Reconstruction of the Two-dimensional Birefringence Map of the Skeletal Muscle Sarcomere in Relaxed and Rigor States by Interference Microscopy Data


Abstract—A new method—automated quantitative interferometry—was proposed for studying the birefringence in striated muscle fibers. On the basis of a Linnik microscope, a setup was designed; this setup gives a phase image, which is a two-dimensional birefringence map. For the first time, two- and three-dimensional maps were obtained for the total birefringence and the birefringence in single sarcomeres in the central part of fibers over a large pixel array. The total birefringence in fibers of the resting length in the rigor state proved to be lower than that in the relaxed state. A calculation of the birefringence by data of the interference patterns of single sarcomeres also showed that the normalized birefringence in A-disks in the relaxed state exceeds that in the rigor state. The results obtained can be explained by a decrease in the anisotropy of the thick filament because of the departure of myosin heads from the molecular core in the fiber rigorization.

Key words: interference, birefringence, muscle, sarcomere, rigor state, relaxed state

INTRODUCTION

Muscle fibers are an anisotropic matter, a highly ordered birefringent system, in which the birefringence characterizes the intrafilamentary periodicity of contractile filaments and also the orientation of myosin cross bridges. Conformational changes in these cross bridges during the ATP cycle lead to generation of force. Therefore, determination of detailed dynamic patterns of the structure of the contractile apparatus on models that most closely simulate physiological conditions is extremely useful for completely understanding the molecular mechanism of force generation. Adequate techniques for solving such problems are noninvasive optical methods of high temporal and spatial resolution. Such is the automated interferometry method we developed.

The purpose of this work was to obtain the interference pattern of the central segment of a single skeletal muscle fiber, to calculate the birefringence from this pattern, and to reconstruct the two-dimensional map of the birefringence in the sarcomere in two stationary phases of the interaction of myosin heads with actin. Unlike previous studies of the birefringence of entire muscle fibers [1–4], this investigation involved local measurements of the birefringence in the central segments of single fibers and single sarcomeres in their structure.

EXPERIMENTAL

The birefringence was analyzed in single demembranized fibers from the rabbit psoas muscle in two stationary phases of the cross bridge cycle, namely, in the rigor and the relaxed states. Muscle preparations were made according to a published procedure [5].
The relaxing solution contained 85 mM KCl, 15 mM Tris-HCl buffer (pH 7.2), 4 mM ATP disodium salt, 2 mM β-mercaptoethanol, 5 mM MgCl₂, 2.5 mM EGTA, and 1 mM NaN₃. The rigor solution contained 95 mM KCl, 15 mM Tris-HCl buffer (pH 7.2), 2.5 mM EGTA, 2 mM β-mercaptoethanol, and 1.0 mM NaN₃. The ionic strength of the solutions was 0.15 M.

A setup was based on a dual-beam laser interferometer [6]. An LG-79 He–Ne laser with a radiation wavelength of 0.63 μm and a power of 5 mW was used as a spatially incoherent light source.

An MII-4 microinterferometer (Fig. 1) consisted of two identical optical arms, viz., a reference arm and an object arm. These arms created two wave systems capable of interfering with each other, since they were produced by splitting the light beam emitted from a point of the broad light source in the focal plane of microlenses.

A single demembranized fiber 40–60 μm in diameter was glued with a BF-6 Butvar-phenolic adhesive to the surface of the object arm mirror, which was the bottom of a flow-through microcell 6 μl in volume. The microcell stood on the object stage. The flange focal length of a 32× lens was about 3 mm. The incident and reflected beams produced a 20 × 20-μm interference pattern of the fiber segment, which was transmitted from a video camera to a computer image analysis system. A phase image was reconstituted using the interference pattern data by the four-phase-step algorithm [7]. The fiber was sequentially exposed to beams polarized parallel and perpendicular to the fiber axis. The birefringence measurement technique was based on phase difference measurements by digital subtraction of two sequential images at different polarizer positions. Experimental data obtained were mathematically processed using a special program package.

RESULTS AND DISCUSSION

Figure 2 shows the phase images of the central part of the fiber, which was obtained on the setup we designed. The phase shifts ΔΦ were determined in two states of the contractile apparatus, specifically, the rigor (Fig. 2a) and the relaxation (Fig. 2b). The measurements were made in the same fiber segment by the rigor–relaxation–rigor scheme. One can readily see regularly alternating sarcomeres. The light and dark regions represent anisotropic myosin A-disks and I-disks, respectively. The digitized scales on the right of Fig. 2 give quantitative characteristics of the phase tapers. Figure 3 presents the three-dimensional maps of the birefringence in the central part of the fiber in the relaxed and rigor states (Figs. 3a and 3b, respectively) as reconstructed from the interference patterns. The birefringence in the relaxed sample is noticeably lower than that in the rigor sample.

