Synchrotron radiation X-ray diffraction studies on muscle: past, present, and future

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Abstract

X-ray diffraction is a technique to study the structure of materials at spatial resolutions up to an atomic scale. In the field of life science, the X-ray diffraction technique is especially suited to study materials having periodical structures, such as protein crystals, nucleic acids, and muscle. Among others, muscle is a dynamic structure and the molecular events occurring during muscle contraction have been the main interest among muscle researchers. In early days, the laboratory X-ray generators were unable to deliver X-ray flux strong enough to resolve the dynamic molecular events in muscle. This situation has dramatically been changed by the advent of intense synchrotron radiation X-rays and advanced detectors, and today X-ray diffraction patterns can be recorded from muscle at sub-millisecond time resolutions. In this review, we shed light mainly on the technical aspects of the history and the current status of the X-ray diffraction studies on muscle and discuss what will be made possible for muscle studies by the advance of new techniques.

Keywords Synchrotron radiation · X-ray diffraction · Muscle · Time-resolved measurements · X-ray microbeam · Coherent diffractive imaging

Introduction

X-rays are electromagnetic waves with wavelengths of typically ~ 0.1 nm, i.e., lengths comparable to the interatomic distances of compounds. As the spatial resolution of a microscope is comparable to the wavelengths of the electromagnetic waves or the particle beams used, X-rays are expected to be used for creating microscopes capable of magnifying objects to visualize individual atoms or molecules. However, X-ray lenses have never been fabricated with accuracy to ensure such a resolution. Instead, the X-rays scattered by the object are directly recorded and analyzed to obtain the structure of the object at molecular or atomic resolutions and its time-dependent changes. This is called the X-ray diffraction technique.

The X-ray diffraction technique has played, plays, and will keep playing an important role in life science studies. Not to

mention protein crystallography, the determination of the double helix structure of DNA owes to X-ray diffraction recording (Watson and Crick 1953). The X-ray diffraction technique also greatly contributed to the understanding of the structure of muscle and the molecular events taking place during contraction.

A century has passed since the establishment of the theory of diffraction from crystals by the Braggs (Bragg and Bragg 1913). In the early 1950s, the theories of diffraction from cylindrically symmetrical objects, including helices, were extensively studied (e.g., Cochran et al. 1952). In terms of structure, a skeletal muscle cell (muscle fiber) can be viewed as a crystal and an assembly of helices at the same time, and as will be explained in more detail, both lines of theories helped to understand the fine structure of the muscle fiber.

Although the basic theories of X-ray diffraction applicable to muscle were established more than half a century ago, the techniques to generate X-rays and to record diffraction continue to develop. The laboratory X-ray generators based on X-ray tubes have largely been replaced by synchrotron radiation facilities. In the early days, X-ray diffraction patterns were photographed by using X-ray film, but today the most widely used detectors are of semiconductor type, capable of both static and dynamic measurements.

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Diffraction data are now digitized and processed by computers. In some time-resolved measurements, thousands of megapixel frames are generated in a second, and the processing of such big data is inconceivable without the use of high-performance computers. Present-day desktop personal computers (high-end models) have sufficient performance to process diffraction data in most cases.

The interpretation of diffraction patterns from muscle required a proper understanding of diffraction theories, and this is true even today. The diffraction theories are often difficult to understand, and this has prevented the X-ray diffraction technique from being widely disseminated among the community of muscle scientists. However, this situation may change in the near future. In the case of light microscopes, researchers can obtain a magnified image of objects without deep knowledge about the theory of light diffraction. Likewise, one day it may be possible to obtain magnified images of objects from X-ray diffraction patterns, when high-performance computers are used as X-ray lenses. The success of this scheme will depend on progress with the software (algorithms) as well as the computational hardware.

As overviewed above, the environment for muscle research using the X-ray diffraction is evolving day by day. This review will deal with the evolution of the X-ray diffraction technique for muscle research in the context of synchrotron radiation and describe the prospect for future development that will be made possible by emerging technologies.

Diffraction theories in brief

This review will describe the minimal requirements for understanding diffraction theories. For more comprehensive description, readers are encouraged to refer to excellent textbooks, such as Cantor and Shimmel (1980) and Squire (1981). A brief introduction has also been published by the author (Iwamoto 2018).

