T.2 Laser micromanipulation of microscopic objects

S. K. Mohanty & P. K. Gupta

Use of lasers for precise manipulation and processing of microscopic objects, such as a single living cell or even objects within a single cell, is finding widespread applications in biological research and technology[1]. The two important tools for laser-assisted micromanipulation of microscopic objects are laser microbeam and laser optical trap. Laser microbeam is essentially a pulsed laser beam coupled to a microscope. The large intensities (~ 10^{13} W/cm^2) generated at the focal point of the large numerical aperture microscope objective can be used to cut, perforate or fuse microscopic objects with sub-micrometer accuracy. As these intensities arise only at the focal point, it is possible to work within the depth of a transparent object without opening it. Optical tweezers or laser optical trap uses the light of a cw infrared laser for precision micromanipulation of microscopic objects. Here, the gradient forces arising due to the large gradient of light intensity in the focused laser beam are used to trap microscopic objects at the focal point of the laser beam. Unlike mechanical micro tools, the optical trap is gentle and absolutely sterile and can be used to capture, move and position single cells or sub cellular particles without direct contact or significant damage.

We have developed a laser micromanipulation set up in which we have incorporated several novel micromanipulation techniques engineered for different applications such as, controlled 2D & 3D rotation of trapped objects and for acceleration and projection of trapped objects. The laser micromanipulation set up has been fully characterized and has already been used for a variety of studies ranging from malaria diagnosis, laser assisted enhancement of neuronal growth cones, 3D viewing of dividing chloroplasts, microinjection of impermeable dyes into cells, transfection, microfluidic applications as well as some studies on interaction between optically trapped dielectric Rayleigh particles. In this article we provide a brief description of the set up as well as some representative studies carried out using the set up.

Laser micromanipulation set up

A schematic of the laser micromanipulation set up[2] is shown in fig.T.2.1. The output of a 1064nm cw Nd: YAG laser (Solid State Laser Division, CAT) and a collinear He-Ne laser beam were expanded using a beam expander (BE) and coupled to the objective of an inverted microscope (Axiovert 135TV, Carl Zeiss) through its base port via a folding mirror (M3). An external lens (EL) placed in the path of these two collinear beams formed a 1:1 telescope with the tube lens (TL). The He-Ne laser beam was used for alignment of trapping beam and detection of position of the trapped object by backscattering. For generating a point tweezers a spherical lens was used as the external lens (EL) and cylindrical lens (or a combination of cylindrical lenses) was used to generate elliptical (line) tweezers. For generating dual tweezers the cw laser beam was split into two beams, which were combined, to result in a small angle between them and coupled to the microscope. The Q-switched pulsed Nd: YAG laser (pulse energy up to 50mJ; repetition rate 1-10Hz, SSLD, CAT) or its harmonics were expanded and coupled through the epi-fluorescence port of the inverted microscope. A 100X Plan Neofluor oil immersion phase objective (MO) was used to focus the laser beam to diffraction limited spot. Both the cw and pulsed lasers were used as optical tweezers for micro positioning of cells in suspension. The pulsed laser and its harmonics were also used for optoporation and other microbeam applications. The dichroic mirror (DM1) combines the pulsed 1064 nm beam and the UV-visible light from the excitation source (mercury lamp) transmitted through the excitation filter (EX). The Dichroic mirror (DM2) reflects the pulsed beam and transmits the fluorescence.

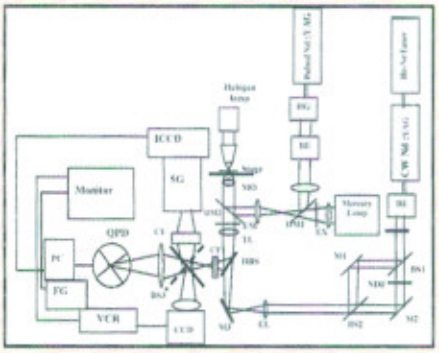

Fig.T.2.1 A schematic of the laser micromanipulation setup developed at CAT

The dichroic beam splitter (DBS) transmits the cw 1064nm and reflects the emitted fluorescence filtered through emission filter (EM) to the CCD. An infrared cut off filter (CF) was placed before the CCD to reject the back-scattered laser light. Bright field and fluorescence images were captured using a video CCD or a cooled CCD and processed using standard image analysis software. The beam splitter (BS3) can be positioned in two orientations to transmit the back scattered He-Ne laser probe beam to quadrant photo detector (QPD) and simultaneously record either image of the object on specimen stage using CCD detector or acquire spectra of trapped objects using an ICCD and Spectrograph (SG) combination.

