checked for by inoculating three bottles of sterile medium with 200 μl of the fixed sample (by PFA, EtOH and heat, respectively). The control flasks were incubated under conditions identical to the culture flasks. If no visible growth occurred after 2 weeks, the fixation was considered complete.

Resonance Raman Measurements

Washed cell pellets (after fixation or live samples) were either mounted on standard microscope slides (ISO Norm 8037/l microscope slides by Menzel-Gläser, Braunschweig, Germany) or slides coated with poly-L-lysine (Polysine™ by Menzel-Gläser), covered with a 0.17 mm cover slip and measured immediately.

Resonance Raman measurements were performed at room temperature with a confocal Raman microscope (CRM200, by WITec GmbH, Ulm, Germany), equipped with an oil-immersion objective (Nikon CFI Achromat) with a magnification of 100, a Numerical Aperture of 1.25, and corrected for cover slips of 0.17 mm thickness. A stabilized, frequency-doubled continuous-wave Nd:YAG laser at 531.9 nm was used for excitation. The system had an ellipsoid measurement volume of ~0.5 μm^3 with a spatial resolution of 300 nm in the horizontal plane and 1.2 μm perpendicular to it. Slit width was 50 μm, realized by a multimode fiber connecting the Raman microscope with the spectrometer (UHTS 300, by WITec). The used grating had 600 lines per millimeter. Spectra were recorded with an emCCD camera (ANDOR DU970N-BV-353), electrically cooled to −69°C. The spectral resolution of the setup was 2 cm^{-1} with a spectral accuracy of 1 cm^{-1}. Recorded spectra covered the range between −80 and 3,710 rel. cm^{-1}.

Laser intensity was adjusted to 25 mW, giving 2.8 MW/cm^2 within the measurement volume on the sample. Measurement time per spectrum was set to 0.1 s. The spectra of 100 different bacteria cells were recorded and averaged for each sample spectrum. Measured cells had a minimal distance of 3 μm from one another to avoid effects of photo-bleaching and thermal damage.

Preparation and analysis of the Raman spectra was done with commercial spectra analysis software (OPUS version 5.5 by Bruker).

The preparation of the sample spectra consisted solely of subtracting ~900 counts of continuous background noise and normalizing to the dominant Raman line in the averaged spectrum of the native bacteria. This was the line at 1,521 rel. cm^{-1} for *Rhodobacter sphaeroides* 158 (normalization interval: [1,501, 1,542]) and the line at 1,509 rel. cm^{-1} for *Rhodopseudomonas palustris* 123 and *Rhodospirillum rubrum* 467 (normalization interval: [1,491, 1,532]). Normalization intervals were set in relation to a horizontal baseline, accounting for the asymmetry of the lines toward higher wavenumbers.

The analysis of the spectra focused entirely on Raman lines clearly visible in the obtained spectra. Very weak lines resulting from the excitation of other chromophores were omitted unless contributing additional information.

In comparative spectra analysis (CSA), the sensitivity settings in the software finding exactly the Raman lines which can clearly be identified by the naked eye in the spectrum of the native sample were applied