Electromagnetic waves (visible light or X-ray) are scattered by an object, and the distribution of the scattered waves is described as a Fourier transform of the object. X-rays are mostly scattered by the electrons in an object, so that the scatter is the Fourier transform of the electron density distribution of the object. This Fourier transform is called the structure factor of the object, and it is expressed in terms of a complex number having both amplitude and phase. The space where the object exists is called the real space, whereas the space where the structure factor exists is called the reciprocal space, where distances are inversely related to those in the real space.

When a Fourier transformation is applied once again to the structure factor (inverse Fourier transformation), it goes back to the real space and the image of the object is restored, often at a different magnification. The lens in a light or electron microscope performs this inverse Fourier transformation. In order to restore the image, both amplitude and phase

information must be preserved. In the case of X-ray diffraction, however, only the amplitudes are recorded by X-ray detectors, and the phase information is lost. As a result, the image of the object cannot be restored by applying an inverse Fourier transformation to the recorded diffraction pattern. This is called the phase problem, and it is the largest obstacle for wide application of X-ray diffraction studies. Because of this problem, it is a common practice among muscle scientists to directly interpret X-ray diffraction patterns, and this requires a proper understanding of diffraction theories.

Bragg diffraction This refers to the diffraction by crystals, and the theory was established by the Braggs (Bragg and Bragg 1913) as mentioned earlier. A 3-dimensional crystal can be regarded as a stack of parallel lattice planes on which constituent atoms or molecules are arranged. The X-rays scattered by all the different planes of the same stack positively interfere with each other to generate a strong reflection when the differences in the X-ray path lengths are multiples of integers of the wavelength (Bragg condition; Fig. 1). There are various ways of defining lattice planes, and they are referenced by the Miller indices h , k , and l , corresponding to the 3 dimensions of the crystal.

In skeletal muscle, the thick (myosin-containing) filaments and the thin (actin-containing) filaments form a hexagonal lattice in the sarcomere, and this is in effect a 2-dimensional crystal. Because of this, Bragg reflections arise from vertebrate skeletal muscle. Because it is a 2-dimensional crystal, the reflections only arise on the equator (in a direction perpendicular to the filament axis), and they are commonly referred to as the equatorial reflections. These equatorial reflections are the strongest reflections that arise from muscle. Only the first two of the Miller indices are used to index the planes from which the reflections arise, such as 1,0 and 1,1 (Fig. 2). Asynchronous insect flight muscle can be regarded as a 3-dimensional crystal. In this case, Bragg reflections also arise off the equator, and they require the 3 Miller indices to index their corresponding lattice planes.

In a monocrystal, when the Bragg condition is met for a particular lattice plane (e.g., 1,0), the Bragg condition is not met for other lattice planes (e.g., 1,1). Because of this, a monocrystal must be rotated to record as many reflections as possible. However, all possible reflections are simultaneously recorded in a single diffraction pattern from muscle. This is because a muscle or a muscle fiber contains a large number of myofibrils with random rotational orientations. Thus, a diffraction pattern from a muscle or a muscle fiber is a rotationally averaged pattern. If one records a diffraction pattern from a single myofibril and the 1,0 reflection is observed, the 1,1 reflection is not observed (Iwamoto et al. 2005).

Diffraction from a helix The contractile machinery of muscle contains thick and thin filaments, as stated above, and both

Fig. 1 Bragg conditions under which reflections are generated from the lattice planes of a crystal. The lengths of X-ray paths for path1, path 2, path 3 differ by the lengths indicated by the bold lines. If these are the multiples of integers of the wavelength, positive interference occurs and a strong reflection is generated. Modified from Iwamoto 2019

have a helical structure similar to a strand of DNA. It is proven that the Fourier transform of a continuous helix is expressed as a series of Bessel functions of the first kind (Cochran et al. 1952) (see Fig. 3). The whole diffraction pattern is X-shaped, and each of the layer-like reflections is called a layer-line reflection. The intensity on the meridian exists only for the 0th order Bessel function (origin, or the center of the pattern). The interval of the two neighboring layer-line reflections corresponds to the inverse of the helical pitch.