For small displacement of optically trapped objects the force field experienced by the object can be expressed as $F = -k x$, where x is the displacement of the particle from trap center and the constant k is known as trap stiffness. The

trapping force and the stiffness parameter of the trap were determined following the standard procedures[1]. A quadrant photodiode based system and a centroid tracking system using an algorithm developed in house were incorporated in the micromanipulation set up to monitor displacement of trapped objects with resolutions approaching few nm. The use of centroid tracking enabled simultaneous monitoring of the position of multiple particles and could facilitate simultaneous characterization of multiple optical traps.

Development of new laser micromanipulation techniques

a) Rotating line optical tweezers

The use of optical tweezers based methods to rotate microscopic biological objects is receiving considerable attention since ability to orient biological objects in a precisely controlled manner can help study interaction between specific regions of these objects. Most of the techniques developed for rotation of objects are limited to either absorbing[3] or birefringent[4] or specially fabricated structures[5] and hence are not suitable for rotation of biological objects. An interferometric approach suitable for rotation of trapped biological object has recently been reported[6]. However, this method has two important drawbacks, poor utilization of the trap laser power due to the loss in the generation of the required interference pattern and high susceptibility of interference pattern to vibrations. It may also be difficult to apply this approach to rotate objects inside biological cells because scattering will degrade the contrast of the interference pattern.

We have demonstrated controlled, continuous rotation of objects using rotating line optical tweezers (RLOT), which was generated by placing a rotating cylindrical lens in the path of the trapping beam[7-9]. In line optical tweezers, object lacking spherical symmetry orients itself along the length of the trap. Therefore, by rotating the elliptic trapping beam the trapped particle can be rotated around the axis of the laser beam. Since most naturally occurring biological objects do not have spherical symmetry these can be rotated with RLOT. It is pertinent to emphasize here that the major axis of elliptic tweezers has to be comparable or larger than the size of the object(s) to be trapped. However, if very large tweezers are used for rotation of small object(s), the laser power will not be utilized efficiently. Further, the object will have freedom to move along the major axis of the elliptic profile. In our set up, use of a 200mm focal length cylindrical lens led to focal spot size at the object plane of ~1μm x 10μm and with 50mm focal length cylindrical lens the focal spot size at the object plane was ~1μm x 40μm. The minimum power, at the trapping plane, required for trapping and rotation of *E. coli* bacteria using line tweezers (10μm x 1μm) was ~ 15mW. Since the drag force of the medium upon the rotating objects determines the limiting speed of rotation, the speed can be increased by an increase in the power of the trapping beam. At 25mW power at the trapping plane, the trapped bacteria could be rotated up to a speed of ~ 9Hz. The bacteria trapped at this power level and rotated at 3Hz for 10 min were motile on being released from the trap. This observation as well other studies on the bacteria confirmed that its viability was not compromised. By using line tweezers (40μm x 1μm) we could trap eight human red and white blood cells along the 40μm long major axis of the elliptical profile. Because of increased trap dimensions larger trap beam power was required to trap and rotate the assembly of cells. At a power level of 40mW at the trapping plane, the assembly could be rotated at speeds up to 3Hz.

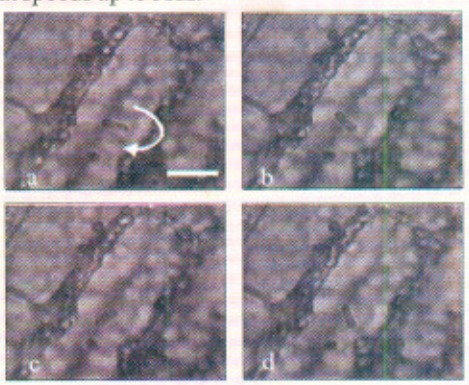

Fig.T.2.2 Rotation of an intra-cellular object inside *Elodea densa* plant cell. Direction of rotation is shown by arrow. (a); rotation by angles of 45° (b); 145° (c); and 235° (d). Scale bar: 20μm

