Non-invasive study of nerve fibres using laser interference microscopy

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This paper presents the results of a laser interference microscopy study of the morphology and dynamical properties of myelinated nerve fibres. We describe the principles of operation of the phase-modulated laser interference microscope and show how this novel technique allows us to obtain information non-invasively about the internal structure of different regions of a nerve fibre. We also analyse the temporal variations in the internal optical properties in order to detect the rhythmic activity in the nerve fibre at different time scales and to shed light on the underlying biological processes. We observe pronounced frequencies in the dynamics of the optical properties and suggest that the oscillatory modes have similar origin in different regions, but different strengths and mutual modulation properties.

Keywords: laser interference microscopy; live-cell imaging; nerve fibres

1. Introduction

The choice of experimental techniques for research in cellular biology is basically a compromise between three different objectives: to gain as diverse information on the cellular processes as the object can provide; to approach the nanoscale level of intracellular structures; and to cause as little damage to the object as possible. The continuous search for this compromise is reflected in the rapid progress in the development of new imaging techniques for research in the areas of cellular biophysics, molecular biology, neurophysiology, etc. How can we learn more about the private life of the cell without disturbing the biological processes or damaging the cell?

It is obviously a problem that the internal processes in most cell types cannot be followed under a conventional microscope without special preparation. One uses microelectrodes to study the membrane potential and electrical activity

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of nerve or muscle cells, and various fluorescent dyes are applied to visualize
the intracellular structures or processes. However, these techniques are all
more or less damaging. Besides, they cannot provide simultaneous infor-
mation on several co-occurring multiple processes. Hence, there is an obvious
need for novel techniques to complement traditional electrophysiology and
fluorescent microscopy.

A first question to address is how we can avoid the use of dyes. Generally
speaking, staining is used to make specific cellular components, such as proteins
or ions, visible or to increase the contrast in cells that are nearly transparent so
that the information available from conventional amplitude microscopy without
staining is restricted. However, the local refractive index (RI), an intrinsic
optical property (IOP) of biological objects, provides additional valuable
information. Although the cell often does not absorb light effectively, various
cellular structures may have different RI values and, therefore, different phase
shifts or delay times for light beams propagating through the object. From the
spatial variation in the phase of the transmitted laser light, it is thus possible to
construct a so-called phase image of the cell. This image contains information
about the local RI and, therefore, about the cellular structures.

The idea of phase shift detection and phase image reconstruction underlies a
number of new microscopy techniques. The main advantage of phase imaging is
obviously that this technique is less invasive than fluorescent microscopy. The
position of laser interference microscopy among other microscopy techniques for
cell research is illustrated in figure 1. The figure clearly shows how laser
interference microscopy covers the extremely important region where processes
in living cells can be studied with subcellular spatial resolution.

Measurement of the phase shifts resulting from the retardation of the
propagating light with subsequent reconstruction of cellular phase images
with submicrometre resolution is achieved in several types of interference
microscopy: laser interference microscopy (Andreev & Indukaev 2003; Sosnovtseva et al. 2005), digital holographic microscopy (Rappaz et al. 2005), Fourier phase microscopy, Hilbert phase microscopy, diffraction phase microscopy, etc (Tychinskii 2001; Popescu et al. 2004, 2005, 2006). These types of interference microscopy represent in some sense a replacement for the once quite popular technique of light scattering (Cohen et al. 1968; Stepnoski et al. 1991; Boppart 2003; Lazebnik et al. 2003). Despite many advantages, light-scattering-based microscopy has its own limitations because the intensity of the scattered light is relatively weak compared with the practical detection limits. Hence, the light is difficult to observe without extensive signal accumulation. By virtue of this limitation, light scattering can only be measured from cells with significant changes in IOP, such as neurons and nerve fibres under electrical excitation.

The pioneering work in this field was done by Hill & Keynes (1949) and Cohen et al. (1968), who observed changes in the light-scattering intensity of a nerve fibre during electrical activity. Later Stepnoski et al. (1991) measured the angular distribution of scattered laser light from the soma, axon and dendrites of Aplysia neurons under electrical stimulation. The change in the light scattering had the same temporal characteristics as the action potentials, and the optically detected electrical activity completely corresponded to the electrically recorded signals. More recently it has been shown that the intrinsic optical properties of a cell also depend on the organization of the cytoskeleton and the location of the various organelles (Haller et al. 2001). It has also become clear that variations in the IOP dynamics can reflect intracellular activity, provided that the applied techniques are sensitive to small changes in the RI values. Interference microscopy is much more sensitive to IOP changes than light-scattering microscopy, and the development of different phase imaging microscopes has made it possible to use the IOP for studies of cells, excitable and otherwise.

