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Seeing the unseen: Imaging rotation in cells with designer anisotropic particles

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Abstract

Cellular functions are enabled by cascades of transient biological events. Imaging and tracking the dynamics of these events have proven to be a powerful means of understanding the principles of cellular processes. These studies have typically focused on translational dynamics. By contrast, investigations of rotational dynamics have been scarce, despite emerging evidence that rotational dynamics are an inherent feature of many cellular processes and may also provide valuable clues to understanding those cell functions. Such studies have been impeded by the limited availability of suitable rotational imaging probes. This has recently changed thanks to the advances in the development of anisotropic particles for rotational imaging. In this review, we will summarize current techniques for imaging rotation using particle probes that are anisotropic in shape or optical properties. We will highlight two studies that demonstrate how these techniques can be applied to explore important facets of cellular functions.

Keywords

Anisotropic particles; Janus particles; Single-particle tracking; Rotational dynamics; Fluorescence imaging

1. Introduction

All cellular processes involve cascades of orchestrated transient and dynamic interactions. The capacity to visualize and measure these dynamic events in real-time would be powerful for understanding them. In some cases, such observations are feasible. Single particle tracking (SPT) is a method that follows the trajectories of individual objects of interest. It has been widely used to measure complex cell dynamics and interactions (Kusumi et al., 2014; Saxton and Jacobson, 1997). Rapid progress in high-resolution optical imaging and real-time tracking techniques has led to many exciting discoveries that would have been impossible to achieve with traditional ensemble-average techniques (Ruthardt et al., 2011; Huang and Xie, 2014; Wang and Tao, 2013). For example, such techniques have revealed the “walking” mechanisms of motor proteins, such as kinesins (Yildiz et al., 2004; Asbury et al., 2003; Visscher et al., 1999), dyneins (Toba et al., 2006; DeWitt et al., 2012), and myosins (Yildiz et al., 2003; Warshaw et al., 2005), along cytoskeletal filaments with unprecedented resolution. Tracking the dynamics of viruses during infection enabled the identification of

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distinct steps in the process. Such knowledge is critical for the development of effective vaccines (Lakadamyali et al., 2003; van der Schaar et al., 2007, 2008; Arhel et al., 2006; Liu et al., 2014). Receptors and lipids on cell membranes were found to undergo anomalous diffusion (Dahan et al., 2003; Ueda et al., 2001; Ritchie et al., 2005; Kusumi et al., 1993). The observation of molecular transient confinements within particular regions of the plasma membrane provided direct evidence supporting the model that cell membranes exhibit compositionally distinct compartments (Douglass and Vale, 2005; Kusumi et al., 2005).

In contrast to the extensive efforts to measure the translational dynamics of molecules and organelles inside cells, their rotational dynamics have rarely been investigated. However, a growing number of studies show that rotational dynamics are a prevalent and important feature in biological systems (Anthony and Yu, 2015). Noji et al. (1997) performed an early study that directly observed the rotation of mitochondrial F₁-ATPase during ATP hydrolysis. They anchored the ATPase on a surface and attached a freely rotating actin filament to the γ -subunit, so that the rotation of the actin filament directly indicated the rotation of the γ -subunit in the protein complex during ATP hydrolysis. Viruses have been shown to undergo a variety of rotational dynamics when they bind to the ligands on lipid membranes (Kukura et al., 2009). Monitoring this rotation revealed ligand-receptor recognition mechanisms that would have been missed if only translational motion was measured. Rotation of particles during cell entry has also been observed. Our group showed in a recent study that ligand-coated microparticles rotate actively during uptake by macrophage cells due to pulling forces exerted by the cell membrane (Sanchez et al., 2015). Rotational dynamics are also an important clue to the mechanisms of intracellular cargo trafficking. Studies have shown that molecular motors that carry cargos along microtubules and actin “walk” in a spiral trajectory around the cytoskeletal track as they move forward (Brunnbauer et al., 2012; Can et al., 2014). During this process, the cargos themselves also exhibit interesting rotational dynamics. Gu et al. (2012) demonstrated that endosomes rotate during axonal transport when they become detached from microtubule tracks. Such studies demonstrate that the rotational dynamics observed in cellular systems can provide a new level of information about the mechanisms underlying intracellular interactions that would be missed if only translational motion was measured.