At 25mW power in the line tweezers (10μm x 1μm) trapping beam, we could also rotate an intracellular object (presumably a calcium oxalate crystal) trapped inside *Elodea densa* plant cell with speeds of up to 4 Hz (fig. T.2.2). To the best of our knowledge, this was the first demonstration of rotation of an intracellular object in living cell of intact tissue. Rotation of intracellular objects might prove useful to explore several aspects of developmental biology. For example, spindle could be reoriented inside cell during cell division and the delay, if any, in the transition from metaphase to anaphase can be monitored. In addition, this technique might also be used to trap and orient probe particle, which may find use in atomic force and photonic force microscopy. A patent application for this approach for rotation of microscopic object(s) has been filed[7].

b) Optical trap with spatially varying Polarization

We have used interference of two orthogonally polarized beams to generate a uniform intensity trap having spatial variation of the polarization vector of the resultant

electric field and demonstrated the use of such a trap for orientation or variable speed rotation of birefringent objects without the need of any moving component in the system[10].

c) Development of techniques for laser assisted 3D rotation of microscopic objects

For many of the biotechnology and MEMS applications, ability to rotate microscopic objects in three dimensions would be of considerable help. To the best of our knowledge there has been only one report addressing this important issue[11]. This approach makes use of a spatial light modulator (SLM) to create a pair of closely separated optical traps to hold different parts of the same object. The pair of traps could be made to revolve around each other, rotating the trapped object with them at speeds of up to 0.2 Hz, which was limited by the maximum update rate of the SLM.

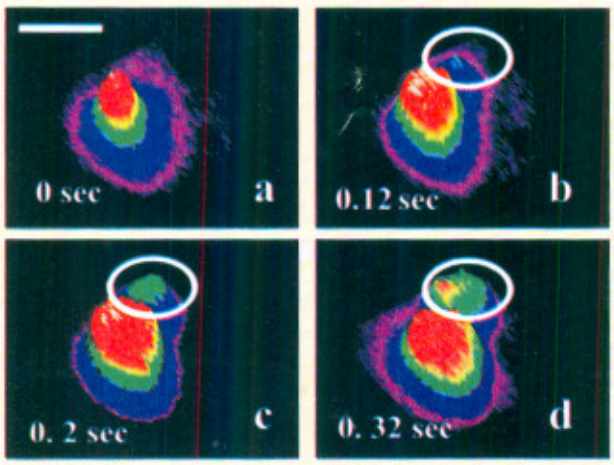

Fig.T.2.3 Intra-cellular 3D orientation and auto fluorescence imaging of motile dividing chloroplasts. (a) Only one of the two sister chloroplasts is visible because the other is in a plane below the plane of the bigger sister chloroplast. (b) to (d); the chloroplast was reoriented from the vertical plane to the horizontal plane facilitating simultaneous visualization of both the sister chloroplasts. The sister chloroplast that was earlier invisible has been enclosed by a circle for clarity

We have developed alternative simpler methods for full 3D rotation of microscopic object(s). In one approach[12] a tangential force was applied on the object to cause its rotation by focusing a pulsed near infrared laser beam at a point on the periphery of the object. With this approach an optically trapped dividing chloroplast could be oriented to permit 3D imaging from different angles of view (fig.T.2.3). The other technique developed[13] relies on the combined use of a point and a line tweezers and exploits the fact that whereas in point tweezers, the transverse gradient force is three to four times stronger than the axial gradient force, in a line tweezers, the transverse gradient force along the major axis is weaker than the axial gradient force. Because of this asymmetry in gradient forces, whereas in a point tweezers an asymmetric object (or assembly of objects) tends to align along the direction of the trapping laser beam, in a line tweezers it aligns with its major axis along the major axis of the elliptical focal spot. Therefore, by controlling the relative powers of each of the tweezers, controlled orientation of microscopic object(s) can be achieved.

d) Transportation and acceleration of particles with laser beam

We have used an optical trap with asymmetric intensity profile in plane transverse to the beam propagation direction to generate an asymmetric transverse gradient force and used it for transportation, and acceleration of microscopic object(s). We could simultaneously transport hundreds of particles along the 40μm long major axis of the elliptical focal spot within 10 seconds and also demonstrate transport of objects varying in sizes from sub-micron to tens of microns. Further, since the asymmetric gradient force depends on the optical and geometrical properties of particles, so do the acceleration and the velocity of projection. The technique may therefore find use for sorting of different particles based on difference in these properties. A PCT patent application[14] has been filed for the approach.