In most cases, helical structures in the cell take the form of a discontinuous helix, consisting of discrete monomers. This also applies to the thick (myosin-containing) and thin (actin-containing) filaments in muscle. A discontinuous helix can be regarded as a product of two functions, a function expressing a continuous helix and a periodicity function expressing the monomer repeat, p (Fig. 3). The Fourier transform of the discontinuous helix is, due to the convolution theorem (see Iwamoto 2018), the convolution of the Fourier transforms of the two functions. It is a series of X-shaped of Bessel functions arranged along the meridian (Fig. 3). A diffraction intensity

Fig. 2 The hexagonal lattice of myofilaments in the cross section of vertebrate skeletal muscle. The green dots represent the myosin-containing thick filaments, and the red dots represent the actin-containing thin filaments. Two representative lattice planes (1,0 and 1,1) are indicated

appears on the meridian with an interval of $1/p$. Therefore, whenever an intensity appears on meridian (except for the origin), it reports the repeat of monomers that constitute a filament.

Before the days of synchrotron

The earliest diffraction recordings from muscle were done in the 1940s (Astbury 1947), long before the emergence of the sliding filament theory of muscle contraction (Huxley and Hanson 1954; Huxley and Niedergerke 1954). X-ray tubes had been used since the early twentieth century, and it is around this time when the powerful rotating anode-type X-ray generator was invented. By the time the diffraction theory of muscle was well established, owing to the contribution by H.E. Huxley (e.g., Huxley and Brown 1967), the rotating anode generators were widely used among muscle X-ray scientists. Although the rotating anode generators are the strongest laboratory X-ray sources even today, it took as long as 50–70 h to record a two-dimensional diffraction pattern from a muscle, demonstrating weak layer-line reflections (Huxley and Brown 1967; Namba et al. 1980). X-ray tubes use the characteristic X-ray beams from the element which the anode is made of. Because of this the choice, the irradiating X-ray wavelength was limited. For muscle research, X-rays from a copper anode ($\text{Cu-K}\alpha$, $\lambda = 0.154$ nm) was preferred. To record diffraction patterns, high-sensitivity X-ray films were used. X-ray films were also used in the early days of synchrotron experiments in the 1980s.

Second-generation synchrotron radiation facilities

A synchrotron is a circular accelerator in which accelerated charged particles (typically electrons and positrons) orbited along a closed path (Fig. 4). To make the orbit circular, bending magnets are placed along the particle beam path to bend the orbit using magnetic fields. Whenever the orbit is bent, strong radiation of electromagnetic waves is emitted in a tangential direction. This is synchrotron radiation. Synchrotron

Fig. 3 X-ray diffraction from a discontinuous helix. A discontinuous helix (top right), a commonly observed form of biopolymers, can be regarded as the product of continuous helix (top left) and a periodicity function representing the repeat of monomers (top middle). The bottom row represents the structure factors (Fourier transforms) of the objects in the top row. The structure factor of a continuous helix is a mathematically

pure series of Bessel functions of the first kind (bottom left). The structure factor of the periodicity function is a row of dots with an interval that is the inverse of the monomer repeat (bottom middle). The structure factor of a discontinuous helix is the convolution of the left two and is a series of a set of Bessel functions stacked in the vertical (along the fiber axis or the meridian) direction. The operator U denotes convolution

radiation has continuous spectra with wavelengths ranging from the infrared to X-rays. In the wavelengths of X-rays, synchrotrons are the brightest light sources that humans have ever created.

Initially, researchers who wanted to use synchrotron radiation needed to visit synchrotron facilities built for particle physics experiments. These are called first-generation synchrotron radiation facilities.

The second-generation synchrotron radiation facilities were designed and built to utilize synchrotron radiation, and they came into operation after 1980. It is this generation of facilities that attracted the attention of muscle researchers. To

stabilize the radiation intensity, the current within the ring was kept as constant as possible. This type of synchrotron is called a storage ring. Thus, second-generation synchrotron radiation facilities use the bending magnets as primary sources of synchrotron radiation. As will be explained later in more detail, the third-generation facilities featured with another type of light source, called an insertion device, but in reality the boundary between these generations is not very clear. For example, the storage ring DORIS at DESY in Hamburg was initially built as an electron-positron collider in 1974, but after 1980 it was used as a second-generation facility. Later, it was modified to accommodate a number of insertion devices.

The features of synchrotron radiation X-rays include (1) an overwhelmingly brighter source of the X-rays than the laboratory generators, (2) it is highly oriented, and (3) its wavelengths are tunable.