Despite those pioneering studies, our understanding of the rotational dynamics in cells remains limited. A major challenge is the lack of methods to precisely and directly measure rotation reliably in a variety of different cellular environments (Anthony and Yu, 2015). Currently existing techniques for measuring rotational dynamics in biological systems rely on the use of anisotropic particles as imaging probes. Two types of such particles have been used so far: those that are geometrically anisotropic, and those that are optically anisotropic. The former type includes colloidal rods (Han et al., 2006; Mukhija and Solomon, 2007), filamentous viruses (Lettinga et al., 2005), gold nanorods (Gu et al., 2013), and quantum rods (Tsay et al., 2006; Ohmachi et al., 2012). The latter includes particles with surface-tethered markers (Kukura et al., 2009; Robbins and Theriot, 2003; Irmscher et al., 2012) and two-faced Janus particles that have different optical properties on opposite sides (Anthony and Yu, 2015; Behrend et al., 2004a,b, 2005; Anker and Kopelman, 2003; Anthony et al., 2006, 2008; Hong et al., 2006). In this review, we assess these techniques for measuring the rotational dynamics in biological systems. In addition to discussing recent major advances in

methodology, we will describe several case studies in detail to demonstrate how different rotational tracking methods were employed to illuminate important biological questions, from cellular entry of particles to trafficking of intracellular cargos. The merits and drawbacks of each technique are discussed throughout the review.

2. General methods for probing rotational dynamics

2.1. Methods based on particle probes that are anisotropic in shape

Particles that are non-spherical, exhibiting a regular and detectably anisotropic shape, are useful for rotational tracking without the need for optical asymmetry. Among them, the most widely used are rod-shaped particles, including gold nanorods (Gu et al., 2012, 2011; Neupane et al., 2016; Chaudhari and Pradeep, 2014; Xiao et al., 2011a), colloidal ellipsoids (Han et al., 2006; Mukhija and Solomon, 2007), quantum rods (Tsay et al., 2006; Ohmachi et al., 2012), filamentous viruses (Lettinga et al., 2005), and dumbbell particles (Uspal et al., 2013; Uspal and Doyle, 2014). Typically, the rotation of rod-shaped imaging probes can be measured along two of the three possible rotational axes: in-plane rotational angle ϕ and out-of-plane rotational angle θ (Fig. 1a) (Anthony and Yu, 2015). The third axis of rotation, longitudinal rotational angle δ around the long axis of a rod, is generally not visible unless optical anisotropy is created along the side of the rod. Rotation of a rod-shaped particle is typically detected optically using techniques based on either interference patterns or intensity patterns.

2.1.1. Gold nanorod-enabled imaging—Gold nanorods (AuNRs) (Chen et al., 2013a; Park et al., 2014) have been used as rotational reporters due to their distinct surface plasmon resonance (LSPR) responses along different axes. Localized plasmonic oscillation from AuNRs can be seen as independent dipoles consisting of longitudinal and transverse modes. The spatial distribution of the scattering electric field of AuNRs is dependent on the orientation of the nanorod (Xiao et al., 2010). The orientation of single AuNRs can thus be determined by measuring the polarized signals from scattered light. To demonstrate the application of this method, Xiao et al. (2011b) used light-sheet scattering microscopy to measure both the in-plane and out of-plane orientation of AuNRs in HeLa cells. Because the unique plasmonic response of AuNRs also results in anisotropic absorption properties, Chang et al. (2010) introduced an absorption-based technique that measures the orientation of AuNRs by detecting refractive index changes of the surrounding medium caused by photothermal heating of the AuNRs. Both the light scattering and the light absorption methods are capable of resolving the orientation of AuNRs in two dimensions with high accuracy. Wang et al. (2010) advanced the rotational tracking of AuNRs to three dimensions by using Nomarski-type differential interference contrast (DIC) microscopy. When AuNRs are illuminated by two orthogonally polarized illumination beams, the phase delay induced by the nanorods gives rise to an interference pattern that consists of a pair of bright and dark spots (Fig. 1b). The periodic changes of the intensities of these bright or dark spots with time depend on changes in the orientation of AuNRs relative to the two polarization directions, enabling the measurement of two rotational angles of the nanorods. This method of detection was used to measure the rotation of ligand-coated AuNRs on cell membranes (Xiao et al., 2012) and the rotation of endosomes in axons (Gu et al., 2012). The AuNR-

based imaging can potentially be used for correlative light and electron microscopy studies, owing to the electron-dense nature of gold nano-particles. Such studies are expected to be informative in revealing the relationship between rotational dynamics measured from live cell imaging and intracellular structures measured from electron microscopy. A potential limitation of the AuNR-based imaging is that the detection of the anisotropic scattering or absorption signals from AuNRs can be compromised in highly light scattering environments. As a result, this rotational imaging method may be limited to certain cell types or biological systems. Moreover, the rotation of a nanorod around its longitudinal axis cannot be measured due to its optical symmetry along that axis.

