Femtosecond Dynamics of Intracellular Water Probed with Nonlinear Optical Kerr Effect Microspectroscopy

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ABSTRACT A nonlinear optical Kerr effect (OKE) microscope was developed and used to elucidate the ultra-fast diffusive motions of intracellular water molecules. In the OKE microscope, a pump-induced birefringence is sensed by a delayed probe pulse within a spatially confined volume that measures 0.5 μm in the lateral direction and 4.0 μm along the axial coordinate. This microscope allows the recording of time-resolved Kerr signals, which reflect the ultra-fast structural relaxation of the liquid, exclusively from intracellular aqueous domains. Because relaxation occurs on a picosecond time scale, only local diffusive motions are probed. The microscopic OKE signal is therefore insensitive to long-time-scale hindered translational motions enforced by intracellular mechanical barriers but probes the intrinsic orientational mobility of water molecules in cells instead. The Kerr response as determined from single intact mammalian cells under physiological conditions shows a structural relaxation time of 1.35 ps, which is 1.7 times slower than the Kerr decay observed in pure water. The data indicate that the mobility of water molecules in cellular domains is moderately restricted due to the high intracellular content of proteins and solutes.

INTRODUCTION

Given the complex composition of solutes and the presence of macromolecular structures in intracellular aqueous domains, the molecular mobility in cell water is expected to differ from the molecular dynamics in dilute aqueous solutions. Several studies have suggested that a certain degree of structural organization of intracellular water molecules, like the structured water layers that accompany proteins (Rupley and Careri, 1991; Denisov and Halle, 1995; Garcia and Hummer, 2000), is likely to persist over distances much longer than the molecular scale, thereby affecting the motional properties of the aqueous matrix (Clegg, 1984; Mastro and Keith, 1984; Cameron et al., 1997). The effective viscosity of cellular water ultimately controls the rate of diffusion-limited reactions and solute transport in the cell. Insight into the rheological characteristics of intracellular aqueous domains contributes therefore to the basic understanding of the functioning of cells (Porter, 1984; Luby-Phelps, 2000).

The translational diffusion of intracellular molecular compounds in the aqueous domains of the cell is influenced both by the effective viscosity of cell water and by mechanical barriers as imposed by the cytoskeletal network and organelle structures (Fushimi and Verkman, 1991). Determination of the effective viscosity by measuring the diffusion constant of either fluorescent probes or electron spin resonance labels is generally hampered by the unknown contributions of cytoskeletal obstructions and nonspecific binding of the probe molecules. As a result, a large diversity is found in the reported intracellular viscosity, ranging from 2–100 times more viscous environment in the cellular domains compared with neat water solutions (Keight et al., 1977; Lepock et al., 1983; Dix and Verkman, 1990). An alternative approach is to measure the diffusional properties of intracellular water directly. By mapping the hydrodynamics that occur on a picosecond time scale (10^{-12} s) only local diffusive motions are probed, excluding perturbations induced by mechanical barriers from the determination of water mobility. Restricted motions of water on a picosecond time scale relate therefore directly to the influence of solutes that induce a short-range structural organization of the water molecules. Experimental evidence for reduced picosecond diffusive motions of water has been given by high-frequency dielectric studies of protein solutions in which little or no rotational freedom was observed during the hydration lifetime of 100–300 ps (Wei et al., 1994). A threefold reduction in the ultra-fast mobility of intracellular water was found using quasi-elastic neutron scattering techniques (Trantham et al., 1984). However, technical limitations and difficulties in the interpretation of these experiments have precluded a conclusive measurement of the ultra-fast motional dynamics of water in cells under physiological conditions.

In contrast with other methods, optical microscopic techniques are generally less invasive and allow inspection of living cells at high spatial resolution (Pawley, 1993). With the advent of robust femtosecond laser systems, time-resolved nonlinear optical methods with sub-picosecond time resolution can now be readily applied in a microscope. Among the vast range of nonlinear optical methods, the optical Kerr effect (OKE) technique is particularly sensitive to the ultra-fast orientational dynamics of transparent liquids and has been extensively used to clarify the nuclear mobility of many organic and inorganic solvents (McMor-
row and Lotshaw, 1991; Back et al., 1992). In the OKE experiment, a polarized ultra-short pump laser pulse induces a transient birefringence in the sample. When a second laser beam that is 45° polarized with respect to the pump passes the focal region, part of its intensity will be depolarized due to the presence of the transient birefringence. The depolarized signal is indicative of the applied anisotropy in the sample. Relaxation of the induced birefringence due to structural changes in the solvent can be followed in time in the OKE microscope by time delaying the probe pulse with respect to the pump pulse. The corresponding time-resolved signal reflects the ultra-fast nuclear motions of the sample molecules. In water, the generated OKE signal is mainly dictated by the dynamics of the hydrogen-bonding network rather than by the response from single molecules (Palese et al., 1994; Santa et al., 1994; Foggi et al., 1997). Therefore, the OKE technique represents a sensitive probe for the short-time-scale dynamics of intracellular correlated water structures. Examination of the OKE in microscopic volumes has, however, never been undertaken.