At present, phase images have been obtained for a variety of cell types, organelles and even whole organisms: neurons (Erokhova et al. 2005; Rappaz et al. 2005; Sosnovtseva et al. 2005; Brazhe et al. 2006; Yusipovich et al. 2006), erythrocytes (Popescu et al. 2004, 2005; Brazhe et al. 2006), mast cells (Brazhe et al. 2006), HeLa cells (Popescu et al. 2004; Choi et al. 2007), epithelial kidney cells (Park et al. 2006), chloroplasts (Tychinsky et al. 2004) and the nematode Caenorhabditis elegans (Choi et al. 2007). Studies of intracellular dynamics by means of interference microscopy are less numerous. Most such studies focus either on the investigation of relatively slow changes in the cell thickness (up to 0.5 Hz; Popescu et al. 2004), on fast measurements (with millisecond resolution) of the optical path length without further frequency analysis (Popescu et al. 2005), or on dynamical morphometry (Rappaz et al. 2005).

We have studied the intracellular dynamics in a broader frequency region and have shown that different types of neurons, erythrocytes and mast cells display regular changes in RI with frequencies in the 0.1–30 Hz range that are specific for each cell type (Sosnovtseva et al. 2005; Brazhe et al. 2005, 2006). However, even if the best approaches to interference microscopy are applied, a significant amount of valuable information about the cell compartmentalization and dynamics may remain undetected. To further understand the cellular processes and reveal their characteristic time patterns and mutual interactions, it is necessary to combine phase imaging of various cell types with advanced
data analysis techniques. At the same time, it is necessary to investigate other biological objects, well studied by means of traditional techniques, and to compare the obtained results.

To our knowledge, myelinated nerve fibres have not previously been studied by interference phase microscopy. This is partly due to the high compactness of myelin and the associated technical difficulties. However, it is apparent that non-invasive studies of nerve fibres and detailed investigations of fibre structure and function are interesting and significant aspects of both fundamental and applied neural research.

Nerves provide information about the outside world to the central nervous system and convey the control commands throughout the body. Failure to perform these functions lies at the basis of several disorders, many of which are related to demyelination, a state that leads to both pain and block of transmission. The morphology of a nerve fibre is quite complex, especially in the paranode–node–paranode (PNP) region (Scherer & Arroyo 2002). A relatively complete understanding of the PNP and myelin structure was formed only several years ago, thanks to the extensive development of confocal microscopy with fluorescent proteins and methods of molecular biology and biochemistry with site-directed mutagenesis. Figure 2 shows a simplified schematic of this region. The scheme was composed on the basis of multiple data of histochemistry, electron scanning microscopy and a combination of techniques from molecular biology and confocal microscopy (Waxman et al. 1995; Scherer & Arroyo 2002; Corfas et al. 2004; Sasaki et al. 2006). Schwann cells form myelin sheaths around the long spans of the axon in the so-called internode regions. The point where two myelinated regions meet—node of Ranvier—is free from myelin; however, the axon is
here surrounded by microvilli (tiny protrusions) of Schwann cells. It is in these nodes that the transmembrane ionic currents primarily take place during the action potentials.

On both flanks of the Ranvier node, there are paranode regions in which other cytoplasm-enriched loops of a Schwann cell—the paranodal loops (non-compact myelin)—fasten the Schwann cell to the axon. Further along the nerve, the paranodal region then blends into the internode region with compact myelin. Layers of compact myelin consist of Schwann cell membranes wrapped around the axon. The body of the Schwann cell lies outside of the myelin. Each region of the myelin and the axon is characterized by its own composition of transmembrane proteins. In mammals, the axon plasma membrane in the Ranvier node is enriched with voltage-dependent Na and Ca channels. Slow K channels are also present there. The paranodal region is enriched with fast voltage-dependent and other potassium channels; adjacent to the node there are also specific proteins that provide junctions between myelin and the axon. Paranode regions also have transmembrane proteins that form tight junctions with cytoskeleton proteins.

In the myelinated nerve fibres of the frog, the distribution of ion channels in the axon membrane is less strict than in mammals. However, the higher concentration of ion channels in the Ranvier node is preserved. Sodium channels are sparse in the internode regions and rich near the nodes. Most of the potassium channels are uniformly distributed.

Paranode loops provide isolation of the extracellular space in the internode and paranode regions. This helps to maintain a low membrane potential of the axonal membrane in the internode during the propagation of an excitation.

It is known that the structure of the nerve fibre is important for the correct function and impulse transduction. The glia-rich structures of the PNP suggest that an active information exchange between the glia and the axon must take place here. Many nerve pathologies are related to deterioration of PNP structure and axoglial interactions. However, up to this time, simultaneous and non-invasive investigations of the detailed PNP morphology and function of myelinated nerve are still troublesome.