2.1.2. Using rod-shaped particles and ellipsoids as imaging probes—

Besides AuNRs, the rotation of other rod-shaped particles has been measured in various experimental systems. For example, Han et al. (2006) created μm -sized ellipsoids by stretching polymeric spheres. They visualized the two-dimensional orientation of the ellipsoids under a bright-field microscope (Fig. 1c inset). They found that ellipsoids tend to exhibit diffusional movements along their long axis on short time scales. On longer time scales translational diffusion becomes random after decoupling from the rotational diffusion (Fig. 1c, d). This work is among the first to directly visualize the strong coupling between translational and rotational motion of colloids. Mukhija and Solomon (2007) used a similar particle fabrication approach to make fluorescent rods that were a few μm in length and had aspect ratios of 3–7. The three-dimensional orientation and position of the μm -sized rods suspended in viscous solutions was directly visualized by using conventional confocal fluorescence microscopy. Uspal et al. (2013) and Uspal and Doyle (2014) synthesized dumbbell-shaped particles using continuous flow lithography and studied how the orientation of these particles affected their translational motion in microfluidic flows. They observed that tadpole-shaped particles with one large lobe and one small lobe quickly rotate to align with the direction of flow, whereas symmetric particles orient at random and oscillate transversely in the flow.

Quantum rods are another type of fluorescence-based probes for rotational imaging. Their orientation and rotation are measured using their linearly polarized fluorescence emission. Like spherical quantum dots, quantum rods have excellent photostability and brightness, both of which are advantageous for biological imaging (Chan et al., 2002). Tsay et al. (2006) measured the translation and rotation of peptide-coated quantum rods following non-specific protein binding using fluorescence correlation spectroscopy. They observed that protein binding interactions cause more changes to the orientation of a rod than to its translational motion. They concluded that measuring the rotational dynamics is much more informative to reveal the binding interactions than measuring translational motion alone. Ohmachi et al. (2012) measured the three-dimensional orientation of quantum rods with an accuracy of 10° and 33 ms by using a polarized fluorescence microscopy technique (Fig. 1e). In this imaging method, four polarization components (at 0° , 45° , 90° , and 135°) were imaged at the same time and used to deduce the orientation of a rod in three-dimensions (Fig. 1f). By attaching quantum rods to myosin V motors, they were able to observe that myosin V motors rotate 90° in-plane between two consecutive steps during actin-dependent transport (Fig. 1g). Information from such high-resolution measurements revealed that the myosin V motors

alternate between a two-head bound state and a one-head bound state as they walk along actin, an important new clue to the mechanism of such motors.

Besides synthetic particles, rod-shaped viruses have also been employed as rotational imaging probes. An example is bacteriophage *fd*, which has a filamentous shape and is about 800 nm in length and 6.6 nm in diameter. Due to this large aspect ratio, the viruses at high concentrations form a nematic liquid crystal phase, in which the filamentous viruses exhibit some orientational order but no translational order. The rotation of single viruses in this densely packed liquid crystal phase was studied, with only a small fraction of viruses fluorescently labeled to avoid interference from neighboring viruses (Fig. 1h, i) (Lettinga et al., 2005). A density-dependent packing was observed in their experiments. However, at lower concentration, the viruses are in isotropic phase, where the virus orientation is random (Fig. 1k).

2.2. Methods using optically anisotropic particle probes

2.2.1. Creating optical anisotropy using surface-tethered fluorescent nanoparticles—Robbins and Theriot (2003) demonstrated an early study in which surface-tethered fluorescent nanoparticles were used for rotational tracking. In order to examine the longitudinal rotation of a Gram-positive bacterium during actin-propelled movement, they attached one to a few fluorescent microspheres to the outer surface of the bacterium. The position of the fluorescent microspheres and both ends of the bacterium were located in optical images. The longitudinal orientation of the bacterium was deduced from the orthogonal projection of the fluorescent microsphere on the long axis of the bacterium. This approach of creating optical anisotropy was also employed by Kukura et al. (2009). To investigate the rotational dynamics of viruses during ligand-receptor binding, they attached a 15–20 nm quantum dot onto the capsid of each 45 nm virus. Single viruses were imaged using interferometric scattering microscopy (iSCAT) and their surface-tethered quantum dots were imaged with fluorescence microscopy. The relative orientation and projected distance between the tethered reporter and the center of mass of the virus were used to calculate the three-dimensional orientation of a single virus (Fig. 2a). Their study showed that virus particles exhibit a range of interesting rotational dynamics during ligand-receptor binding (Fig. 2b). Irmscher et al. (2012) combined magnetic particle actuation, in which particle movement was induced by an external magnetic field, with rotational tracking to measure the local stiffness of cell membranes. Fluorescent nanoparticles were attached to the surface of magnetic particles to enable rotational imaging and tracking of the particles. Magnetic particles bound to cell-surface receptors were actuated by an in-plane oscillating magnetic field. Translation and rotation of the particles were simultaneously measured and used to calculate the mechanical coupling between the particle and the cell membrane. Using this method, the authors observed that the local stiffness of the contact area between the particle and the cell membrane varies depending on the properties of the ligand-receptor binding. This method can be useful for understanding mechanical response of cell membranes during important biological processes such as endocytosis. It is straightforward to fabricate such probes, but they are subject to some major limitations. The first limitation is that the fabrication method does not allow one to control the number of surface-tethered nanoparticles on given objects of interest. Some particles will have too many or too few