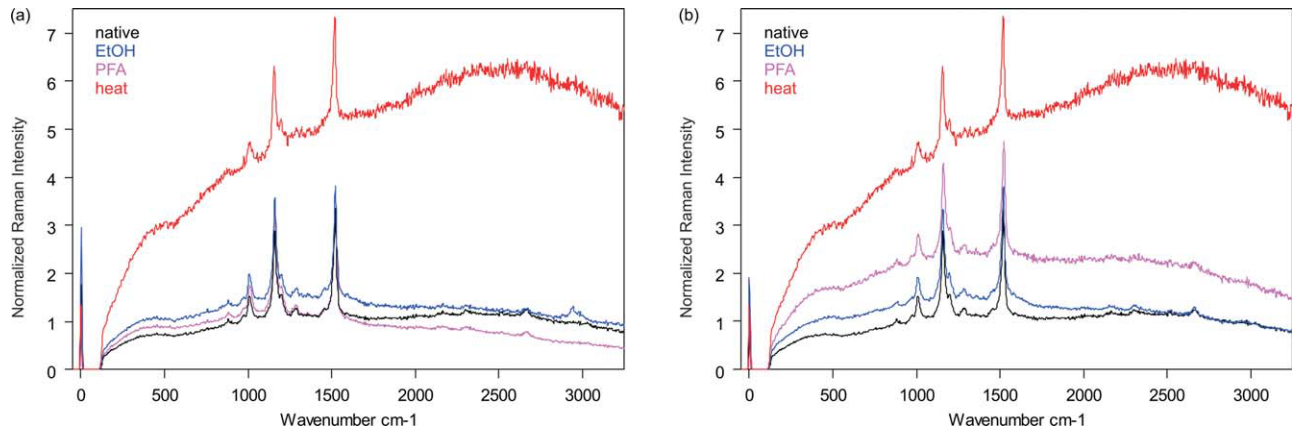

Fig. 2. Resonant Raman spectrum of native *Rhodobacter sphaeroides* 158 and *Rhodobacter sphaeroides* 158 fixed with ethanol, paraformaldehyde, or heat (70°C), respectively. Samples were mounted on an uncoated microscope slide (**a**) or on a microscope slide coated with poly-L-lysine (**b**). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

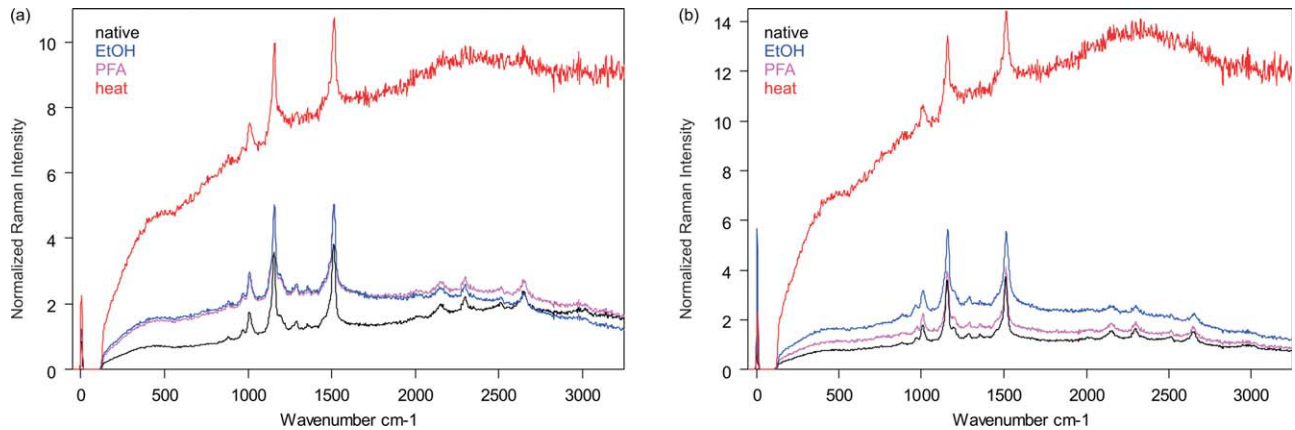

Fig. 3. Resonant Raman spectrum of *Rhodopseudomonas palustris* 123 (**a**) and *Rhodospirillum rubrum* 467 (**b**) native or fixed with ethanol, paraformaldehyde, or heat (70°C), respectively, and mounted on uncoated microscope slides. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

without variance to the fixed samples. In addition, it was determined which Raman lines could be identified with optimal settings for the spectrum of the fixed samples without causing false positives.

RESULTS AND DISCUSSION

Confocal resonance Raman microscopy is used primarily to analyze native, i.e., living, samples of bacteria. Therefore, the resonant Raman spectra of the native bacteria were taken as reference for the evaluation of fixatives for subsequent Raman measurements.