In this contribution we report on ultra-fast structural relaxation measurements of cell water in applying the OKE technique under high-numerical-aperture (NA) conditions. The nonlinearity of the Kerr process guarantees a high three-dimensional resolution of the OKE microscope, permitting the registration of Kerr signals that originate exclusively from intracellular aqueous domains. We show that time-resolved OKE profiles can be obtained noninvasively from single mammalian cells. In comparing the Kerr response of cell water with dilute water solutions, an intrinsic measure of the motional properties of the cell medium is obtained.

### MATERIALS AND METHODS

#### Essentials of the microscope

The layout of the OKE microscope system is based on the configuration of an optically heterodyne-detected (OHD) OKE experiment (McMorrow et al., 1988) and is depicted in Fig. 1. Several modifications compared with regular OHD-OKE experiments were found to be essential in the microscope arrangement. First, because the best possible focusing characteristics are obtained by overfilling the back aperture of a high NA objective, the noncollinear beam geometry that is commonly employed in OHD-OKE experiments leads correspondingly to a reduction of the spatial resolution, most prominent in the axial direction. Therefore, a collinear excitation geometry is adopted to ensure optimal spatial confinement of the optical radiation near the focal volume. In conjunction with the collinear geometry, a spectral shift between pump and probe pulses is introduced that allows us to suppress the pumping radiation at the detector stage by means of a spectral filter. Finally, a coherent detection scheme is adopted to compensate for the polarization changes that are inherently induced by the high-numerical-aperture microscope objectives (Higdon et al., 1997).

#### Light source

Multicolor pulsed radiation is provided by a visible femtosecond optical parametric oscillator (OPO) system (Potma et al., 1998). The OPO is pumped by the second harmonic of the 800-nm pulsed radiation from a mode-locked Ti:sapphire laser. A cavity-dumping unit is inserted into one of the arms of the OPO providing a means to vary the repetition rate of the output pulses. Tunability of the repetition frequency forms an additional handle for controlling the balancing between high peak intensities and low average illumination doses, as is required for biological samples. The experiments reported here are conducted at a dumping frequency of 800 kHz. In the OKE experiment, the visible femtosecond output of the OPO is used in conjunction with the remainder of the 800-nm beam. To synchronize both beams, the infrared radiation is pulse picked at the same rate by a second Bragg cell driver to the dumpper unit of the OPO. A prism compressor is introduced in the optical paths of both beams to minimize the temporal width of the pulses at the sample position, which typically measures ~100 fs. Adjustment of the time delay between the visible and near-IR pulses is realized with a computer-controlled translation stage. Both beams pass a telescope that acts as a spatial filter and expands the beams to a diameter of 8 mm, as defined by the distance between the points along the radial coordinate where the intensity has fallen to e⁻¹ of its initial value.

#### Microscope configuration

In the OKE excitation scheme, the 800-nm radiation is employed as the pump whereas the wavelength of the probe was set to 620 nm. A set of polarizers (Karl-Lambrecht, Chicago, IL) fixes a relative polarization angle of 45° between the expanded pump and probe beams. An additional quarter wave-plate is placed in the optical path of the probe beam. The beams are collinearly combined on a dichroic mirror (Chroma, Brattleboro, VT) and directed to a 20×, 0.5-NA (Zeiss, Plan-Neofluar, Oberkochen, Germany) objective lens that confines the beams to a diffraction-limited focal spot in the sample. The sample is mounted on a piezo transducer (Piezosystems Jena, MiniTritor, Jena, Germany) for providing the three-dimensional scanning. Transmitted radiation is collimated by a parfocally positioned 40×, 0.75-NA objective (Zeiss, Plan-Neofluar). We have selected this particular objective pair because of their beneficial low depolarization of the laser light. In principle, higher-numerical-aperture-lenses with the same polarization characteristics may be used as well. The transmitted light is guided through an analyzer that blocks all components of the probing radiation that have retained their original polarization. The depolarized part of the probe consists of the pump-induced contribution and a fraction that originates from polarization changes introduced by the high-NA lenses. To isolate the pump-induced contribution, the remaining radiation is focused with a 10×, 0.25-NA (Melles Griot, Irvine, CA) lens into a single-mode fiber (Newport, FSV, 4.6-μm mode field diameter, Irvine, CA) that effectively averages over the spatial amplitude pattern of the beam. Theoretically, this coherent detection scheme is capable of compensating for the polarization variations raised by the microscope objectives (Higdon et al., 1997).