The present study for the first time applies laser interference microscopy to visualize and study the dynamics of myelinated nerve fibres in the PNP region. In the first part of §4b, we focus on fibre morphology and compare phase images with conventional microscopic photographs. In the second part of the section, we study the IOP dynamics in different nerve fibre regions and by means of wavelet analysis in order to estimate the characteristic frequencies of the IOP variations. Our results indicate that the details of the fibre structures are resolved better in phase images than in the conventional microphotographs. We also observe pronounced frequencies in the dynamics of the fibre optic properties and suggest that these frequencies have similar origin in different fibre regions but different modulation properties.

2. Principles of laser interference microscopy

Laser interference microscopes differ in the details of their design, principles of operation and algorithm of the phase image reconstruction (Bennet et al. 1951; Allen 1985; Tychinsky 1989, 1991). However, they all have a number of
important features in common. The phase image of the object is reconstructed
by measuring the optical path difference (OPD) between a control (or reference)
laser beam and the laser beam transmitted through the object and retarded
by its different structures. As discussed above, the OPD value depends on the
intrinsic optical properties of the cell, and records of the spatial and temporal
variations in OPD provide information on cellular structure and dynamics. In the
following, we shall first discuss some of the basic principles of laser interference
microscopy and thereafter describe the operation of the phase-modulation laser
interference microscopy that we used to study the dynamics of nerve fibres and
the phase-stepping interference microscope that we used to obtain phase images
of the nerve fibres.

\( a \) Dynamics of intrinsic optical properties

The phase-modulation laser interference microscope MIM 2.1 was developed
by Amphora Laboratories in Moscow, Russia (Andreev & Indukaev 2003, 2005).
The MIM 2.1 set-up is a modified Mach–Zehnder interferometer working in the
reflected light mode (figure 3) and equipped with a 532 nm He–Ne laser with an
output power of 40 mW. The choice of the reflected light mode stems from the
original application of the set-up to examine the surface of nanotechnological
structures. The basic principle of MIM 2.1 is the following: a beam of collimated
light from the laser (L) is divided on the polarizing beam splitter (PBS) into
the object beam (OB) and the reference beam (RB). The RB, reflected from
the reference phase-modulation mirror (PM), and the OB, transmitted through
the sample and reflected from the bottom mirror layer, interfere on the detector
(D). The ratio of light intensity distribution between OB and RB can be adjusted
by rotating the half-wave plate (1/2 WP) inserted in front of the beam splitter.
The OB goes through the objective O1, the non-polarizing beam splitter BS1 and the telescopical system T. The RB is reflected from the PM and forms the reference wavefront via the objective O2, the non-polarizing beam splitter BS2 and the telescopical system. The PM is located near the focal plane of the objective O2. Object and reference wavefronts form the interference image on the CMOS photosensor plate D. The pixelwise analysis of the image is operated by a personal computer. The operating software was also developed at Amphora Laboratories.

While in most interference microscopy imaging systems the phase profile is reconstructed from the full interference image or a series of images, the phase profile reconstruction algorithm in the MIM set-up is pixelwise. That is, for each pixel of the detector the phase height is determined independently. The PM is harmonically modulated with 500 Hz frequency by means of a piezoelectric crystal, and, therefore, the length of the RB is also modulated. In this way, the light intensity measured in a pixel is seen as a function of the RB length. The intensity of the pixel varies with the movement of the PM, and the position of the PM, which corresponds to the point of maximal rate of intensity change, is found by the computer system. The use of a calibrated piezomodulator allows us to determine the precise position of the PM, and this position is used as a measure of the local and instantaneous OPD. For more details, the reader is referred to the description of the principles of design and function in the recent paper by the constructors of the microscope (Andreev & Indukaev 2005; Andreev et al. 2005).

The OPD values, obtained for each pixel, are described by

$$\Phi_{\text{obj}} = \frac{\phi_0 - \phi_{\text{obj}}}{2\pi} \frac{\lambda}{2} - \Phi_0.$$  (2.1)

Here, $\phi_0$ and $\lambda$ are the initial phase and the wavelength of the laser beam, respectively; $\phi_{\text{obj}}$ is the phase in the presence of the object; and $\Phi_0$ is a constant phase shift determined by the choice of the phase reference point. Together all the values of $\Phi_{\text{obj}}$ constitute a surface that we refer to as the OPD relief or, in other words, the phase image of the object.