surface reporters to be usable for imaging. But we expect this issue to be solved in the near future as new particle fabrication methods are being discovered. The second limitation is that attachment of a nanoparticle to the surface of an object of interest inevitably changes the shape, which may bias the rotational measurement. The third limitation is that this method, like most others, is limited by two-dimensional projection. It can only resolve the out-of-plane rotational angle (θ) in the range of 0–90° instead of 0–180°.

2.2.2. Optically anisotropic Janus particles—Optical anisotropy may be created by asymmetrically functionalizing the surfaces of uniform spherical particles. The resulting particles have distinct chemical or material properties on opposite hemispheres. These particles are commonly referred to as Janus particles, named after the two-faced Roman god Janus (Granick et al., 2009; Walther and Muller, 2013; Jiang et al., 2010). Janus particles that exhibit different optical properties in their two hemispheres were first employed for rotational imaging by Kopelman et al. They coated a thin layer of metal on one hemisphere of μm -sized fluorescent particles, such that the Janus particles were fluorescent on one side and opaque on the other (Behrend et al., 2004a,b, 2005; Anker and Kopelman, 2003). Due to this optical anisotropy, such particles vary in intensity with rotational orientation in fluorescence imaging, enabling direct visualization of particle rotation (Behrend et al., 2004b). However, the particle intensity only contains information about out-of-plane rotation (Behrend et al., 2005; Hong et al., 2006). Anthony et al. (2006) later developed single-particle rotational tracking methods for measuring both the in-plane and out-of-plane orientation of single metal-coated Janus particles, particle clusters (Hong et al., 2006), and particles in close proximity (Anthony et al., 2008). Section 3.2 of this review contains a detailed discussion. Our group has recently applied this imaging technique to track endosome trafficking in living cells. We have succeeded in fabricating optically anisotropic Janus particles that do not have metal coatings (Sanchez et al., 2015). Our particles display two fluorescent patches on opposite sides of a single particle, enabling simultaneous measurement of both in-plane and out-of-plane rotation, though with the usual ambiguity for the out-of-plane angle due to two-dimensional projection. A more detailed discussion on using such dual-color Janus particles for detecting particle rotation during cellular up-take is included in Section 3.1 of this review.

2.2.3. Nanodiamonds—Valued for their exceptional photostability and biocompatibility, fluorescent nanodiamonds are an emerging class of biological imaging probes (Vaijayanthimala et al., 2015; Hsiao et al., 2016; Fu et al., 2007). They are nano-sized diamond crystals that fluoresce due to naturally occurring nitrogen–vacancy defects. The special physical properties of these defects allow the orientation of the nanodiamond crystals to be readily measured. The quantum states of the nitrogen-vacancy centers split along the crystallographic direction, which defines an intrinsic quantization axis (Fig. 3a) (Maclaurin et al., 2012; McGuinness et al., 2011). The separation between these split quantum states determines the fluorescence intensity of each nanodiamond. The separation changes depending on the orientation of the intrinsic quantization axis with respect to an externally applied magnetic field (Fig. 3a) (McGuinness et al., 2011; Geiselman et al., 2013). By measuring these changes in fluorescence intensity, the orientation of each nanodiamond can thus be determined. McGuinness et al. (2011) were the first to track the

orientation of fluorescent nanodiamonds in cells using this phenomenon. They used a technique known as optically detected magnetic resonance (ODMR) to detect the location and orientation of single nanodiamonds with an angular precision of 1° over a period of up to 16 h (Fig. 3b, c). This study was focused purely on demonstrating the feasibility of the technique. No particular biological process was investigated. One limitation of using nanodiamonds for rotational tracking is that only the angle between the nitrogen-vacancy quantization axis and the direction of an external magnetic field can be resolved. Since fully determining the rotational orientation of an object requires specifying orientation components along three rotational axes, the capability of this technique may be limited.