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**FIGURE 1** Schematic layout of the OKE microscope. A, analyzer; MO, microscope objective; LIA, lock-in-amplifier; Pol, polarizer; SF, spatial filter; SMF, single mode fiber.
In the present configuration we obtain an extinction coefficient of \(1.6 \times 10^{-2}\) for the probe beam by careful alignment of the analyzer and the quarter wave plate. For comparison, without the use of the coherent detector the extinction amounts only \(5.0 \times 10^{-3}\). A spectral filter (Omega, Brattleboro, VT) rejects any remaining pump radiation before the signal is detected with a photomultiplier.

The yield of the Kerr response depends critically on both the spatial and temporal overlap of the pump and probe pulse. To facilitate the alignment procedure, the much stronger nonresonant coherent anti-Stokes Raman scattering (CARS) signal of the coverglass is optimized. A photodetector is placed in between the collimating objective and the analyzer. A 500-nm interference filter (Omega) separates the CARS signal from the excitation beams. Because the CARS signal relies on the multicolor interaction of the excitation beams (Zumbusch et al., 1999), it constitutes a sensitive probe for optimizing the overlap of the pump and probe pulses.

**Optical heterodyning of the signal**

Introducing a mechanical chopper into the pump beam and amplifying the amplitude modulations on the signal using a lock-in amplifier increases the OKE detection sensitivity. Optical heterodyning of the signal is accomplished by rotating the input polarizer over \(-1^\circ\) to create a small depolarized probe component that interferes with the signal. The amplified signal is dominated by the cross product of the signal and the heterodyning field. The heterodyne contribution to the signal thus scales linearly with the heterodyning field (McMorrow et al., 1988). Because the latter is much stronger than the signal field, optical heterodyne detection (OHD) provides a means to boost up the detection sensitivity significantly.

**Intracellular OKE microspectroscopy**

African green monkey kidney (COS-1) cells were grown on glass coverslips in 90% Dulbecco’s modified Eagle’s medium and 10% fetal bovine serum at 37°C. Before the experiment, cells were rinsed and immersed in PBS (20 mM). The cells were subjected to microscopic investigations after an adjustment period of 30 min at room temperature. All experiments were performed at room temperature within a time span of 1 h after cell preparation. For intracellular OKE measurements, average power levels of the pump beam were always kept below 100 \(\mu W\). The power of the probe beam never exceeded 30 \(\mu W\). A single time-resolved OHD-OKE measurement takes \(\sim15\) s. At the power levels used, no signs of photodamage were observed by inspecting the cells after OKE microspectroscopic examination.

**Chemicals and materials**

Carbon disulfide was purchased from Aldrich (Milwaukee, WI). Small micron-sized glass beads were bought from SPI supplies (West Chester, PA). The glass beads were washed in a 1:9 water:ethanol solution, suspended on number 1 borosilicate coverslips, and dried in air.

**RESULTS AND DISCUSSION**

**Performance of the OKE microscope**

The signal measured in OKE microspectroscopy stems from a third-order nonlinear interaction \(\chi^3\) between the incident photons and the sample molecules. The Kerr signal comprises two interactions with the pump beam and one interaction with the probe beam and scales as:

\[
S_{\text{Kerr}} \propto I_{\text{pump}}^2 I_{\text{probe}}
\]  

In Fig. 2 \(a\), the OKE signal of carbon disulfide (CS\(_2\)), a strong Kerr scatterer, is shown as a function of the power of the incident laser beams. In accordance with Eq. 1, the registered nonlinear signal depends quadratically on the pump beam intensity whereas a linear dependence on the probe power is observed. Compared with CS\(_2\), the Kerr response of water is more than two orders of magnitude smaller. For a successful registration of water in the Kerr microscope, the use of an optical heterodyne detection scheme is a prerequisite. In contrast with Eq. 1, the heterodyne signal intensity scales linearly with both the pump and probe beam powers (McMorrow et al., 1988). Fig. 2 \(b\) shows the Kerr signal when the OHD technique is adopted. The linear dependence found confirms that the amplified signal is dominated by the heterodyne contribution.