The resonant Raman spectrum at 532 nm of *Rhodobacter sphaeroides* shows the strongest lines at 1,519, 1,157, and 1,007 cm^{-1} (order by intensity) and additional, but considerably weaker lines at 880, 1,192, and 1,289 cm^{-1} all associated with spheroidene (see Fig. 2a).

The resonant Raman spectra at 532 nm of *Rhodopseudomonas palustris* and *Rhodospirillum rubrum* have dominant lines at 1,509, 1,151, and 1,004 cm^{-1} (order by intensity) and weaker lines at 878, 967, 1,282, and 1,350 cm^{-1}, all of which are associated with spirilloxanthin (see Fig. 3).

Additional Raman lines above 2,000 cm^{-1} are found in the resonant Raman spectra of all three types of bacteria: at 2,163, 2,296, 2,517, and 2,669 cm^{-1} in the spectrum of *Rhodobacter*, and at 2,002, 2,149, 2,293, 2,508, and 2,638 cm^{-1} in the spectra of *Rhodopseudomonas* and *Rhodospirillum*. These are due to second order processes.

Medium DSM 27 produces no Raman lines or remarkable background between 200 and 3,700 cm^{-1} at 532 nm excitation (data not shown).

Comparison of EtOH, PFA, and Heat Fixation

Figure 2a shows the resonant Raman spectrum of native *Rhodobacter sphaeroides* 158 in comparison with the spectra of *Rhodobacter sphaeroides* 158 fixed

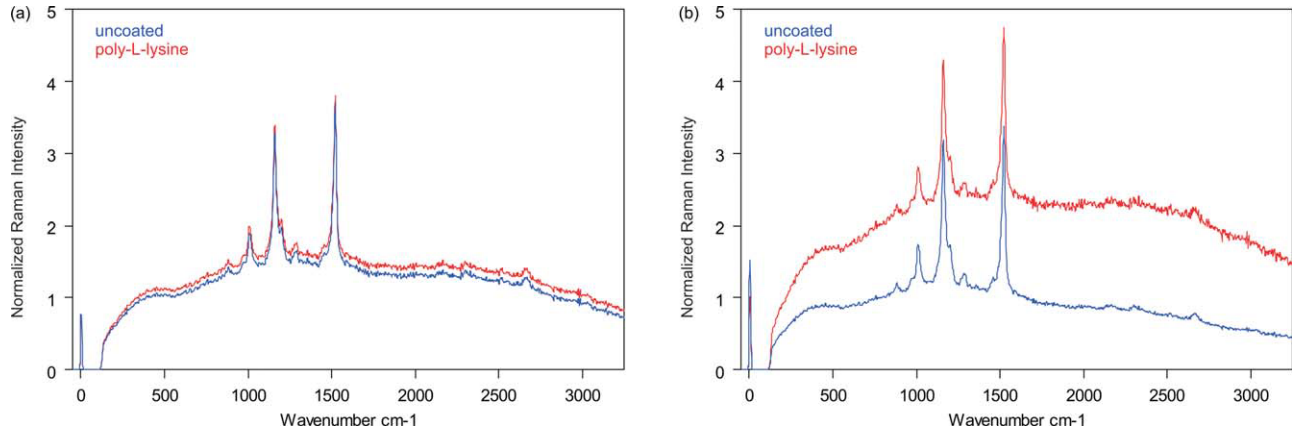

Fig. 4. Resonant Raman spectra of native and PFA fixed *Rhodobacter sphaeroides* 158 mounted on uncoated (**a**) and poly-L-lysine coated (**b**) microscope slides. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