2.3. Digital holographic microscopy (DHM) for rotational imaging

Digital holographic microscopy (DHM) is a rotational imaging technique that does not require the use of optically anisotropic probes. In this imaging method, interference patterns, i.e. the holograms, between a scattered light beam that passes through the sample and a reference light beam are digitally reconstructed to uncover the 3D structure, position and orientation of μm -sized objects (Yu et al., 2014a,b). A two-step process is generally involved in DHM based rotational tracking. In the first step, an object beam is passed through the sample. The scattered light from the sample interferes with a reference beam to form interference patterns, which contain both amplitude and phase information (Yu et al., 2014a). In the second step, the digitally recorded interference patterns are fitted to simulated holograms that were numerically reconstructed based on known properties of the sample, including refractive index, particle size and shape. An optimal match between the experimental holograms and simulated ones returns information about the translational and rotational motion of particles within the sample. This technique was used by Wang et al. (2016) to investigate the tumbling motion of freely swimming *E. coli*. Because the recorded intensity of the scattered light also contains information about the mass of the sample, Goldfain et al. (2016) were able to use DHM to measure not only the position and orientation, but also the DNA content of individual bacteriophages. Overall, DHM is a unique technique that can be used to measure the translational and rotational dynamics of particles of known sizes and shapes without the use of imaging probes. However, large tracking inaccuracy could be introduced in samples that have complex structural variations such as inside living cells (Dixon et al., 2011).

3. Fluorescently anisotropic particles for imaging rotation in biological systems

In this review, we seek not only to summarize the development of rotational imaging and tracking techniques, but also highlight how such techniques can be used to advance our understanding of the biological systems. However, most of the studies so far have been heavily focused on the development of techniques rather than on biological applications. In the following section, we discuss in detail two of our recent studies that used optically anisotropic Janus particles to measure rotational dynamics in living cells during important biological processes, including cellular uptake of particles and intracellular trafficking of endosomes.

3.1. Measuring rotation of particles during cellular entry

We recently investigated the rotation of Janus particles during their phagocytosis by macrophage cells (Sanchez et al., 2015). Information obtained from these measurements of rotational dynamics was used to deduce cell-particle interactions during this process. Phagocytosis is a cell uptake process used by immune cells, such as macrophages, to engulf and degrade pathogens to fight against infection (Rodriguez et al., 2013). Immune cells also use phagocytosis to remove foreign particles, including those intended for intracellular drug delivery (Blanco et al., 2015; Fang et al., 2014). Therefore, understanding the phagocytosis of particles has significant implications for both fundamental understanding of cell biology and biomedical innovation. Single-particle imaging and tracking methods have been used to dissect the complex interactions involved in the phagocytosis process (Chen et al., 2013b; Li et al., 2017). But, studies prior to our own measured only the translational motion of particles. We investigated the rotation of particles during cellular uptake by creating Janus particle rotational probes that displayed two patches of fluorescent proteins on opposite poles (Fig. 4).

We used a “sandwich” microcontact printing method to prepare the dual-color Janus particle probes (Fig. 4a). This procedure involves three key steps. The first step is to prepare a monolayer of particles. The next step is to “ink” the polydimethylsiloxane (PDMS) stamps with proteins. Molecules to be transferred onto particles, such as proteins, are nonspecifically adsorbed onto the surface of pre-cleaned PDMS stamps during this “inking” step. The last step is to transfer the “ink” onto particle surfaces by pressing the PDMS stamp against the particle monolayer. We have used this method to successfully print proteins (bovine serum albumin, fibronectin and IgG) and small molecules (si-lanes) onto particles (Sanchez et al., 2015; Gao and Yu, 2015). We have noticed a few critical steps for a successful microcontact printing procedure. First, a uniform closely packed particle monolayer is necessary. Second, the transfer of proteins is most efficient from low-surface energy hydrophobic surfaces to high-surface energy hydrophilic surfaces (Bernard et al., 2000). Therefore, making the particle surface hydrophilic greatly increases the success of microcontact printing. Third, some moisture on the PDMS stamps after protein “inking” helps the “printing” of proteins (Bernard et al., 2000). This may be related to the denaturation of proteins, but the exact mechanism is unclear (Ricoult et al., 2014). One major advantage of this microcontact printing fabrication method is that it allows the precise control over the surface coverage and pattern of the protein patch printed onto particles (Gao and Yu, 2015). A drawback of this procedure, however, is the low-throughput yield of particles and the inevitable particle-to-particle variation in the printed patches.

In our study, the Janus particles were also coated with immunoglobulin G (IgG), which serves as a ligand for the Fc receptors on macrophage membranes that initiate phagocytosis of particles. Rotation of the particles was clearly observed during live cell imaging (Fig. 4). We then quantified the in-plane rotation of the dual-color Janus particles by locating the positions of each pair of protein patches on single particles. The single-particle rotational tracking algorithm contains three steps. The first step is to reduce noise by smoothing the images using a Gaussian function (Crocker and Grier, 1996). The second step is to identify the center of each protein patch using a polynomial-fit Gaussian weight method (Rogers et