The obtained interference image is prone to many forms of external disturbance. To eliminate the influence of such disturbances, a channel for the subtraction of artificial low-frequency vibrations was used. The active vibro-isolation is an additional photodiode placed near the CCD array. It receives light from a region adjacent to the working view area and its signal is subtracted from the CCD signal. Thus, any vibrations, inducing movements of the object as a whole, are subtracted out.

We used a $20 \times$ objective with a numerical aperture $\text{NA} = 0.15$, as a low-aperture objective provides a better depth of focus. The lateral resolution of the object was 2.16 $\mu$m, and the visible field size was 52.3 $\mu$m. The laser power impinging on a region with diameter approximately 50 $\mu$m was less than 1 mW.

As the OPD measurements can be made independently for each pixel, we adopted the following procedure for the measurements: a line of 13 nm with 32 uniformly distributed pixels was chosen (a scan line), and the OPD in these pixels was measured in cycles. Since the processing of each pixel takes 20 ms, the sampling rate for each data track on a scan line was 15.6 Hz.
The OPD depends on both the RI and the cell height and can be described by the following equation:

$$\Phi(x, y) = \int_0^Z (n_{\text{obj}}(x, y, z) - n_s) \, dz - \Phi_0,$$

with $n_s$ being the (constant) RI of the physiological saline surrounding the biological object; $n_{\text{obj}}(x, y, z)$ the RI of the cell in the point $(x, y)$ at distance $z$ from the mirror; and $Z$ a point somewhere above the cell.

To estimate the sensitivity of the technique, consider the general form of the phase-height differential, $d\Phi = Z \, dn + (n - n_s) \, dZ$. As a typical value for $n$, we can take $n = 1.38$ (Rappaz et al. 2005). The RI of the physiological solution is $n_s = 1.34$, while the fibre diameter could be $Z \approx 10\, \mu m$. If we now compare the effects of a 1 per cent change in $Z$ and $n$, we obtain $Z \, dn \approx 0.14\, \mu m$ and $(n - n_s) \, dZ \approx 0.004\, \mu m$. Thus, as long as the changes in the fibre thickness are relatively small, the OPD dynamics is mainly determined by variations in $n$, i.e. in the cellular optical properties. As mentioned, the local RI and, therefore, the OPD values reflect many different cellular processes (e.g. changes in the membrane potential, movements of vesicles along the cytoskeleton elements, changes in the membrane fluidity and in the clustering of membrane-bound channels, etc.). We suppose that observable rhythms in the OPD dynamics can be ascribed to specific cellular processes and to their mutual interactions.

(b) Phase imaging

A phase-stepping interference microscope, developed by the Russian Scientific Research Institute of Optical and Physical Measurements (VNIIOFI; Vishnyakov & Levin 1998), Moscow, Russia, was used for the nerve fibre imaging. This device is based on the MII-4 Linnik interferometer (LOMO, Russia) with a solid-state 650 nm laser light source providing a continuous-wave total power of 5 mW, which is less than 1 mW per object. After a number of initial experiments, we decided to use this set-up for the nerve fibre imaging in the present study because the fibre is too thick for good imaging under the green laser of MIM 2.1. In the VNIIOFI set-up, there is a red laser that allows us to obtain phase images for larger and thicker objects.

We used the microscope with a $20 \times$ objective with the numerical aperture $NA = 0.65$. The size of the working interference view area was $150 \times 100 \, \mu m$. To obtain a phase image, five interference fringe images at different phase values of the RB were made; each of the five took 1.5 s to make, so the whole phase image of an object took 7.5 s. The OPD profile reconstruction was made by the method of phase steps on a generic PC by means of the software TVMicro-2p, developed at VNIIOFI and provided with the device (Vishnyakov & Levin 1998).

3. Experimental procedure for nerve visualization

Our study was performed on individual myelinated nerve fibres from the sciatic nerve of the grass frog Rana temporaria. The sciatic nerve was isolated after anaesthesia of the animal and was placed in a Petri dish with the appropriate physiological solution (100 mM NaCl, 2 mM KCl, 1.08 mM CaCl$_2$ and 11 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid)) for 30 min. Hereafter, individual nerve fibres were isolated from the sciatic nerve with a thin glass
needle, and the isolated nerve fibres were placed in a containment chamber with a mirror bottom layer and filled with physiological solution. In order to verify that the laser illumination did not cause photodamage, we measured the absorbance of the nerve in the 532 nm region. No light absorption was found in this region and photodamage is therefore unlikely to take place. The nerve fibres were stable under illumination of both lasers (532 and 650 nm) for at least 3 hours, which is significantly longer than the duration of the experiments. We did not test the absorption of the 650 nm laser light by nerve fibres. It is known, however, that red light in general is less damaging to cells than blue and green light (Hamblin & Demidova 2006). All experiments were done on nerve fibres attached to the chamber bottom layer, and any change in the observed OPD value can thus be ascribed to processes in the fibre, not to its displacement as a whole.