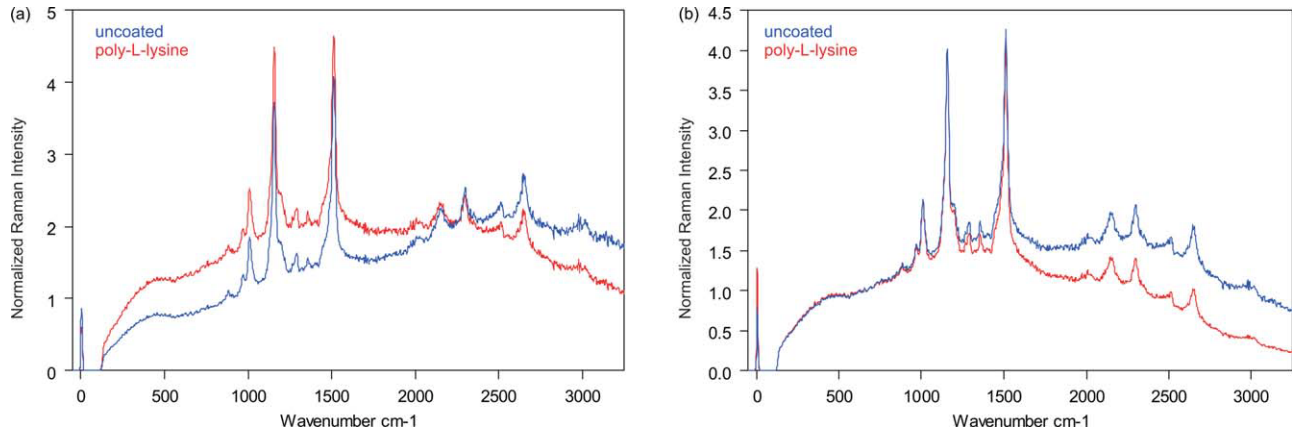

Fig. 5. Resonant Raman spectra of PFA-fixed *Rhodopseudomonas palustris* 123 (**a**) and PFA-fixed *Rhodospirillum rubrum* 467 (**b**) mounted on uncoated and poly-L-lysine coated microscope slides. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

with EtOH for 30 min, PFA for 1 h, and heat fixation at 70°C for 15 min, respectively. A similar comparison for *Rhodopseudomonas palustris* 123 (a) and *Rhodospirillum rubrum* 467 (b) is given in Figure 3. All samples were mounted on uncoated microscope slides (for the effect of poly-L-lysine coated slides, see below).

As can be seen, even gentle heat fixation causes the Raman spectrum to deteriorate with a strongly increased background and a complete loss of the weaker Raman lines independent of bacteria sample and type of microscope slide. Only the three dominant carotenoid lines are clearly visible, of which the two strongest lines were allocated correctly. The third strongest line above 1,000 cm^{-1} is broadened and shifted by 10 cm^{-1} to higher wavenumbers. Heat fixation is clearly detrimental to subsequent Raman measurements.

The fixatives EtOH and PFA both preserve the Raman spectrum of the bacteria well on uncoated microscope slides. Shape, spectral position, and relative intensity of the Raman lines are unchanged in comparison to the spectrum of native samples.

However, signal as well as background intensity is diminished by 25 to 50% in PFA fixation compared with when the sample was native or fixed with EtOH. Although this overall reduction in intensity has no influence on the quality of the spectrum itself, it may prove a problem in case of weak Raman spectra or less sensitive detectors.

Effect of Poly-L-lysine Coated Microscope Slides

As can be seen in Figure 2, the use of poly-L-lysine coated microscope slides in comparison to uncoated slides has little to no discernable effect on the Raman spectrum of native samples or samples fixed with EtOH or heat, but it is clearly detrimental to the Raman spectrum of samples fixed with PFA.

All spectra of PFA fixed samples recorded on poly-L-lysine coated slides show a distinct loss of signal and background intensity with increasing wavenumbers. The weaker Raman lines above 1,800 cm^{-1} are typically lost entirely, while the Raman lines at 880 and 1,289 cm^{-1} are still clearly visible. A comparative spectrum analysis with the analysis software settings suitable for the Raman spectrum of a native sample caused an average of 20 or more false positives throughout the spectrum. The same happened with the software set-