al., 2007). Each pair of patches belonging to a single particle was identified based on a distance threshold, using the assumption that patches on the same particle would be closer than those on different particles. The final step is to draw a vector to connect the centers of each pair of patches. The orientation of this vector indicates the in-plane angle of the particles and the midpoint of the vectors indicates the particle centroid (Fig. 4b). From this single-particle rotational tracking, we discovered that the particles exhibited a heterogeneous mixture of rotational motions at different stages during their entry into macrophage cells (Fig. 4c). During initial particle-cell contact the particles underwent rapid directional rotation, indicating a process during which cells actively scanned the particle surface for ligand recognition and at the same time pulled particles toward the cell body. After ligand-receptor recognition, particle rotation slowed down to random jiggling during cell entry. After sometime, however, particles again exhibited fast directional rotation, indicating the active rotation of particles within the cell cytosol after phagocytosis. The obvious correlation between translational and rotational dynamics of particles revealed new information about cell-particle interactions during the different stages of phagocytosis.

3.2. Measuring rotational dynamics during intracellular trafficking using metal-coated Janus particles

Janus particles that are fluorescent on one hemisphere and opaque on the other (due to a thin metal coating) enable the measurement of two of the three components of rotational orientation, as described in the previous section. We have recently started to use these rotational imaging probes to investigate the dynamics during intracellular trafficking. Intracellular trafficking is the active transport of proteins, vesicles and organelles by molecular motors along microtubules and actin (Gross, 2004; Hancock, 2014). It is an important cellular process that regulates a multitude of cell functions, from cell division to organization of organelles (Gross, 2004; Soldati and Schliwa, 2006; Karki and Holzbaur, 1999). Single-particle imaging and tracking have been widely used to study the translational dynamics of cargos during active transport (Yildiz et al., 2004; Asbury et al., 2003; Toba et al., 2006; Yildiz et al., 2003; Warshaw et al., 2005). However, little is known about the rotation of cargos. We have recently found that endosomes exhibit a range of interesting rotational dynamics in living cells, by using single-particle rotational tracking techniques with metal-coated fluorescent Janus particles.

Fabrication of metal-coated Janus particles is straightforward (Fig. 5a). A monolayer of fluorescent particles is first formed on a flat substrate (glass microscope slides or silicon wafers) by a solvent evaporation process. The quality of the particle monolayer is critical for creating Janus particles with satisfying half-and-half optical morphology (Chen et al., 2011). After formation of a particle monolayer, layers of metal coatings, each in the thickness of 10–50 nm, are sequentially deposited onto the particle monolayer using thermal or e-beam evaporators. Under high vacuum, the evaporated metal is deposited directionally and as a result it only covers the top hemisphere of the particles in a closely packed monolayer. An important consideration during the particle fabrication procedure is the choice and sequence of the metal coatings. First, a thin layer of chromium or titanium is typically required to increase the adhesion between other metal coatings on silica or polymer particles. Metal coatings without the chromium adhesion layer can peel off easily. Second, when weight

imbalance between the two hemispheres of a Janus particle is a potential concern, light metals, such as aluminum, are preferred over others. However, aluminum has important disadvantages, by comparison with gold, for some applications. A top coating of aluminum does not allow surface functionalization, whereas a gold coating does. Moreover, aluminum can dissolve in acidic environments such as the interior of many intracellular compartments. Therefore, we used gold-coated Janus particles for imaging endosomes in living cells. Lastly, the metal coatings must be thick enough to completely block the fluorescence emission from the other hemisphere, but thin enough to minimize the shape and weight difference between the two sides.

Under a wide-field fluorescence microscope, the shape and fluorescence intensity of the Janus particles change with the orientation of the particle, with the shapes of the fluorescent profiles resembling the moon in different phases (Fig. 5b). These changes enable the direct visualization and measurement of two of the three angles describing the rotational orientation of single particles. In principle, the in-plane rotational angle (φ) can be measured from the orientation of the fluorescent region with respect to the particle centroid, and the out-of-plane rotational angle (θ) can be measured from the brightness change of the Janus particle profiles (due to change in “phase”, i.e., new, crescent, half, gibbous, full) (Behrend et al., 2005). A half-coated Janus particle is symmetric around the axis pointing from the centroid of the fluorescent hemisphere to the centroid of the metal cap. As a result, rotation around that axis is invisible. However, it has been shown that the measurements of in-plane and out-of-plane angles are sufficient to determine the rotational angle variation and rotational diffusion coefficient (Perrin, 1934).