Figure 4 demonstrates the scanning patterns of the two-dimensional birefringence map in the rigor and the relaxed states (Figs. 4a and 4b, respectively), which enable one to evaluate the contributions of the A- and I-zones of the sarcomere to the total birefringence. The obtained scanning patterns allow one to calculate the ratios $BF = BF_A / BF_I$ between the...
Fig. 2. Phase images of muscle fiber in the (a) rigor and (b) relaxed states.
birefringences in various parts of the sarcomere in the studied physiological states. In the rigor and the relaxed states, these ratios are $0.0912 \pm 0.0171$ and $0.2790 \pm 0.0312$, respectively ($n = 7$).

In all the tested fibers at a sarcomere length of $L_s = 2.5 \, \mu m$ (the resting length), the total birefringence in the sarcomere in the rigor state proved to be lower than that in the relaxed state and was $2.34 \cdot 10^{-5} \pm 0.78 \cdot 10^{-5}$ as against $4.24 \cdot 10^{-5} \pm 1.01 \cdot 10^{-5}$, respectively (data averaged over all the sets of experiments, $n = 8$).

It follows from the above that the main contribution to the total birefringence in the sarcomere is made by the A-disk as such and also myosin heads oriented in a certain manner. This is supposedly due to a decrease in the anisotropy of the thick filament because of the departure of myosin heads from the molecular core in the fiber rigorization.

The obtained birefringences are consistent with the previously published values [1–4] in the direction of their changes during the rigor–relaxation transitions but differ from them in absolute value. This difference is explained by the difference in the methods of mathematical determination and normalization of the birefringence.

Unlike polarization methods [1–4], the method we used allows separate recording of two phase images $F(x, y)$ of the fiber, which are obtained when the radiation polarization plane is parallel ($\parallel$) and perpendicular ($\perp$) to the fiber axis:
Fig. 4. Scanning patterns of the two-dimensional birefringence map of fiber in the (a) rigor and (b) relaxed states.

\[ F_\parallel(x,y) = \int [n_\parallel(x,y,z) - n_{\text{m}}]dz, \]

\[ F_\perp(x,y) = \int [n_\perp(x,y,z) - n_{\text{m}}]dz. \]

Physically, these phase images at each point \((x,y)\) are equal to the transmitted light path lengths. Here, \(n\) and \(n_{\text{m}}\) are the refractive indices of the fiber and the environment (immersion liquid), respectively; the \(x\) axis coincides with the fiber axis; the \(y\) axis is perpendicular to the fiber axis; and the \(z\) axis coincides with the beam direction.

Polarization methods measure, one way or another, only the optical path difference; therefore, the fiber birefringence \(BF(x,y)\) is usually determined as

\[ BF(x,y) = \frac{F_\parallel(x,y) - F_\perp(x,y)}{nD}, \]

where the normalization is performed to the optical thickness \(nD\) of the fiber. Measurements of \(nD\) (the refractive index \(n\) of the fiber and its geometrical thickness \(D\)) at each point \((x,y)\) are impossible. Therefore, as the normalization factor, the average fiber diameter is chosen. With such normalization, the birefringence measurement result depends on the choice of the measurement point, and the measurements are possible only within a narrow region near the fiber axis.

The interference method of measuring the birefringence, which is proposed in this work, allows separate recording of two phase images \(F_\parallel(x,y)\) and \(F_\perp(x,y)\). From these images, their difference and sum are obtained, which are then used for calculating the birefringence by the expression

\[ BF(x,y) = 2 \frac{F_\parallel(x,y) - F_\perp(x,y)}{F_\parallel(x,y) + F_\perp(x,y)}. \]

In such determination of the birefringence, the normalization at each point \((x,y)\) is made to its own value of the optical path length (the half-sum at different polarization), which renders the birefringence measurement independent of the choice of the measurement point.

Another consequence of determining the birefringence in such a way is as follows. The fiber consists of a large number of densely packed myofibrils (up to a hundred or more). The optical path length is an additive quantity; therefore, the total phase taper in light transmission through the fiber is the sum of the phase tapers across each of the myofibrils. Under the assumption that these tapers are identical for all the myofibrils, the fiber birefringence determined thereby is actually the birefringence in a single myofibril.

Thus, the birefringence calculated by the above expression is independent of the number of myofibrils in the fiber and is the average birefringence in a single myofibril. This explains the difference in absolute value between the birefringences found in this work and other studies [1–4]. The total birefringence in the entire fiber can be estimated by multiplying the obtained birefringence in a single myofibril by the average number of myofibrils in the fiber. Since the fiber contains about a hundred of myofibrils, our results agree in the order of magnitude with the data obtained earlier [1–4].

Note also the following. If myofibrils in the fiber are packed regularly (i.e., are in register with one another), then the depth of modulation of the wave front between the A- and I-disks increases proportionally to the number of myofibrils in the fiber. If the packing is irregular, there can be no wave front modulation between the disks altogether. This is because of the fact that adjacent light rays pass through the equal numbers of the A- and I-disks, and their total phase tapers are the same. Thus, the optical path difference is contributed to only by regularly packed myofibrils.

Computer interpretation of interference patterns also allows one to monitor and record the dynamic patterns of structural transformations of the contractile apparatus during force generation in muscle in real time over a large pixel array.
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REFERENCES