3D Manipulation of Protein Microcrystals with Optical Tweezers for X-ray Crystallography

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Abstract. In some synchrotron facilities such as SPring-8, X-ray microbeams have been utilized for protein crystallography, allowing users to collect diffraction data from a protein microcrystal. Usually, a protein crystal is picked up manually from a crystallization droplet. However it is very difficult to manipulate the protein microcrystals which are very small and fragile against a shock and changes of temperature and solvent condition. We have been developing an automatic system applying the optical tweezers with two lensed fiber probes to manipulate the fragile protein microcrystal. The system succeeded in trapping a crystal and levitating it onto the cryoloop in the solvent. X-ray diffraction measurement for the manipulated protein microcrystals indicated that laser irradiation and trap with 1064nm wavelength hardly affected the result of X-ray structural analysis.

1. Introduction

We have constructed and are operating a micro-focus beamline, BL32XU at SPring-8 with the objective of collecting diffraction data from protein crystals in the micron size range [1]. Users who deal with microcrystals have special difficulties in preparation of frozen samples, namely picking up a microcrystal from the solution with a cryoloop, freezing, and packing it into a cryo-sample container. To reduce the difficulty, we have been developing an automatic system, adopting optical tweezers as a manipulation tool of fragile protein microcrystals. The optical tweezers can manipulate a micron-sized dielectric object without touching that object [2-4]. It was also reported that optical tweezers in the near-infrared region can trap and manipulate a living cell without critical photodamage [5]. We applied optical tweezers composed of two lensed fiber probes [6] to manipulate a protein microcrystal in solution. A schematic drawing of two-lensed fiber probes and the principle by which they can be used to trap a dielectric object are shown in figure 1. The probes of the optical tweezers with the lensed fibers are small enough to be inserted into a crystallization droplet within various crystallization plates and have an advantage for manipulation of the object with lower emission power than with laser beams.

Figure 1. Optical forces acting on a dielectric object. Near the cross-focus point of the laser beams, the scattering force, $F_{\text{scat}}$, and gradient force, $F_{\text{grad}}$, act on the dielectric object. The total force acts on the object as buoyancy (shown by red arrow). The object is captured and held at equilibrium point where the force of gravity and of buoyancy are equal [6].
optical tweezers based on a conventional condensing lens. We evaluated the optical tweezers system with two lensed fiber probes for X-ray protein crystallography.

2. Experimental

The optical tweezers system is composed of two lensed fiber probes attached to two electric $xyz$ micro-manipulators M200 (Suruga Seiki Co., Ltd.), an inverted microscope ECLIPSE TE2000-U (Nikon Co., Ltd.), and two Ytterbium Fiber Laser module YLM-1-1064 (IPG Laser GmbH) with an emission wavelength of 1064nm. Each laser module is connected to each lensed fiber probe. A single-mode tapered lensed fiber for NSOM probe (Nanonics Imaging Ltd.) is applied to the probe of the optical tweezers. The numerical aperture of the probe is 0.22 and the spot diameter is 5µm in the theoretical specification. Photographs of the system are shown in figure 2. A cryoloop attached on the top of a manual micro-manipulator is inserted into the crystallization droplet. The optical tweezers pick up and put a microcrystal into the cryoloop in the droplet. The droplet is cooled at 20°C by the temperature-controllable cooling plate to avoid vaporization. The emission power at the sample position is calibrated using a laser power meter OPM-370 (Sanwa Electric Instrument Co., Ltd.).

In addition, we set up another optical tweezers system with a large condensing lens as a probe to investigate the tolerance of protein crystals to high power irradiation from a near-infrared laser. The large condensing system could not levitate a protein crystal in the solution, but could move it horizontally and irradiate the sample with near-infrared laser light of emission power up to 760mW.

Two protein crystal sample types were prepared in this study. Microcrystals of lysozyme were for 3D manipulation by the optical tweezers with two lensed fibers and rod-like crystals of thermolysin were for the investigation of irradiation tolerance. After manipulation by the optical tweezers, the crystals were pulled out from the crystallization drop together with cryo-protectant and were rapidly frozen in liquid nitrogen. X-ray diffraction data for the lysozyme and thermolysin crystals were collected at BL41XU [7] and BL45XU [8] at SPring-8, respectively. Data collection protocols, such as the total X-ray dose and oscillation region, were optimized to minimise X-ray radiation damage to the crystals. The diffraction images were processed using HKL2000 [9]. Initial phase was derived by molecular replacement. The molecular structures were refined using the program CNS [10].