As described above, the myelinated nerve fibre represents a complicated structure with several functionally different regions. Ordinary microscopy in the transmitted light configuration usually does not provide sufficient information.

Figure 4. (a) Photograph of the myelinated nerve fibre in reflected light. (b,c) Phase images in the (b) pseudo-three- and (c) two-dimensional projections of the nerve fibre in the region marked by the white square in (a). Scale bars in (a) and (c) represent 10 µm. In (b), (i) points to the axon in the node of Ranvier, (ii) to the microvilli of Schwann cells, (iii) to the paranodal region, and (iv) to the myelinated internode region. (d) Phase profiles along the scan lines 1 and 2 as shown in (c). The scan line 1 crosses the nerve fibre in the myelinated internode region, and the scan line 2 in the region of the Ranvier node. The length scale of the horizontal axis is in micrometres and the vertical axis shows optical path difference in nanometres.
about the morphology of the nerve fibre. Figure 4a shows a photograph of the myelinated nerve fibre in the reflected light set-up. Ranvier nodes are easily identified as the more narrow regions while the internode regions are thicker, but it is difficult to see any difference in the thickness and the compactness of the node and internode regions. We should also emphasize that the presented photograph in reflected light provides better contrast than is usually the case for conventional photographs in transmitted light. Figure 4b,c represents phase images of the same nerve fibre; figure 4b is a pseudo-three-dimensional phase image, constructed from the two-dimensional phase image shown in figure 4c. White colour in figure 4b,c indicates the areas with higher OPD values corresponding to regions with higher RI and/or higher geometrical height. The Ranvier nodes are clearly identified in the phase image and the lamellae of the Schwann cell that surround the axon are resolved better than in figure 4a. Paranoide regions in the phase images have an uneven, hilly structure, presumably due to the paranodal loops attached to the axon. The OPD value in the paranodal region is larger than in the node owing to the greater thickness and, therefore, higher OPD of the paranode. The phase image in the myelinated internode region has an unusual structure: it is higher at the boundaries and lower in the centre. It is also seen that the OPD value along the whole fibre is the highest on the boundary of the myelinated internode region. This is due to the structure of myelin, which is thicker and more compact than the axon plasma membrane and cytoplasm. Again, we would like to stress that the differences between paranode, node and myelin regions are much more pronounced on the phase image than on a conventional microphotograph. Besides, phase images give quantitative information about redistribution of RI, which is unavailable from the amplitude microscopic images.

4. Dynamic properties of the myelinated nerve fibre

(a) Wavelet and double-wavelet transform

Spectral analysis of biological time series is ever more often based on the application of a wavelet transform to the data (Grossmann & Morlet 1984). The advantages of this approach in comparison with the classical Fourier transform have been widely discussed in the literature (Addison 2002). Generally speaking, the wavelet transform allows us to follow the temporal variation of the amplitudes and frequencies of the various spectral components in a non-stationary time series. The continuous wavelet transform of a signal \(x(t)\) is given by

\[
T_x(a, t) = \frac{1}{\sqrt{a}} \int_{-\infty}^{\infty} x(u) \psi^* \left( \frac{u - t}{a} \right) du,
\]

where \(\psi\) is a ‘mother’ wavelet function. This function should be soliton-like with zero average, and the wavelet transform is obtained by convoluting the signal with a dilated and translated replica of the mother function.

In equation (4.1) \(T_x(a, t)\) are called the wavelet coefficients and \(a\) is the time-scale parameter. The details of this transform (e.g. the choice of \(\psi\)) depend on the application. In the analyses of rhythmic components, the Morlet function is
typically chosen. A simplified expression of the Morlet function has the form

\[ \psi(\tau) = \pi^{-1/4} \exp(j2\pi f_0 \tau) \exp \left[ -\frac{\tau^2}{2} \right]. \] (4.2)

The value \( f_0 \) allows us to search for a compromise between the temporal localization of the wavelet and its sharpness in the frequency domain. We used \( f_0 = 1.5 \) in this work. The relation between the scale \( a \) and the central frequency for the mother function \( f \) is then \( f \approx f_0/a \).

The wavelet transform coefficients are complex and contain information about both the phase and the amplitude at different frequencies. To study the rhythmic components in the OPD dynamics for myelinated nerve, we used the total energy contained in the signal: \( E_x(a, t) \propto |T_x(a, b)|^2 \).