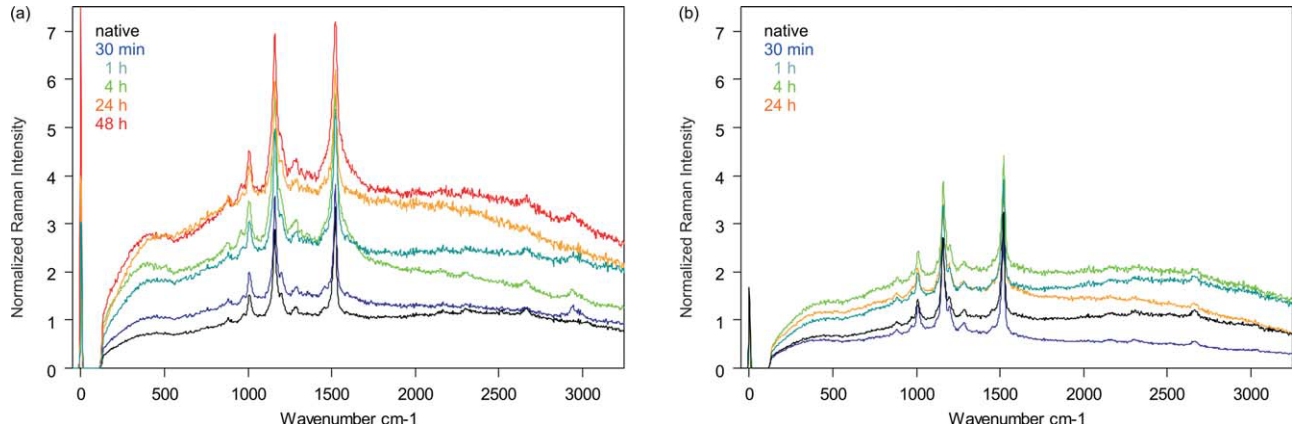

Fig. 6. Effects of extended ethanol (**a**) and paraformaldehyde (**b**) exposure on the resonant Raman spectrum of *Rhodobacter sphaeroides* 158 mounted on uncoated microscope slides. The spectra of *Rhodobacter* fixed with ethanol for 4 and 24 h show a different course above 1,600 cm^{-1} due to a different culture of the bacteria being used. The comparison with the spectrum of the corresponding native sample confirmed the variant course to be caused by the bacteria not the applied fixative. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

tings suitable for a PFA-fixed sample mounted on an uncoated microscope slide.

In addition, for PFA-fixed *Rhodobacter*, the signal intensity throughout the spectrum is 50% or less of that typically detected on uncoated slides. In the normalized spectra, this shows as an increase in background and noise compared with the measurement on uncoated slides. The same can be seen to a lesser extent in the Raman spectrum of PFA fixed *Rhodopseudomonas*, which yields ~20% loss on poly-L-lysine-coated slides, whereas no such effect is seen in the Raman spectrum of *Rhodospirillum* (see Fig. 4 for *Rhodobacter sphaeroides* and Fig. 5 for *Rhodopseudomonas palustris* (a) and *Rhodospirillum rubrum* (b)).

We assume the loss of signal intensity throughout the spectrum to be a surface effect, because it clearly decreases with increasing cell size. The cells of *Rhodobacter* with an average cell size of 1 μm are slightly smaller than the height of our measurement volume (1.2 μm). A flattening of these cells against the poly-L-lysine coated surface causes an effective loss in the cross-section of sample and measurement volume, which is also seen to a lesser extent for *Rhodopseudomonas* with its ellipsoid cells of up to 2 μm length and not at all for the up to 5 μm large *Rhodospirillum*.

At this point in time, we may only speculate about the reason for this interesting effect restricted to the combination of PFA fixation and poly-L-lysine-coated slides. We currently assume an increased interaction of the polycationic poly-L-lysine coating with anionic sites on the surface of PFA-fixed cells. The increased interaction may be caused either by a decrease in cationic sites on the cell surface due to methylene bridges formed in the bacterial protein matrix, or by a higher affinity of methylene bridged proteins to L-lysine. The latter might also be responsible for the loss in intensity specifically at higher wavenumbers, which is independent of cell size.