Fig. 3 shows the OHD Kerr signal that is obtained when a 1-\(\mu\)m glass bead is scanned laterally through focus. Because air is the surrounding medium, the Kerr signal is generated only when the focal spot overlaps with the glass material. The nonresonant CARS signal of the same glass bead is also indicated in the figure. The close resemblance of the profiles reveals that the imaging properties of both techniques are comparable. Taking the size of the glass bead into account, a lateral resolution of 0.5 \(\mu\)m is estimated, which agrees nicely with the size of the illumination volume.

**FIGURE 2** Logarithmic plot of the Kerr signal intensity as a function of incident beam powers. (a) Homodyne-detected optical Kerr effect signal; (b) Optically heterodyne-detected Kerr signal; ——, variations in the pump intensity; ■, dependence of the signal on the probe beam intensity; ——, least-square fits to the experimental data.
as calculated from focal intensity distribution corresponding to the NA of the objective lens. Here we have assumed that the accumulation of signal proceeds in an incoherent manner. Although the Kerr signal results from a purely coherent process, it is not expected that the incoherent approximation leads to rigorously flawed predictions concerning the resolution in the lateral direction (Potma et al., 2000). An estimate of the axial resolution is obtained by scanning a thick layer of carbon disulfide axially through focus (Fig. 4). Based on a model that recognizes the coherent signal accumulation (Potma et al., 2000), a spatial resolution of 4.0 μm is deduced from the axial edge scan. Cultured COS-1 cells are used in the experiments. To ensure that the signal originates solely from the cell’s interior, we have selected those cells exhibiting a height in the 7–9-μm range. The much thinner cellular extensions and lamellipodial regions are also excluded from the measurements. Because the probed volume extends over several microns, the measured OKE signal represents an effective measure of the spatially averaged response of the aqueous intracellular environment to a transient birefringence.

Note that the OKE technique is only sensitive to the pump-induced birefringence. Hence, inherent birefringence of the cell does not interfere with the measurement. Light scattering by particles on the order of the optical wavelength is circumvented by positioning the confined focal spot in those regions of the cell that show minimal scattering activity. Moreover, randomly scattered contributions are significantly reduced by the coherent detector that acts like a confocal filter. We observe no significant particle-induced background signal over a sampling range of three orders of magnitude.

**Ultrafast motions of intracellular water**

In Kerr microspectroscopy the ultra-fast dynamical properties of the sample molecules are monitored within a microscopic probing volume. The high temporal resolution of the femtosecond excitation pulses is exploited to time resolve the structural dynamics of the liquid. Instead of utilizing the magnitude of the signal as a contrast parameter, the OKE microscope maps a structural relaxation time as function of focal position. The inset of Fig. 5 shows the OHD-OKE response of pure water that is obtained when the probe pulse is delayed with respect to the pump. The initial part of the signal is primarily dominated by the electronic response of the sample molecules and mimics the cross-correlation of the pulses. A much weaker tail is observed for longer delays, which is governed by nuclear motions and reflects the structural relaxation processes of the liquid. For delays longer than 500 fs, the data are adequately described with a single-exponential decay time of 0.79 ps, which agrees well with previously reported values (Santa et al., 1994). A similar OKE profile is observed when the focal spot is positioned within the interior of the cell. The Kerr response presented in Fig. 5 is obtained by averaging over 100 individual measurements taken from five different cells. Unlike the OKE response of pure water, the structural relaxation of the intracellular medium is characterized by a

**FIGURE 3** OHD Kerr signal measured (●) when scanning a 1-μm glass bead laterally through the focal volume of a 20×, 0.5 NA dry objective lens. ——, CARS signal intensity as observed from the same bead.

**FIGURE 4** Axial edge response of a glass/CS₂ interface measured in the OHD-OKE microscope.

**FIGURE 5** Intracellular optically heterodyne-detected OKE signal as a function of the delay between pump and probe. Single traces are recorded from different positions in the cell cytoplasm. The profile presented is the average of 100 individual measurements taken from five different cells. A least-square fitting procedure gives an exponential relaxation time \( \tau = 1.35 \) ps. The dotted line refers to the signal obtained from the supporting glass coverslip, 10 μm below the cells, and is indicative of the temporal resolution of the OKE experiment. The dashed line represents the fit to the response of pure water, also shown in the inset, with a relaxation constant of \( \tau = 0.79 \) ps.
decay time of 1.35 ps. Within the accuracy of the experiment, the time-resolved signals of separate cells are comparable.