For muscle research, what new features could these developments offer? One of the largest benefits was that the exposure time needed was dramatically reduced so that it came within the time range of physiological events of contraction. Now, one could conduct a fast dynamic (time-resolved) diffraction recordings. H.E. Huxley and colleagues published a series of the first time-resolved diffraction recordings from muscle by using DORIS (Huxley et al. 1980, 1981, 1982, 1983; Kress et al. 1986), which had a major influence on the muscle community. In their very first paper published in 1980, they state that it took only a few minutes to record on film a

Fig. 4 Schematic drawing of the basic structure of a synchrotron (storage ring). There are many other magnets and components along the ring, but they are omitted for simplicity

good diffraction pattern that would have taken 24 h using a high-power rotating anode generator. For time-resolved measurements, they initially used a combination of an image intensifier and a TV camera, but in later studies they used a position-sensitive proportional counter (PSPC, see Fig. 5). This device counts the ionization events caused by the radiation and has a configuration in which multiple wires are connected by a delay line so that the position of the ionization event is determined by detecting the time difference of output pulses of the two amplifiers connected to the two ends of the delay line (Kikuta 1992).

Although it is 1-dimensional, the output from a PSPC is easily digitized and is suited for fast time-resolved measurements because it was not limited by the time frame of a video camera. Thus, the PSPC became the detector of choice in second-generation facilities. The spatial resolution is limited by the minimum interval between the wires. Two-dimensional wire detectors were fabricated by wiring in two layers (including those using the advanced microstrip and microgap technologies; Lewis 1997), but they were replaced by semiconductor-type area detectors before being widely used.

Another benefit of using synchrotron radiation is that the distance between the sample and the detector (camera length) can be made very long owing to the very bright and well-oriented nature of the radiation. By extending the camera length, closely spaced reflections can be separated, but the X-ray dose on a unit area of the detector is inversely proportional to the square of the camera length, so that longer exposure times were required. In contemporary synchrotron radiation facilities, camera lengths of 5–10 m or more are common (e.g., ID02 beamline of ESRF, ID18 beamline of APS, and BL40B2 beamline of SPring-8) and in one special case, it can be made as long as 200 m (BL20XU beamline of SPring-8).

By using a long camera length, one can determine the spacing of the monomers in protein filaments very accurately.

Fig. 5 Principle of operation of a position-sensitive proportional counter (PSPC). The pulse generated by the ionized gas molecules is conducted in both directions through the delay line to reach the two amplifiers at different timings (modified from Kikuta 1992)

The non-invasive nature of X-ray diffraction allows the spacing to be determined in situ, free of artifacts from chemical fixation or distortion from thin-sectioning. Using this merit, Huxley et al. (1994) and Wakabayashi et al. (1994) determined the elastic extension of myofilaments during contraction, which is much less than 1% of the spacing at rest. Before this achievement, myofilaments were regarded as rigid structures, and practically all of the muscle fiber compliance was believed to come from myosin cross bridges. The proof of filament compliance fundamentally changed how the results of mechanics experiments on muscle are interpreted (e.g., Linari et al. 1998).

To record static diffraction patterns, photographic films were still used in second-generation facilities, but they were later replaced by a new type of detector, called imaging plate (IP). This was developed by the Fuji Film Company, Japan, and was much more sensitive than photographic films and had a high dynamic range ($\sim 10^5$). An IP is a flexible plastic plate coated with fine phosphor crystals (BaFBr:Eu^{2+}) (Amemiya et al. 1987). When irradiated with X-rays, the phosphor forms color centers, which are later stimulated by He-Ne laser beams to emit photostimulated luminescence with intensities proportional to the number of absorbed X-ray photons (Amemiya et al. 1987). The intensity of the luminescence is digitized by the IP reader. IPs can be reused repeatedly after residual color centers are erased by exposure to visible light. They were first used for muscle research by Amemiya et al. (1987) in Photon Factory, a second-generation synchrotron radiation facility in Japan.

Unlike some other detectors, IPs do not distort the recorded images, and the pixel size can be made as small as 50 μm , and they have a high dynamic range. For these reasons, IPs are still used today for some applications. However, IPs are gradually being replaced by semiconductor-type area detectors because of the slow readout and the need to erase after each exposure.

Third-generation synchrotron radiation facilities

The third-generation synchrotron radiation facilities have a new type of light source, called an insertion device. This is a double array of strong magnets with alternating polarities that apply alternating magnetic fields to the electron orbit with a short periodicity (Fig. 6). Each time the electrons go through the alternating magnetic field, synchrotron radiation is generated, and as a result, very intense, well-oriented radiation beams are generated. In general, the radiation from an insertion device is ~ 10 times brighter than that generated by a bending magnet.