Effects of the Duration of Fixative Exposure

The effect of extended fixation on the resonant Raman spectrum of *Rhodobacter sphaeroides* 158 is shown in Figure 6. As can be seen, EtOH fixation causes additional fluorescent background in the spectrum, the intensity of which is increasing with the time the sample was exposed to the fixative. The overall signal intensity and the spectral position of the Raman lines is not affected within the first 24 h. However, the peaks of the intense Raman lines at 1,007, 1,157, and 1,519 cm^{-1}, associated with the carotenoid spheroidene, are found to be slightly broadening with time. The Raman line at 1,157 cm^{-1} shows this most clearly. This may be due to EtOH being a solvent to spheroidene.

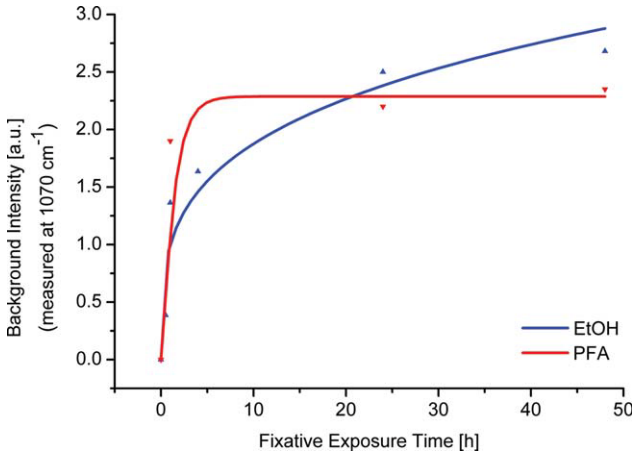

Fig. 7. Development of fluorescent background intensity in the Raman spectrum of *Rhodobacter sphaeroides* 158 measured at 1,070 cm^{-1} as a function of the time the sample was exposed to the fixative. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The resonant Raman spectrum of bacteria fixed with PFA for an extended period of time also shows an increased fluorescent background. However, this is due to a reduction of the signal intensity rather than an increase in background intensity, which causes weaker Raman lines to be lost in the noise or below the detection level. In addition, the background intensity

was fairly constant after 4 h. We strongly assume this to indicate that the formation of all possible methylene bridges within the protein matrix of the cell is completed after 4 h and additional fixative exposure has no further effect on the samples.

To illustrate the effects of both fixatives, Figure 7 shows the additional background intensity at 1,070 cm^{-1} in the normalized spectra as a function of the fixation time. Additional background caused by EtOH fixation follows a root function, whereas the background from PFA resembles a hyperbolic tangent. This indicates clearly that for short time fixation of less than a day, EtOH may be a preferable fixative, whereas in case of required longer exposure times, the results obtained with PFA may be superior.

CONCLUSIONS

EtOH and PFA fixation are both compatible with confocal resonance Raman microscopy, preserving the overall form and structure of the Raman spectrum well in case of short exposure to the fixative and use of uncoated microscope slides.

However, the signal intensity of Raman spectra of PFA-fixed samples is reduced in comparison to those of native or EtOH fixed samples. This may be critical in case of weak Raman lines. Mounting PFA fixed samples on poly-L-lysine coated microscope slides may cause additional loss of signal intensity increasing with higher wavenumbers. Losses above 1,800 cm^{-1} may become critical. In addition, the increased flattening of PFA fixed samples against poly-L-lysine coated surfaces is to be considered in case of small sample cells, which may not fill the measurement volume completely.

The time for which the sample is exposed to the fixative proved important for choosing the fixative with respect to subsequent resonance Raman microscopy. For short exposure times below 24 h, the results with EtOH fixed samples may be superior to those obtained with PFA fixed samples. However, if the sample is to be kept in the fixative for an extended amount of time, PFA may be preferable, because degeneration of the spectrum saturates after a few hours of exposure.

Heat fixation was found incompatible with subsequent resonance Raman microscopy. It causes a large increase in fluorescent background independent of the type of bacterial sample and a complete loss of all but the strongest Raman lines.