3. Results and discussion

The optical tweezers with two lensed fiber probes succeeded in trapping protein crystals with sizes between 5 to 30µm and manipulating them in the horizontal and vertical directions. Crystals over 30µm in size could not be levitated by the optical tweezers. The emission power to manipulate the crystals is less than 10mW per probe at the sample position, which is remarkably smaller than the few hundred milliwatts of the optical tweezers with the conventional condensing lens system [11]. The system also succeeded in manipulating a 1µm polystyrene bead. The continuous images captured during the manipulation of a 15µm lysozyme crystal are shown in figure 3. The crystals were soaked in a crystallization droplet including 30% glycerol as cryo-protectant. The crystal on the bottom of the drop was trapped by the optical tweezers, as shown in figure 3(a), and then levitated and moved onto a
cryo-mount loop, LithoLoops™, with 0.02mmϕ, as shown in figures 3(b)-(d). The crystal was released over the loop and allowed to sink down into the loop, as shown in figure 3(e). Just after the crystal caught in the loop, the loop was pulled out from the droplet, as shown in figure 3(f). Manipulation by the optical manipulation was complete within 30sec. After the manipulation, no damage was observed to the shape of crystals. On the other hand, the loop made of polyimide film was sometimes burned out by irradiation of the focused beam. The statistics of X-ray diffraction data collection for the manipulated lysozyme crystals are shown in table 1. Tweezers 1 and 2 labeled in dataset column indicate the crystals picked-up by the optical tweezers, and manual 1 indicates the crystals picked-up manually as references. Each dataset was collected from different lysozyme crystals. As total X-ray dose in the data collection was limited to less than 0.5MGy, X-ray radiation damage of each crystal would be negligible. There was no significant difference in the statistics of crystals manipulated by the optical tweezers and by hand. In addition, there was no difference in the structure refinement statistics of the datasets (data not shown). In particular, the relative isotropic $B$ factor is a key criterion for X-ray radiation damage. On the other hand, there is no criterion to evaluate the photodamage of biomolecules by the near-infrared laser, because the detailed influence of the laser on biomolecules is not clear. A composite omit electron density map generated by CNS suggested that there was no photodamage at amino-acid side chains and disulfide bonds compared to those of the molecular structure without near-infrared laser irradiation. It is indicated that manipulating by the optical tweezers hardly affected the X-ray crystal structure analysis.

Thermolysin crystals which were used in the investigation of the tolerance of protein crystals to high irradiation from a 1064nm laser were rod-like crystals with the longest dimension ~ 300μm. The crystals were soaked in cryo-protectant solution including 30% PEG400. One of two edges of a crystal was trapped by the optical tweezers with high emission power of 760mW for 60sec at room temperature. Two X-ray diffraction datasets were collected for a single crystal at both edges with and without irradiation of the laser, respectively. The total X-ray dose in the data collection was restricted to less than 5MGy. The statistics of X-ray analysis are shown in table 1. Datasets of trap1, free1 and trap2, free2 were collected from the same crystal, respectively. There was no significant difference in the statistics between the datasets with and without irradiation by the high power laser. R.m.s. deviations between the molecular structures in the single crystal are smaller than 0.04Å, indicating that the laser irradiation hardly affected the molecular structure. It was reported that aerobic conditions increased the photodamage of living cells by a near-infrared laser compared to anaerobic conditions.

![Figure 3](image_url)

**Figure 3.** Lysozyme crystal (indicated by red arrow) was trapped by the optical tweezers and levitated onto a cryo-mount loop in (a) - (g). The time displayed at left-down in each frame shows an elapsed time after trapping the crystal. In the last frame (f), the loop which caught the crystal had been pulled out from the droplet.
[5]. However, the result of our structure analysis suggested that there was no influence of oxidative damage in the crystals irradiated with a strong near-infrared laser. Therefore, it was estimated that the irradiation of near-infrared laser with lower emission power hardly affected the molecular structure of proteins.

4. Concluding remarks
Optical tweezers with two lensed fiber probes succeeded in trapping protein crystals smaller than 30μm and levitating them onto the cryoloop in the solvent. X-ray diffraction measurement of the manipulated protein crystals indicated that laser irradiation and trap with 1064 nm wavelength hardly affected the result of X-ray structural analysis. The optical tweezers will be a powerful tool to manipulate a fragile protein microcrystals for X-ray crystallography. Based on the results, we are developing an automatic protein microcrystal pick-up and freezing system combining the optical tweezers with an automatic cryo-sample exchanger, SPACE [12].

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Table 1. Crystal size and X-ray diffraction data collection statistics.

| Crystal sample | Lysozyme | Thermolysin |
|----------------|----------|-------------|
| Dataset        | tweezers1| tweezers2   |
| Crystal size(μm³) | 15x15x20 | 15x15x10    | 25x25x25 | 300x30x30 | 280x30x30 |
| Data Collection Statistics | | | | |
| Resolution (Å) | 50–1.70 (1.76–1.70) | 50–2.08 (2.15–2.08) |
| Space group    | P4,2,2   | P6,22       |
| Cell dimension: | 79.3, 79.9, 79.2, 92.7, 92.8, 92.8, 92.6, | |
| a, c (Å)       | 37.1, 37.0, 37.0, 129.3, 129.3, 129.5, 129.7, | |
| R_sym (%)      | 6.1 (31.5), 6.7(37.0), 6.5(33.7), 8.7(15.6), 8.6(14.8), 8.7(15.7), 8.4(16.1), | |
| I / σ          | 35(7), 23(4), 34(7), 45(18), 46(19), 48(20), 45(16), | |
| Isotropic B factor (Å²) | 14.1 | 14.3 | 15.1 | 9.1 | 8.7 | 8.3 | 9.4 |
| Mosaicity (°)  | 0.40–0.43 | 0.25–0.30 | 0.23–0.35 | 0.127 | 0.100 | 0.091 | 0.122 |

*R_sym = ∑|I| -<|I>| / ∑|I|*, where I is the intensity of an observation and <|I>| is the mean value for that reflection and the summations are overall reflections.

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