The Kerr signal results from a nonresonant nonlinear process and is as such not exclusively selective for water. Any molecular species with an appreciable Kerr coefficient might contribute to the signal. However, because water constitutes the most abundant intracellular molecular compound (~70% by volume weight), the signal should predominant originate from the cell water. A strong indication for this assumption is provided by the observation that the absolute OKE signal yield measured in intracellular domains is similar to the yield monitored in extracellular regions. A significant contribution of other molecular Kerr scatterers implies a corresponding rise of the OKE electronic response (Santa et al., 1994). In addition, in both the extracellular space and in the cellular interior, the relative nuclear contribution to the signal is identical. Given these observations, we conclude that the majority of signal measured in the intracellular regions is dominated by the response of water.

For comparison, we have performed similar experiments on protein solutions of bovine serum albumin (BSA). Again, the amplitudes of the electronic and nuclear OKE response indicate that water is the predominant Kerr scatterer. At a concentration of 100 mg of protein/ml, which is representative for the protein content in cells (Lodish et al., 1999) a relaxation time of 0.90 ps was found. Fig. 6 summarizes the results of the diffusion experiments for the different conditions examined. The slowdown of the relaxation dynamics in the protein solution relative to pure water indicates that the presence of the protein has an effect on the orientational mobility of the water molecules. The even longer decay times that are observed from intracellular regions suggest that the orientational dynamics of water structures in the cell be affected by the presence of multiple solutes. The slow relaxation rate in the aqueous cellular domains insinuates that the hydrogen-bonding network in the intracellular environment is more rigid than in bulk water (Santa et al., 1994).

From the OKE measurements we deduce that the ultrafast dynamics of the cell water is slowed down by a factor of 1.7 relative to pure water. Compared with the severely hindered rotational motions of water structures in the hydration shell of proteins (Garcia and Hummer, 2000; Wei et al., 1994), the spatially averaged dynamics of cell water are characterized by only a moderate reduction of the motional molecular freedom. This observation hints at an interpretation in which the structural organization that is reminiscent of strongly restricted orientational dynamics does not persist throughout the bulk of the cell water. If we assume that the effective volume occupied by a water molecule is given by \( V_{\text{eff}} \), the reduced dynamics of cell water may be extrapolated to a macroscopic description of the liquid, using the Stokes-Einstein relation (Berne and Pecora, 1976):

\[
\tau_{\text{rot}} = V_{\text{eff}} \eta / KT,
\]

where \( \eta \) is the viscosity of the medium, \( K \) represents the Boltzmann constant, and \( T \) is the temperature. Following this relation, the aqueous parts of the intracellular environment are characterized by an effective viscosity that is 1.7 times as high as the viscosity of pure water. Although Eq. 2 refers to a greatly simplified hydrodynamic approach, the predicted effective viscosity is highly instructive and underlines the modest stiffening of the hydrogen-bonding network of the intracellular medium relative to pure water at room temperature.

**CONCLUSION**

A multicolor nonlinear optical microscope has been constructed that allows the measurement of ultra-fast orientational motions of sample molecules that reside within a microscopic probing volume. Employing OHD, we were able to resolve the weak Kerr response of water in an optical microscope. The OKE microspectroscopic technique constitutes a unique approach to study the structural dynamics of the aqueous domains of intact mammalian cells. In the OKE experiment, only local diffusive motions are probed that relate directly to molecular environment of the water molecules. In mapping the short-time-scale dynamics, the influence of mechanical obstructions that usually interfere with long-time molecular diffusion measurements is circumvented. The slowdown of the orientational motions of the water molecules with a factor of 1.7 relative to dilute aqueous solutions indicates that the complicated biochemical composition of the cell liquid exerts influence on the mobility of water assemblies. Under the assumption that the reduced diffusive motions of intracellular water may be extrapolated to an effective macroscopic viscosity of the cell medium, the viscosity of the cell liquid is only 1.7 times as high as that of pure water at room temperature.
this relatively low viscosity of the aqueous cytomatrix in mammalian cells, it is unlikely that the structural dynamics of cell water as probed by the optical Kerr effect will have a dramatic effect on the rate of intracellular diffusion-limited enzyme reactions and solute transport. In this light, heavily restricted diffusive motions of macromolecules is induced by mechanical barriers rather than by the intrinsic physical properties of the liquid cell medium. In selecting even higher NA objectives with appropriate polarization characteristics, OKE microspectroscopic experiments may eventually lead to the measurement of water mobility in specific subcellular domains and organelles.

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