Best results, independent of microscope slide, were obtained from samples fixed with EtOH for 30 min and from samples exposed to PFA for 1 h, when mounted on uncoated slides.

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REFERENCES

Ballesteros SA, Chirife J, Bozzini JP. 1993. Antibacterial effects and cell morphological changes in *Staphylococcus aureus* subjected to low ethanol concentrations. J Food Sci 58:435–438.

Cogdell RJ, Gall A, Köhler J. 2006. The architecture and function of the light-harvesting apparatus of purple bacteria: From single molecules to *in vivo* membranes. Q Rev Biophys 39:227–324.

Denyer SP. 1995. Mechanisms of action of antibacterial biocides. Int Biodeterior Biodegrad 35:227–245.

Denyer SP, Steward GSAB. 1998. Mechanisms of action of disinfectants. Int Biodeterior Biodegrad 41:261–268.

Eads DD, Moser C, Blackwood ME, Jr, Lin C, Dutton L, Spiro TG. 2000. Selective enhancement of resonance raman spectra of separate bacteriopheophytins in *Rb. sphaeroides* reaction centers. Biopolymers (Biospectroscopy) 57:64–76.

Fox CH, Johnson FB, Whiting J, Roller PP. 1985. Formaldehyde fixation. J Histochem Cytochem 33:845–853.

Harz M, Rösch P, Popp J. 2009. Vibrational spectroscopy—A powerful tool for the rapid identification of microbial cells at the single-cell level. Cytometry A 75:104–113.

Huang WE, Stoecker K, Griffiths R, Newbold L, Daims H, Whiteley AS, Wagner M. 2007. Raman-FISH: combining stable-isotope Raman spectroscopy and fluorescence *in situ* hybridization for the single cell analysis of identity and function. Environ Microbiol 9:1878–1889.

Imhoff JF. 2006. The phototrophic alpha-proteo bacteria. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E, editors. The prokaryotes. New York: Springer. pp. 41–64.

Imhoff JF, Trüper HG, Pfennig N. 1984. Rearrangement of the species and genera of the phototrophic "purple nonsulfur bacteria." Int J Syst Bacteriol 34:340–343.

Koyama Y. 1991. Structures and functions of carotenoids in photosynthetic systems. J Photochem Photobiol B 9:265–280.

Koyama Y. 1995. Resonance Raman spectroscopy. In: Britton G, Liaaen-Jensen S, Pfander H, editors. Carotenoids, Vol 1B: Spectroscopy. Basel: Birkhäuser. pp. 135–146.

Kudelski A. 2008. Analytical applications of Raman spectroscopy. Talanta 76:1–8.

Larson EL, Morton HE. 1991. Alcohols. In: Block S, editor. Disinfection, sterilization, and preservation. Philadelphia: Lea and Febiger. pp. 191–203.

Mackenzie C, Eraso JM, Choudhary M, Roh JH, Zeng X, Bruscella P, Puskás A, Kaplan S. 2007. Postgenomic adventures with *Rhodobacter sphaeroides*.. Annu Rev Microbiol 61:283–307.

Mazia D, Schatten G, Sale W. 1975. Adhesion of cells to surfaces coated with polylysine. J Cell Biol 66:198–200.

Metz B, Kersten GFA, Hoogerhout P, Brugghe HF, Timmermans HAM, de Jong A, Meiring H, ten Hove J, Hennink WE, Crommelin DJA, Jiskoot W. 2004. Identification of formaldehyde-induced modifications in proteins. J Biol Chem 279:6235–6243.

Russel AD. 1991. Principles of antimicrobial activity. In: Block S, editor. Disinfection, sterilization, and preservation. Philadelphia: Lea and Febiger. pp. 27–58.

Rutala WA. 1989. Draft guideline for selection and use of disinfectants. Am J Infect Control 17:24–38.

Varotsis C, Babcock GT, Garcia-Horsman JA, Gennis RB. 1995. Resonance Raman spectroscopy of the heme groups of cytochrome cbb3 in *Rhodobacter sphaeroides*. J Phys Chem 99:16817–16820.