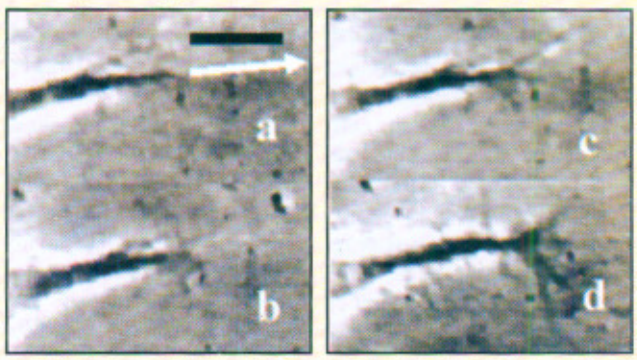

Fig.T.2.4 Time-lapse digitized video images of optical enhancement of the growth of a neuronal growth cone. The time interval between successive images was 5 min. The asymmetric gradient force was used to transport intracellular proteins in the direction marked by arrow. Scale bar:10μm

The laser-guided transport of intracellular proteins has been used for controlled enhancement and guidance of neuronal growth cones. Even at room temperature (~ 25 ^{0}C) where the neuronal growth is known to be very slow significant enhancement of neuronal growth (32 ± 6μm/h) could be achieved (fig.T.2.4). We have also been able to make use of this technique in inducing artificial growth cones from neuronal cell body and establishment of connection between two neurons at room temperature.

Studies using the laser micromanipulation set up

a) Laser assisted microinjection into targeted cells

Laser-assisted microinjection (optoporation) of exogenous material (genes, fluorochromes, or photo-activable compounds) into cells is receiving considerable attention. Compared to conventional techniques, optoporation is more efficient and less tedious and offers the advantage that it can be used on cells in suspension as well as attached cells. Because the constituents of the cell membrane have strong absorption in the UV spectral range, lasers in this spectral range were the first to be investigated for optoporation. However, the use of UV light raises concern about the damage to cells or even exogenous biological material being transferred into the cell. Use of lasers with wavelength in near infrared region would be more desirable for optoporation due to significantly lower absorption by cellular components in this wavelength range. We have demonstrated[15,16] the use of a nanosecond 1064nm Nd:YAG laser for microinjection of impermeable fluorochromes as well as transfection of GFP encoding plasmid into Human breast adenocarcinoma (MCF-7) cells.

The Nd:YAG laser beam (17ns, 10Hz) was focused using a 100X Plan Neofluor oil immersion objective at the edge of the membrane of targeted cell which was suspended in a medium containing impermeable dye merocyanine 540. The energy of the laser pulse at the sample plane was 150μJ / pulse resulting in an energy density of ~ 2.4 x 10^4J/cm^2. The dye gets injected into the cell through the point of irradiation and its uptake increased with increasing laser exposure. Optoporation of propidium iodide, another impermeable dye, into MCF-7 cells could also be carried out. Further the fact that even 10 min after termination of the irradiation, no significant increase in PI fluorescence from nucleus was observed indicates that membrane integrity of the microinjected cell remained intact. Transfection of GFP-plasmid into the cell using nanosecond pulsed laser was also confirmed by monitoring the fluorescence of GFP expressed in transfected cells (fig.T.2.5).

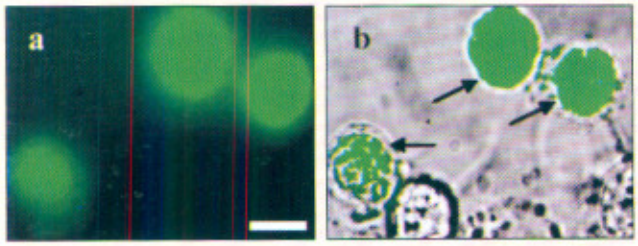

Fig.T.2.5 (a) Fluorescence image of MCF-7 cells 24h after transfection with green fluorescent protein (GFP) coding plasmid. (b) Bright field and fluorescence images of cells are overlaid to show targeted cells (shown by arrows). The GFP in transfected cells was excited using 395-440nm band pass filter and emission was monitored using 470 nm long pass filter. Scale bar:10μm

b) Use of optical tweezers for microfluidics applications and diagnosis of malaria