Owing to the frequency distribution in the wavelet function, the time-averaged power spectra are somewhat broadened compared with the power spectra obtained from a Fourier transformation. Particularly, for higher frequencies the peaks associated with some components in the signal become broader and lower. Since we trace only the amplitude of the local maxima within the rhythm, we need to correct for this peak amplitude attenuation. By comparing the transforms of a series of artificial harmonic signals, we ended up with an empirical frequency-dependent correction term. In all the following figures we worked with the surface

\[ S_x(f, b) = \frac{2f^2}{\pi} |T(f, b)|^2, \] (4.3)

in which all the peaks related to the frequencies were of similar heights.

(b) Results and discussion

Regular cooperative processes inside the cell are assumed to lead to regular changes in the RI. In the early days it was supposed that the electrical activity of neurons and the propagation of an excitation along the nerve fibres would affect their intrinsic optical properties more strongly than most other cellular processes. As previously mentioned, Stepnoski et al. (1991) observed light scattering from stimulated neurons and their fibres, caused presumably by a reorientation of dipoles in the membrane and the resulting change in the RI. Under rest conditions, a nerve does not undergo rapid changes in membrane potential and related reorganizations in the cytoplasm. However, the nerve needs to maintain its rest potential, the specific clusterization of the plasma membrane proteins (channels, transporters and ATPases) and the localization of mitochondria in the cytoplasm. Hence, it seems natural to suggest that the intrinsic optical properties of the nerve fibre will undergo continuous changes even without any external excitation. Our working hypothesis was therefore that the OPD dynamics would display rhythmic components that could be ascribed to specific cellular processes and their interactions and that these rhythms would differ from region to region in the fibre. The following section represents the characteristic data of OPD dynamics in the different nerve regions. Similar observations were made in 10 independent experiments.

Figure 5a presents the evolution in time of the OPD along the scan line crossing the Ranvier node. Data are presented without detrending, so there is a slight background increase of OPD with time in all fibre and chamber points.
However, this does not affect the frequencies observed in figure 5b. Figure 5c,d shows schematics of the nerve fibre in the region of the Ranvier node. Points 1–6 and points 22 and 23 of the scan line lie in the chamber (regions (i)) and points 7–21 lie in the nerve fibre (regions (ii, iii)). The ‘Ranvier node’ and the ‘chamber’ points are clearly distinguishable, as their OPD values are significantly different. Figure 5b represents power spectra of the OPD variations in all points of the scan line, obtained by means of the standard procedure of fast Fourier transform (FFT). As we were limited by the sampling frequency and recording time, we could analyse OPD variations only up to 7.0 Hz. However, we did not observe any frequencies in the range 2.0–7.0 Hz, and, for this reason, we present only the results from the frequency range up to 2.5 Hz. It is important to note that the boundaries of the Ranvier node can be easily distinguished from the chamber by the higher intensity of the OPD variations. Remarkably, the centre of the node does not display pronounced rhythmic components at frequencies higher than 0.2 Hz, but it does exhibit intensive frequencies lower than 0.15 Hz. In the case of the internode regions (both myelinated and paranodal regions), FFT reveals less power spectral density than at the node boundary (data not shown).
To investigate the time dependence of the frequencies in the observed OPD variations in different nerve fibre regions (myelinated internode, paranode, boundary and centre of the Ranvier node), we applied the technique of the wavelet transform. Figure 6 presents the obtained spectrogram, i.e. a matrix of wavelet coefficients showing the power spectral density of the OPD variations at the boundary of the Ranvier node (21st point of the scan line shown in figure 5). The overall variation of OPD with time is shown in figure 6. Several pronounced rhythms of approximately 0.1, 0.3, 0.8, 1.0 and 1.5 Hz can be seen in the spectrogram. All rhythms except that at 0.1 Hz are highlighted by semi-transparent white curves. These curves were drawn with a simple rhythm-tracing algorithm: a piecewise frequency band of interest is roughly selected, and then at each time point the local maxima of the spectrogram are automatically detected. These constitute a ridge on the surface—a rhythm. Indeed, in an extremal case of the narrow frequency band, this procedure tends to be subjective. At present, we cannot provide a precise measure of the objectiveness of the procedure, but we tried to avoid too subjective rhythm following. The frequencies of the low-frequency rhythms 0.1 and 0.3 Hz remain relatively stable during the period of observation, whereas the rhythms of 0.8, 1.0 and 1.5 Hz change with respect to both their power and their frequency. This non-stationarity of the rhythmic components arises at least partly because the cellular processes interact with one another, with the result that the amplitudes and frequencies of the high-frequency modes are modulated by the low-frequency modes.