There are two types of insertion devices: undulators and wigglers. The difference is that the extent of bending of the electron orbit is smaller for an undulator than for a wiggler. An undulator is suited for generating monochromatic X-rays, while a wiggler is suited for generating intense white X-rays

Fig. 6 Schematic drawing of an undulator. It consists of two rows of magnets with alternating polarities (blue and red colors), sandwiching the electron orbit (magenta). Each time the electron orbit is bent by the magnetic field, strong radiation is generated (yellow)

with a wide bandwidth of wavelengths. For this reason, beamlines with undulators are preferentially used for diffraction studies. Because the third-generation synchrotron still requires bending magnets to make the orbit circular, bending magnet beamlines also exist in these facilities.

Today, many third-generation facilities are operated all over the world, but three large-scale facilities have become operational since 1994. The first one is ESRF (European Synchrotron Radiation Facility) in France, the second one is APS (Advanced Photon Source) in the USA, and the third one is SPring-8 (Super Photon Ring-8 GeV) in Japan. These three facilities have made major contributions to the advancement of muscle research.

In this third-generation era, PSPCs are no longer used. Instead, semiconductor-type 2-dimensional detectors are widely employed, and they usually are capable of time-resolved measurements. They use several kinds of such detectors. One is a CCD (charge-coupled device) detector with fiber optics. This detector has a phosphor plate in front, which converts the X-ray image to a visible light image, which is led by fiber optics to the CCD chip. By using tapered fibers, the effective area of the detector can be made larger than the area of the CCD chip. The effective area can be even larger using an array of (e.g., in a 3×3 matrix) CCD chips. Even in this case, the area can be made seamless if tapered fiber optics are used.

The second type of detector is an image intensifier combined with a semiconductor-type 2-dimensional detector. This configuration is similar to the one used in the first experiment by H.E. Huxley et al. (1980), but semiconductor-type detectors have digitized outputs that are convenient for later processing. An image intensifier converts the X-ray image to a visible light image and intensifies the image at the same time. Once the image is converted to visible light, it can be recorded by any type of video cameras for visible light images.

Although an image intensifier suffers from a characteristic distortion at the periphery of the image, the merit of this

combination is that one can conduct high-resolution static measurements or high-speed time-resolved measurements simply by exchanging the video cameras to be connected.

Flat panel silicon detectors use amorphous silicon instead of monolithic silicon crystal, and the detector area can be made much larger than the size of usual CCD chips. These detectors are free of distortion, but the choice of exposure times is limited and is not very suitable for high-speed time-resolved measurements.

More recently, photon-counting-type direct silicon detectors, named Pilatus (Dectris, Switzerland) have become increasingly popular. These detectors are free of distortion or dark current, have a high dynamic range, and make millisecond time-resolved measurements possible. Models with large detector areas are available ($> 400 \times 400$ mm). In the large-area models, however, smaller detector modules are tiled, and there is a wide gap between two neighboring modules. The relatively large pixel size ($172 \times 172 \mu\text{m}$) is also a limitation. Researches using these detectors include the one by Perz-Edwards et al. (2011).

In the rest of this section, we will describe a few types of experiments which were made possible for the first time, or made much easier, by using the third-generation facilities.

Interference fringe measurements on myosin meridional reflections

These experiments refer to the precision measurements of the myosin meridional reflections, notably the one at $1/14.3 \text{ nm}^{-1}$ (often called M3). This reflection is considered to report the orientation of myosin heads attached to actin during contraction. Along the meridian, this reflection appears to consist of a single peak under normal recording conditions. However, using a long camera length, this M3 reflection and the higher-order M6 reflection at $1/7.2 \text{ nm}^{-1}$ turned out to consist of closely spaced multiple fine peaks (Linari et al. 2000), which used a 9.85-m camera length. These fine peaks originate from the interference between the structure factor of each myosin head and that of the two arrays of myosin heads on both sides of the bipolar myosin filament. Actually, the peaks are the product of these two functions, as is evident from the convolution theorem as described earlier. The peak heights change during shortening or in response to perturbations such as a step length change of muscle fibers. By analyzing them, one can obtain detailed information about the behavior of myosin heads during the mechanical transients after such a perturbation (Piazzesi et al. 2002; Reconditi et al. 2004; Huxley et al. 2006a, b). It should be noted, however, that it would be more informative if one could obtain the profile of unmodified structure factor of myosin head (i.e., before it is multiplied by the structure factor of the thick filament).