It is known that the shape of normal red blood cell (RBC) depends on the osmolarity of the medium in which it is suspended. In isotonic buffer (290mOsm/Kg) it is bi-concave in shape; in a hypotonic buffer (having an osmolarity of ~ 150mOsm/Kg) it gets swollen and becomes spherical and in hypertonic buffer RBC takes a meniscus shape. We have found that the meniscus shaped RBC acts as a natural motor when optically trapped. The rotation of the meniscus shaped normal red blood cell arises due to the torque generated on the cell by transfer of linear momentum from the trapping beam. The rotational speed was observed to increase linearly at lower trap beam powers and more rapidly at higher powers. Our results suggest that deformation of the RBC caused by the radiation pressure of the trap beam is responsible for this observation.

Since RBC can be easily transported through microfluidic channels, optical manipulation of RBC can provide a convenient means to perform microfluidics functions. We successfully used a RBC micromotor to pump a liquid with flow rates of few nl/sec to few μl/sec. The direction of flow was determined by the direction of rotation of the RBC motor and the flow rate by the speed of the motor, which could be controlled by varying the power of the trapping beam.

Another interesting observation was that under the same trapping conditions where normal RBC rotates, RBC having malaria parasite (as confirmed by fluorescence staining) did not rotate, and the rotational speed of other RBCs from malaria infected sample, were an order of magnitude smaller and also increased much more slowly with an increase in trap beam power than that for normal RBC. The difference in rotational speeds could be exploited for detection of malaria. Our observations are consistent with the fact that the rigidity of malaria-infected cell membrane is an order of magnitude larger than that for normal[17]. Further, it is known that mature parasites release exo-antigens that increase the rigidity of the uninfected RBCs in the sample. An important advantage of the present approach is that even those cells from malaria parasite infected blood sample, which do not show acridine orange fluorescence, show large difference in rotational speed as compared to normal cells.

c) Effect of laser micromanipulation on biological objects

Even with lasers operating in the near infrared spectral range (800nm–1100nm) where cellular constituents do not have significant absorption, the possibility of adverse effects on the cells being manipulated is a matter of concern and requires careful monitoring. With a view to find the

wavelengths which would lead to minimal damage we carried out studies on single strand DNA breaks in optically trapped cells. Over the wavelength range of 750 – 1064nm, the threshold for single strand breaks in DNA was observed to be lowest at around 760nm and for the irradiation conditions investigated (60-240mW, 10-50J/m^2; 30-120s irradiation) the fraction of damaged DNA was a factor of two larger in the wavelength range of 750-780nm as compared to that in the wavelength range of 800-1064nm. Interestingly, in the plot of logarithm of damage threshold as a function of wavelength, while the damage threshold value at 760nm fitted well on the line extrapolated from the values of UV radiation induced damage, the values for damage in the wavelength range 800-1064nm fell on a line that had a different slope. The observed[18] change in the slope of the damage around 780nm is suggestive of a change in mechanism of DNA damage beyond 780nm. With a view to understand the mechanism of damage at 1064nm, we have carried out investigations on generation of oxidative stress and alterations in mitochondria of different cells of epithelial origin (HeLa, MCF-7) exposed to 1064nm cw and pulsed Nd:YAG laser tweezers. Our results suggest involvement of photo-thermal effects in the 1064nm laser irradiation induced changes. Mechanistic studies using quenchers for reactive oxygen species suggest that laser irradiation leads to generation of hydroxyl radicals.

d) Study of matter-matter interaction in presence of light

Although optical binding between dielectric particles in the Rayleigh range (particle dimensions less than the wavelength of the interacting light) has been predicted theoretically[19], it has not yet been demonstrated experimentally, presumably because faster Brownian motion for smaller particles makes tracking of these more involved. Using the centroid tracking system incorporated in our set up we have demonstrated[20] optical binding between two dielectric particles (polystyrene spheres with radius 150nm) optically trapped in a line tweezers using 1064 nm trapping beam. Our experiments also confirmed the theoretically predicted dependence of inter particle separation on the polarization of the interacting light. The phenomenon of optical binding is of interest not only from the point of view of understanding interaction between objects in an optical field but it can also provide a means to arrange particles in two as well as in three dimensions.