Figure 6. (a) Spectrogram for the OPD dynamics in the 21st point of the scan line shown in figure 5. The selected point corresponds to the boundary of the Ranvier node. Pale curves show identified rhythmic components. Colours indicate the spectral power of various frequencies. (b) Time dependence of the OPD variations in the 21st point of the scan line. The vertical axis is OPD in nanometres.
In order to compare rhythmic components in the OPD dynamics between different nerve fibre regions, we analysed the time-averaged power spectra of the OPD variations in the four studied regions (figure 7). One can observe significant differences in the intensity and structures of the spectra. Power spectra for the myelinated internode region and the paranode do not exhibit clear peaks, but a broad ‘tail’ from 0.1 up to 1.0 Hz (figure 7a,b). However, in the corresponding spectrograms rhythms at approximately 0.1, 0.8 and 1.0 Hz can be distinguished, though they have lower intensity than the same rhythms at the node boundary. The node boundary displays both the highest power spectral density and the most pronounced structure of its spectrum (figure 7c). Frequencies at approximately 0.1, 0.3, 0.8, 1.0 and 1.5 Hz can be seen in the power spectrum as well as in the spectrogram, as shown in figure 6. In the power spectrum of the node centre, there are similar frequencies, however, less pronounced than in the node boundary. This difference in the intensity of the rhythms between the node boundary and node centre could be due to the fact that the most intense changes in intrinsic optical properties come from membrane processes. Apparently, on the edges of the nerve fibre the column of integration of OPD incorporates less height of cytoplasm than in the fibre centre. Therefore, the membrane/cytoplasm ratio is higher in the boundary region than in the fibre centre and, consequently, the contributions of the membrane and submembrane processes to the OPD dynamics are also higher in the node boundary.

In previous papers, we discussed how neurons under rest conditions can display rhythmic changes in the OPD dynamics due to variations in the RI (Sosnovtseva et al. 2005; Brazhe et al. 2006). We also suggested the origin of these variations to be subthreshold changes in the membrane potential, ion channel activity and vesicular transport in the cytoplasm, and we showed that the 1.0 Hz rhythm relates to K fluxes through the plasma membrane (Brazhe et al. 2006). It is known that in the absence of external excitation all ion channels in a nerve fibre except the slow voltage-dependent K channels are inactivated, and that the rest membrane potential remains nearly constant. We suppose that the activity of K channels and low-amplitude changes in the membrane potential

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Figure 7. Time-averaged power spectra of the OPD dynamics in (a) the centre of the internode region, (b) the centre of the paranode region, and (c) and (d) the boundary and centre of the Ranvier node, respectively. Note how the spectral amplitudes and frequencies change from region to region.
may contribute to the 1.0 Hz rhythm in nerve fibres as in neurons (Brazhe et al. 2006). However, in contrast to neurons, for which the shape and volume remain constant during the whole period of observation, nerve fibres have small movements of Schwann cell lamellae near the Ranvier node. The structures of the paranodal and myelinated internodal regions are firm due to the multiple joints between myelin and the axon and, hence, do not change their volume or shape. It is therefore natural to assume that the pronounced frequencies observed at the node boundary are caused at least partly by the movements of the flexible parts of a fibre—by Schwann cell lamellae that are not attached to the axon plasma membrane. We should also note that such movements are not visible during ordinary microscopic monitoring of the nerve fibre.

We exclude a spatial displacement of the nerve fibre from the possible causes for the observed frequencies in the node boundary, as the experiments were performed on nerve fibres that attached themselves to the bottom layer of the chamber and similar results were obtained in all independent experiments. Besides, any spatial displacement would have been seen in the evolution of the OPD in time as a displacement of the region with high OPD to the right or to the left side, and we did not observe such a displacement.

To investigate if the temporal variations of the observed rhythms could be caused by nonlinear interaction of the cellular processes, we traced the frequencies and powers of the selected rhythms and applied the wavelet analysis to them. Rhythm tracing was done with the help of the iWand software, developed by one of the present authors (see http://iwand.sf.net). A rhythm is determined as a continuous succession of local maxima in the instantaneous power spectra. The time dependence of the power and frequency of the selected rhythm is then considered as an input signal for the second wavelet analysis. As a result, we obtain a set of frequency and amplitude modulation (FM and AM) spectra.

Figure 8 shows time-averaged spectra of the AM for the rhythms at 0.8, 1.0 and 1.5 Hz as measured in the centre of the internode region (thick black dash-dotted line), the centre of the paranode region (grey line) and the boundary and the centre of the Ranvier node (black solid line and black dashed line, respectively). Note how the intensity and frequency of the modulation vary from region to region.