Fig. 7 End on diffraction pattern from a single myofibril from the flight muscle of a beetle, *Aulacophora femoralis*. This pattern was recorded by using X-ray microbeams with a 2- μm diameter. The features of the hexagonal lattice are evident. Taken from Iwamoto et al. 2006

X-ray microbeam experiments

X-ray microbeams are literally beams with micrometer-sized diameters. X-ray microbeams can be achieved by various ways including the use of focusing optics like Fresnel zone plates or a pair of bent mirrors (Kirkpatrick-Baez or K-B mirrors), but more conveniently, by using pinholes. The use of X-ray microbeams became practical after the advent of the third-generation facilities. In the case of pinholes, the practical lower limit of the beam size is $\sim 2\ \mu\text{m}$, and under this beam size, the spread of the beams will be prominent due to Fraunhofer diffraction. In the case of focusing optics, the beam size may be reduced to $\sim 100\ \text{nm}$ or less in ideal cases, but if the sample is placed at the focus, the beams will be spread at the detector position.

The merit of using X-ray microbeams is that one can record diffraction patterns from local structures. An example is the diffraction pattern from a single myofibril from the asynchronous flight muscle of a beetle, *Aulacophora femoralis* (Iwamoto et al. 2006 see Fig. 7). This pattern originates from a single hexagonal lattice, indicating that the filament lattice orientation of a myofibril from asynchronous flight muscle is consistent throughout its entire length.

Fast time-resolved measurements

Millisecond time-resolved measurements have been possible for some of the intense reflections by using the second-generation facilities, but the advent of the third-generation facilities, along with advanced detectors, has made it possible to do fast time-resolved measurements for weaker reflections with better precision. Fast time-resolved measurement by using 2-dimensional area detectors is a common practice among the third-generation facilities.

In the context of fast time-resolved measurements, the BL40XU beamline of SPring-8 (Inoue et al. 2001) is worth noting. This is a high-flux beamline that can deliver X-ray beams 1000 times more intense than those from standard undulator-based beamlines (10^{15} photons/s vs. 10^{12} photons/s). The BL40XU beamline is equipped with a helical undulator, in which the magnets are arranged so that the magnetic field rotates. The radiation from a helical undulator contains a strong fundamental wave component, which can be used as quasi-monochromatic beams without using a monochromator, after the higher-order harmonics are cut off by multilayer-coated mirrors. The X-ray beams generated in this way have a wavelength

Fig. 8 Diffraction patterns recorded from a bundle of rabbit skeletal muscle fibers at the BL40XU high-flux beamline of SPring-8. **a** Relaxed. **b** Rigor. Recorded by using an imaging plate and the exposure time was 1.4 ms

bandwidth of $\sim 2\%$, whereas in the standard beamlines with a monochromator, it is $\sim 0.01\%$. The high X-ray flux owes to this wider bandwidth. This means some deterioration of the quality of diffraction patterns, but it is tolerable for most of the applications. The diffraction patterns as shown in Fig. 8 were taken from a bundle of rabbit skeletal muscle fibers during the commissioning of the beamline. An imaging plate was used as a detector and the exposure time was 1.4 ms.

A number of 2-dimensional fast diffraction recording experiments were done on muscle using this beamline. In all experiments, a CCD or a CMOS (complementary metal-oxide-silicon) video camera was combined with an image intensifier with a fast-decaying phosphor. In earlier experiments, a fast 3-CCD video camera (model C7770, Hamamatsu Photonics, Japan) was used. This had 3 CCD chips that were alternately used after the light path was split into three by prisms (Yagi et al. 2004). This model had a maximal frame rate of 291 frames/s at its full frame size (640×480 pixels). A number of studies were published by using this detector (Wakayama et al. 2004; Yagi et al. 2005, 2006; Yagi 2007; Matsuo and Yagi 2008; Tamura et al. 2009; Iwamoto et al. 2010). A problem with this video camera was that the 3 CCD chips were not perfectly uniform. As a result, the sensitivity slightly varied from frame to frame. For this and other reasons, this model was later replaced by newer high-speed CMOS video cameras.