Determining the in-plane orientation of Janus particles involves several steps. The fluorescence image of a Janus particle profile is first converted into a contour plot, in which each closed circle represents a string of pixels of the same fluorescence intensity (Fig. 5d). A few outmost contours are then fitted with circles and the average of the centers of the fitted circles indicates the centroid of the entire particle. Orientation of the vector drawn from the particle centroid to the centroid of the fluorescent crescent of the particle in the image indicates the in-plane orientation of the particle (Fig. 5d). Because the intensity of a fluorescent hemisphere follows an approximate cosinusoidal relationship with the out-of-plane angle (θ), angle θ can be calculated using the following equation (Hong et al., 2006):

$$\cos \theta = \frac{2(I_0 - I_{\min})}{I_{\max} - I_{\min}} - 1 \quad (1)$$

In Eq. (1), I_0 is the measured intensity of the fluorescent hemisphere at any given particle orientation. $I_{\max}$ and $I_{\min}$ are the intensity when the fluorescent side (the “full moon” phase) or the metal-coated side (the “new moon” phase) faces the detector.

To use metal-coated fluorescent Janus particles for imaging rotation of intracellular endosomes, those particles were incubated with epithelial cells to allow endocytosis. At the end of the incubation, the Janus particles were tightly encapsulated in endosomes. The translation and rotation of the Janus particles in cells were imaged with wide-field

fluorescence microscopy (Fig. 6). Our results from single-particle tracking show bidirectional movement of the Janus particles (i.e. directional movement in either direction along the cytoskeleton), which is characteristic of active transport in intracellular trafficking. More importantly, we also observed frequent particle rotation as indicated by the changes in both the shape and intensity of the fluorescent hemisphere in the images (Fig. 6 inset). Such rotational dynamics are an aspect of intracellular cargo transport that has been overlooked previously.

There are two significant advantages of using metal-coated fluorescent Janus particles for rotational tracking in comparison with other methods. First, the metal coating method yields Janus particles with uniform and consistent half-half morphology. The small particle-to-particle variation is important for minimizing the tracking inaccuracy. Second, imaging the rotation of the Janus particles only requires wide-field microscopy as opposed to the complex optical setups needed for imaging rotation of gold nanorods and nanodiamonds. Both considerations make the Janus particle-enabled rotational tracking attractive to researchers without specialized knowledge or equipment for imaging. One drawback of this method, however, is that the tracking inaccuracy increases with smaller particle probes due to the difficulty in resolving the crescent shape of the fluorescent hemisphere in diffraction limited images. The smallest Janus particle probes we have successfully used for live cell imaging are 500 nm in diameter. They may still be too large to detect dynamics of some biological processes.⁴

4. Concluding remarks

In this review we summarized recent advances in the imaging and tracking of the rotational dynamics of nano- and micro-particles, and particularly discussed the application of those techniques for understanding interactions in biological systems. As noted, all existing methods except for one rely on the use of anisotropic particles as imaging probes. Those particles could be anisotropic in shape or in optical properties. Shape anisotropic particles used for rotational imaging include gold nanorods, ellipsoidal colloids, and filamentous viruses. The advantage of using those particle probes is that no additional labeling is required to report the rotational motion. Janus particles that have distinct optical properties on two hemispheres are a unique type of rotational imaging probe. They have the advantage of being simple to fabricate and their rotational tracking requires no complex optical setups. We also included two of our group's recent studies that demonstrated the use of Janus particles for dissecting interactions during cellular entry and during intracellular trafficking, both important biological processes. These two studies showcase the richness of information one can obtain from measuring rotational dynamics.

While the studies discussed in this review showcase different rotational imaging methods, most of them so far have simply focused on the development of techniques. In contrast, little has been done to explore the potential of using those methodologies for uncovering the rich and complex interactions that go on within biological systems. One possible limitation comes from the relative large size of the anisotropic probes that are currently available. With the rapid advancement in the synthesis of anisotropic particles, new rotational imaging techniques are expected to emerge. Methods that use nanosized rotational probes will be

more useful for probing dynamics in biological processes than ones using micron-sized particles. Going beyond technique development or studies that simply report rotational dynamics, a particularly important question to answer is the biological significance or function of rotational dynamics in different processes. Combining rotational imaging techniques with other characterization methods, such as electron microscopy, could be powerful for providing information to answer this question. The main goal of this review has been to shine a spotlight on the tremendous potential offered by single-particle rotational imaging techniques for fundamental biological studies. We hope to inspire more studies across multiple disciplines to use rotational measurements to advance our understanding of biological interactions.

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Fig. 1.