Conclusion

A versatile experimental set up for laser micromanipulation of microscopic objects has been developed. The set up incorporates some new techniques developed by us for guided multiple particle transport, and for controlled rotation, orientation, acceleration and projection of trapped microscopic objects. Use of the set up for some representative applications like 3D viewing of trapped objects, intracellular orientation of objects, transfection of plasmids into cells, laser assisted growth of neurons, and studies on optical binding of dielectric Rayleigh particles has been presented to showcase the wide applicability of the developed set up for basic research and technological applications. Work is also in progress to make use of the facility for nano-technology applications and to extend the capability of the set up to probe processes at single molecule level.

References

1. K. O. Greulich, Micromanipulation by Light in Biology and Medicine, Basel, Switzerland: Birkhäuser Verlag, 1999.
2. S. K. Mohanty, N. Ghosh and P. K. Gupta, Proceedings of National Laser Symposium, LASTECH, Delhi, Dec. 13-15, 2000, pp. 49-50.
3. H. He, M. E. J. Friese, N. R. Heckenberg, and H. Rubinsztein-Dunlop, *Phys. Rev. Lett.* 75, 826-829, 1995.
4. M. E. J. Friese, T. A. Nieminen, N. R. Hewckenberg and H. Rubinsztein-Dunlop, *Nature* 394, 348-350, 1998.
5. P. Galajda and P. Ormos, *Appl. Phys. Lett.* 78, 249-251, 2001.
6. L. Paterson, M. P. MacDonald, J. Arit, W. Sibbett, P. E. Bryant and K. Dholakia, *Science* 292, 912-914, 2001.
7. S. K. Mohanty, R. Dasgupta and P. K. Gupta, patent application no. 0935/MUM/2002.
8. R. Dasgupta, S. K. Mohanty and P. K. Gupta, Biotechnology Letters, 25, 1625-1628, 2003.
9. R. Dasgupta, S. K. Mohanty and P. K. Gupta, Optics & Photonics News (Newsletter of Optical Society of America) end-of-year special issue, "Optics in 2003", Dec. 2003, page 16.
10. S. K. Mohanty, K. Divakar Rao and P. K. Gupta, accepted for presentation at Conference on Lasers and Electro Optics (CLEO), to be held at California, USA, May 16-21, 2004.
11. V. Bingelyte, J. Leach, J. Courtial and M. J. Padgett, Appl. Phys. Lett. 82(5), 829-831, 2003.
12. S. K. Mohanty and P.K. Gupta, Proceedings of International Conference on Laser applications and Optical Metrology (ICLAOM-03), December 1-4, 2003, IIT Delhi, Delhi, pp.600-603.
13. S. K. Mohanty, R. Dasgupta and P. K. Gupta, Proc. DAE-BRNS National Laser Symposium, IIT, Khargpur, Dec, 2003, pp. 643-644.
14. S. K. Mohanty and P. K. Gupta, PCT patent application no. PCT/INO3/00277.
15. S. K. Mohanty, M. Sharma and P.K. Gupta, Biotechnol. Lett. 25, 895-899, 2003.
16. S. K. Mohanty, M. Sharma and P.K. Gupta, Optics & Photonics News (Newsletter of Optical Society of America) end-of-year special issue, "Optics in 2003", Dec. 2003, page 17.
17. S.K. Mohanty, M.Parlikad, A. Uppal and P. K. Gupta, Proceedings on CD, Photonics 2002, Sixth Int. Conf. on Optoelectronics, Fiber Optics and Photonics, TIFR, Mumbai, Dec. 16-18, 2002; see also abstract book page 15.
18. S. K. Mohanty, A. Rapp, S. Monajembashi, P. K. Gupta and K. O. Greulich, Rad. Res.157, 378-385, 2002.
19. F. Depasse and J. M. Vigoureux, *J. Phys. D: Appl. Phys.* 27, 914-919, 1994.
20. S. K. Mohanty, J. T. Andrews and P. K. Gupta, Proceedings on CD, Photonics 2002, Sixth Int. Conf. on Optoelectronics, Fiber Optics and Photonics, TIFR, Mumbai, Dec. 16-18, 2002; see also abstract book page 29.