CMOS video camera chips can be made faster than CCD chips because the CMOS technology allows a higher degree of circuit integration on the chips so that each pixel can have its own amplifier. A CMOS video camera used in BL40XU (model SA5, Photron, Japan) achieves a maximal frame rate of 7000 frames/s at a megapixel frame size (1024×1024 pixels). Because it uses only one CMOS sensor chip, there are no sensitivity fluctuations among different frames.

By using this and other models of Photron cameras, a number of fast diffraction recording experiments were done on frog intact skeletal muscle (Matsuo et al. 2010) and insect flight muscle (Iwamoto and Yagi 2013; Iwamoto 2017). In Iwamoto and Yagi's paper, diffraction patterns were recorded from live bumblebees at a frame rate of 5000 frames/s.

In high-flux applications, radiation damage to specimens is a serious problem. To reduce radiation damage, axially uniform samples such as muscle fibers may be continually moved during the exposure. In the experiments by Matsuo et al. (2010) and Iwamoto (2017), the fibers or muscles were moved at a speed of 100 mm/s.

Fourth-generation synchrotron radiation facilities (X-ray free-electron laser facilities)

The X-ray free-electron laser facilities (currently 3 facilities are operational in the world) are sometimes called the fourth-generation synchrotron radiation facilities. The principle of the generation of synchrotron radiation is identical to that of

the third-generation facilities. However, an X-ray free-electron laser has a linear structure, and a linear accelerator is connected to a very long undulator. When bunches of electrons travel along the undulator, they are longitudinally compressed due to the interaction between the electrons and the photons they have generated, and very short (order of femtoseconds) and very intense pulses of light are emitted.

The beams from an X-ray free-electron laser is highly coherent. This property is suitable for the purpose of imaging, as will be explained later. Another feature of the beams from an X-ray free-electron laser is that their peak flux is extremely high. In SACLA, an X-ray free-electron laser, 5×10^{11} photons are

Fig. 9 The hybrid input-output (HIO) algorithm of phase retrieval. The iterative calculation started by giving a random set of phases to the recorded diffraction pattern (top left). The data are brought back and forth between the reciprocal and real spaces by applying Fourier transformation (FFT) and inverse Fourier transformation (FFT⁻¹). In the real space, an approximate area of the sample is assumed (support, the smaller square in the bottom right), and the densities produced outside the support is slashed. In the reciprocal space, the amplitudes are adjusted to the observed values while retaining the restored phases. Quoted from Iwamoto 2018 (adopted from Miao and Sayre 2000)

Fig. 10 Example of phase retrieval by using the HIO algorithm (computer experiment). In this example, the SPPring-8 logo was used as the sample. The calculations converged within a few hundred iterations as judged from the error function (below, purple). In addition to the original HIO algorithm, the shrink-wrap procedure was incorporated (the area of the support is updated to fit the actual shape of the sample; Marchesini et al. 2003). The area of the support relative to the whole area is also

plotted (below, green). Top left, the original object from which the starting diffraction pattern was calculated (whole area: 512×512 pixels). The phase information was discarded and replaced by a random one. Top middle, restored the image. Top right, the final area of support (the red area is outside the support). The update of the support was stopped when the error went below 1%

delivered in a single pulse lasting for 30 fs. When irradiated with these extremely intense beams, the materials are destroyed in the process of Coulomb explosion, in which the outer electrons are stripped off, leaving the molecules positively charged. Then, the molecules break apart because of electrostatic repulsion. However, the X-ray photons are diffracted before destruction, leaving information about the structure of the intact molecules. Through this mechanism, the X-ray free-electron laser is expected to be used for imaging of single protein molecules at an atomic resolution without crystallizing them, but currently this attempt has not been successful. Instead, the X-ray free-electron laser can be successfully implemented for “femtosecond serial protein crystallography,” in which the beams are irradiated to a jet stream of protein microcrystals that are too small for conventional protein crystallography. The X-ray free-electron laser has not been used for muscle research, but in the future, it can be one of the tools for studying the fine structure of muscle, depending on the development of techniques to effectively use the X-ray free-electron laser.

Emerging techniques for imaging fine structure of molecules by using X-ray diffraction

As has been overviewed in this review, the X-ray diffraction data from muscle specimens have been directly analyzed and

interpreted in reciprocal space, similar to diffraction from other fibrous materials. However, it would be much easier to grasp structural information contained in the diffraction patterns if they can be brought back to the real space, i.e., if they can be converted to real-space images. This would also reduce the risk of misinterpreting the diffraction patterns.