(a) A schematic illustration of the three rotational angles of a rod-shaped imaging probe: In-plane rotation (ϕ), out-of-plane rotation (θ), and longitudinal rotation (δ). (b) A gold nanorod in differential interference contrast (DIC) images changes from bright and dark as the in-plane orientation of the rod changes from 0° to 180° in 15° increments. Replicated with permission from Zhao et al. (2017). (c) Tracking the orientation and translational motion of an ellipsoid. The initial rod location is shown in green and the rod orientation at individual elapsed times is shown in yellow. The translational motion of the ellipsoid is strongly coupled with its orientation on a short time scale. Inset: bright-field image of an ellipsoid. (d) The translational diffusion of the ellipsoid becomes random on a longer time scale due to its random rotation. Images (c) and (d) are replicated with permission from Han et al. (2006). (e) Use of quantum rods to track the rotation of single myosin V motor proteins. The quantum rod was attached to the head region of the motor protein as it traveled on an actin filament. (f) The intensities of four polarization components are recorded simultaneously. The ratios between the components depend on the quantum rod orientation. (g) The in-plane (blue) and out-of-plane (red) rotational angles of a quantum rod attached to myosin V are plotted against time. Images (e)–(g) are replicated with permission from Ohmachi et al. (2012). (h) Fluorescent image showing filamentous bacteriophage *fd* in a closely packed nematic liquid crystal phase. Only a small fraction of the viruses were labeled. (i) The viruses are about 800 nm in length and 6.6 nm in diameter. They appear rod-like in optical imaging, from which in-plane orientation was obtained. (j) Trajectories of fluorescently labeled viruses show that the viruses in the nematic phase prefer to diffuse in a direction parallel to their long axis. (k) The orientation-dependent diffusion in nematic liquid crystal phase becomes random in isotropic phase, as shown by the in-plane angle distribution in isotropic phase (gray) and in nematic phase (black). Images (h)–(k) are

replicated with permission from Lettinga et al. (2005). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Rotational tracking of optically anisotropic particles. (a) Schematic illustration of tracking orientation of a single virus that has a surface-attached quantum dot. (b) Diffusion trajectory of a quantum dot-labeled virus on a supported lipid bilayer. A rocking motion of the virion during a confinement domain was observed. Replicated with permission from Kukura et al. (2009). (c) The out-of-plane angle (θ , black) and in-plane rotational angle (ϕ , red) are plotted against time for a 10 μm metal-coated fluorescent Janus particle freely diffusing in phosphate buffer. Inset: a pseudo-colored fluorescence image of a Janus particle. The plot is replicated with permission from Anthony et al. (2008) and the inset is replicated with permission from Anthony et al. (2006). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Rotational tracking of nanodiamonds. (a) Schematic illustration of the quantum state separation of the nitrogen-vacancy center in each nanodiamond. The extent of the split between quantum levels changes depending on the orientation of the nanodiamond crystal with respect to an external magnetic field. (b) A representative optically detected magnetic resonance (ODMR) spectrum of a nanodiamond moving in cultured HeLa cancer cells. (c) Calculated orientation of the nitrogen-vacancy center relative to the direction of the magnetic field as a function of time. Replicated with permission from McGuinness et al. (2011).

Fig. 4.

(a) Dual-color Janus particles were fabricated using a sandwich microcontact printing method. In step one, a PDMS stamp with protein ink (shown in red) is pressed against a monolayer of particles. In step two, the PDMS stamp is peeled off with particles partially embedded. In step three, a second PDMS stamp with a different protein ink (shown in green) is pressed against the exposed surface of the particle monolayer. Particles are harvested via sonication in the final step. The resulting Janus particles display two patches of proteins of different fluorescence emission on opposite sides. (b) Single-particle rotational tracking of the in-plane rotational angle (ϕ) of the dual-color Janus particles. Scale bar: 5 μm (c) In-plane rotational angle ϕ of a single dual-color Janus particle is plotted as a function of time. The three stages of interactions, particle-cell binding, cell entry, and dynamics inside cells, are highlighted by the gray, red, and blue shades, respectively. Replicated with permission from Sanchez et al. (2015). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

(a) Schematic illustration of the fabrication of metal-coated fluorescent Janus particles. The procedure includes: making a particle monolayer on flat substrate, sequential metal coating on the particle monolayer, and removing particles by sonication. (b) Upper panel: fluorescent images of a Janus particle at different out-of-plane orientations. Lower panel: images of the moon at different phases (from left, crescent, half, gibbous, and full). The shape of the fluorescent hemisphere of the Janus particle resembles the illuminated side of the moon. (c) Schematic illustration of the determination of the out-of-plane rotational angle. Emission from the fluorescent hemisphere (shown in red) of a Janus particle can be partially or completely blocked by the metal-coating (shown in black). The overall fluorescence intensity of the particle in a projection image changes with the out-of-plane orientation. Replicated with permission from Anthony et al. (2006). (d) Tracking the in-plane orientation of a Janus particle from a contour plot (left), which is computed from the fluorescence image. The vector pointing from the centroid of the particle towards the centroid of the fluorescent hemisphere indicates the in-plane orientation. Replicated with permission from Anthony et al. (2008). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6.

A representative trajectory of a metal-coated fluorescent Janus particle in a live cell. The translational trajectory is color-coded with time. Pseudo-colored fluorescent images of the Janus particle at different times show the frequent orientation change during intracellular transport. Scale bar: 5 μm . Scale bars in insets: 1 μm .