Figure 8. Time-averaged spectra of the amplitude modulation of the rhythms at (a) 0.8, (b) 1.0 and (c) 1.5 Hz as measured in the centre of the internode region (thick black dash-dotted line), the centre of the paranode region (grey line) and the boundary and the centre of the Ranvier node (black solid line and black dashed line, respectively). Note how the intensity and frequency of the modulation vary from region to region.
especially, the intensity of modulation is much higher than in the myelinated internode and paranode regions. We suggest that the studied rhythms have similar origin, but they are more expressed at the node boundary. We should note that the calculated spectra of FM for the same rhythms have similar intensity in all regions.

Figure 9 shows time-averaged spectra of (a) AM and (b) FM of the rhythms at 0.8 Hz (black solid line), 1.0 Hz (grey line) and 1.5 Hz (black dashed line) in the boundary of the Ranvier node. We can clearly see that different rhythmic activities have different modulation properties.

5. Conclusion

We showed that interference microscopy can be applied to the study of nerve fibre morphology and dynamics. Phase images reveal details of the fibre structure more clearly than do ordinary microscopic photographs, especially in the PNP region. We suppose that early stages of demyelination that do not yet affect nerve fibre shape and thus are indistinguishable in amplitude microscopic images will be well expressed in phase images. Let us finally note that, by virtue of its excellent temporal resolution, laser interference microscopy can also be useful for detailed non-invasive studies of the nerve fibre structure and myelin compactness under excitation. In particular, one can study the effect of neurotransmitters.
We showed, presumably for the first time, that nerve fibres under rest conditions display regular changes in their optical properties. We also determined the characteristic frequencies of the OPD variations. We analysed OPD dynamics in four different regions: the myelinated internode and paranode regions and the boundary and the centre of the Ranvier node. The most pronounced rhythms were observed at the node boundary. The intensity of power spectra in the myelinated internode and paranode regions was very low, suggesting that the intensity of the processes that affect the local RI values is small. In the node boundary, we observed rhythms of approximately 0.1, 0.3, 0.8 and 1.0 Hz; these are similar to the RI changes in the neurons (Sosnovtseva et al. 2005; Brazhe et al. 2006). However, instead of frequencies near 2.0–4.0 Hz, as characteristic for neurons (Brazhe et al. 2006), we observed a rhythm with a frequency of approximately 1.5 Hz. This rhythm was highly pronounced in the node boundary and nearly absent in the internode and paranode regions. We suggest that one of the possible origins for this rhythm could be ‘microvilli breathing’, i.e. small movements of Schwann cell microvilli. This hypothesis must obviously be tested in further experiments.

In the previous work, we have shown that the 1.0 Hz rhythm is related to the $K^+$ flux through the plasma membrane of neurons and its modulation properties can be modified by valinomycin or under $K^+$ depolarization (Brazhe et al. 2006). As in nerve fibres under rest conditions $K$ channels are slightly activated and there is a small current of $K^+$ ions through the plasma membrane, we suppose that the activity of $K$ channels and low-amplitude changes in the membrane potential contribute to the 1.0 Hz rhythm in the OPD dynamics.

The low-frequency rhythms in the OPD dynamics (0.1 and 0.3 Hz) resemble similar rhythms (0.1 and 0.2–0.5 Hz) in neurons. According to the results of electrophysiological experiments (Szűcs et al. 1999) and our results obtained by means of fluorescent microscopy, we suppose that these rhythms in neurons originate from reorganizations in plasma membrane and ion channel activity. Taking into account the $K^+$ flux through the membrane of the nerve fibre and the maintenance of the specific distribution of ion channels in the axon plasma membrane, it seems natural to suggest that the 0.1 and 0.3 Hz rhythms are also related to reorganization in the plasma membrane and ion currents through the membrane, as they are in neurons.

We also studied the amplitude and FM of the relatively fast rhythms at 0.8, 1.0 and 1.5 Hz. We found that the modulation properties for all of these rhythms are common for the four studied regions. This indicates a similar origin for the corresponding rhythms in different fibre areas. However, for all the considered rhythms in the node region, the degree of AM was significantly higher than in the internode and paranode regions.

By comparison, the modulation spectra for the rhythms at 0.8, 1.0 and 1.5 Hz showed that they have common components, but that these components have different contributions. From this result we concluded that the rhythms at 0.8, 1.0 and 1.5 Hz are affected by the same processes, but the relative influence of each of these processes differs among the various rhythms. We should also note that the structure of the modulation spectra (both AM and FM) in the fibres was more complicated and had more components than the modulation spectra of similar rhythms in neurons (for comparison see Sosnovtseva et al. 2005). From these observations we conclude that the rhythms at 0.8, 1.0 and 1.5 Hz in nerve
fibres are likely to be influenced by more processes than in the case of neurons. However, additional experiments are necessary to understand the origin of the observed frequencies and their modulation properties.

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References


Non-invasive study of nerve fibres