Currently, the most promising method to realize this would be a modification of what is called coherent diffractive imaging (CDI). CDI refers to algorithms to recover the once lost phase information and finally restore the real-space image of objects from diffraction patterns. Complete recovery of the phase information is possible if the amplitude information is adequately sampled (e.g., Fienup 1982), and later it was experimentally shown that the image of a microfabricated pattern can be restored from its diffraction pattern (Miao et al. 1999).

CDI is basically a method to restore the real-space image of isolated objects. For this method to work successfully, the entire objects must be illuminated by a coherent domain of X-ray beams (hence coherent diffractive imaging). There are several variations of the phase retrieval algorithms for CDI, and the most widely used one is called the hybrid input-output (HIO) algorithm (Fienup 1982). The principle and the details of the HIO algorithm are available elsewhere (e.g., Miao and Sayre 2000), and are described here only briefly.

It is an algorithm containing iterative calculations (Fig. 9). It starts with a diffraction pattern from an object (reciprocal space) from which the phase information is lost. As an initial guess, a set of random phases is given to the pattern. By applying a reverse Fourier transformation, one obtains an electron density distribution of the object (real space). Of course, it is far from the right answer because of the wrong set of phases. However, we can correct it because we know something about the shape of the object. For example, if we know the approximate size of the object, we can determine if the restored densities are distributed outside the area in which the object is likely to exist. This area is called support. Then, the unlikely densities (outside the support) are trashed, and the densities are subjected to a Fourier transformation to obtain their diffraction pattern (back to the reciprocal space). This diffraction pattern has a set of phases, which is probably more correct than the initial random one. Next, the amplitudes are adjusted to the experimental values, and by applying a reverse Fourier transformation again, one obtains an electron density distribution which is likely more correct than the previous one. After repeating this cycle many times, the densities stop appearing outside the support, and the image converges to the correct one provided the conditions are right. The convergence is judged from the value of the error function, defined as (density outside the support)/(density inside the support). In computer experiments in which flawless diffraction patterns can be calculated, the error function goes down to 0.1%.

In order for the HIO algorithm to work, the diffraction data must be sampled at a frequency at least double the Nyquist frequency, which is an inverse of the size of the object (Miao

and Sayre 2000). For a 2-dimensional object, this requirement applies to both X and Y directions, so that at least $4\times$ sampling is required (oversampling).

The HIO algorithm works very well for objects with sharp boundaries. Figure 10 is an example of a computer experiment using the SPring-8 logo, for which the calculation converges quickly. In the real experiments, objects such as metal nanoparticles are good targets for the HIO algorithm (e.g., Takahashi et al. 2011). It has also been applied to some of the isolated biological samples such as bacterial cells, viral particles, and chromosomes (Nishino et al. 2009; Giewekemeyer et al. 2010; Seibert et al. 2011) but it has not been applied to the muscle.

It remains to be seen whether the HIO algorithm can be extended to fibrous samples like a muscle. A fiber, such as a myosin filament or an actin filament, is a structure continuous in one direction. In computer experiments, the calculations for such a continuous structure do not converge, apparently because the condition of oversampling is not met for the axial direction. The error function levels stay at not very low levels (a few percent) and are not reduced further, indicating that the recovered image is not far from the right answer but is still not satisfactory. However, the behavior of the HIO algorithm is not fully understood. It works poorly for objects with non-sharp boundaries (as often occurs in biological samples), and calculations often fail to converge even when the oversampling criteria are fully met. It is also not true that the HIO algorithm fails for fibrous objects of any type. Figure 11 shows an example of a fibrous object that can be converged (a continuous helix with an infinite length).

Fig. 11 Example of an infinitely long fibrous specimen that can converge in HIO calculations (a simple continuous helix). The way of presentation is the same as in Fig. 10

To summarize, CDI is an emerging technique that may be used for imaging muscle structures out of their diffraction patterns in the near future. At present, the most popular phase retrieval algorithm (HIO) does not work well for fibrous samples such as muscle. However, phase retrieval algorithms with better convergence are being pursued by mathematicians, and some of them incorporate the trendy techniques of artificial intelligence (e.g., Goy et al. 2018). Optimization of phase retrieval algorithms for fibrous materials will fundamentally change the way of X-ray diffraction studies on muscle.

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Conflict of interest Hiroyuki Iwamoto declares that he has no conflict of interest.

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