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Single molecule optical measurements of orientation and rotations of biological macromolecules

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Abstract

The subdomains of macromolecules often undergo large orientation changes during their catalytic cycles that are essential for their activity. Tracking these rearrangements in real time opens a powerful window into the link between protein structure and functional output. Site-specific labeling of individual molecules with polarized optical probes and measuring their spatial orientation can give insight into the crucial conformational changes, dynamics, and fluctuations of macromolecules. Here we describe the range of single molecule optical technologies that can extract orientation information from these probes, we review the relevant types of probes and labeling techniques, and we highlight the advantages and disadvantages of these technologies for addressing specific inquiries.

Keywords

polarization; single molecule microscopy; super-resolution microscopy; fluorescence microscopy; scattering

Introduction

Many of the enzymes that catalyze essential cellular reactions contain several compact protein or RNA domains linked by relatively flexible hinges [1, 2]. The structural dynamics that lead to their functional outputs may involve changes of distance between these subunits and rotations about the hinges [3]. Figure 1 shows three examples of prominent rotations and hinging motions that are established from structural studies. Domains of topoisomerase II (figure 1(A)) undergo large tilting as the complex unwinds DNA after becoming twisted during replication. Elongation factor Tu (EF-Tu, figure 1(B)) shows an almost 90° rotation of its GTP binding domain relative to the rest of the structure after it delivers an amino-acid-tRNA complex into the ribosome during protein synthesis [4]. The large and small subunits of the ribosome also rotate relative to each other in the following step in peptide elongation [5]. The gamma subunit of F₁-ATP synthase (figure 1(C)) rotates through a full circle to power synthesis of ATP from ADP and phosphate, driven by a proton gradient across the inner mitochondrial membrane. These motions have often been inferred from x-ray

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crystallography and cryo-electron microscopy, but those high resolution methods are generally limited to the analysis of static conformational states. In order to understand the relationship between motions of the various segments of the molecule and formation of its end products, the kinetics of the reaction pathway, interconversion rates, and equilibria between the states need to be determined. Other ensemble structural methods, such as NMR spectroscopy, FRET, and low-angle x-ray scattering can provide kinetic information, especially if the reaction is synchronized, for instance by a temperature jump or rapid introduction of the substrate [6]. However, synchronization is lost after a few reaction steps due to stochastic fluctuations of the kinetic transitions.

Single molecule structural methods remove the requirement for synchronizing a population of molecules as they report the reaction trajectory directly for each molecule, including reversals and brief occupancy of states. Rather than averages which blur detail, single molecule experiments produce actual distributions of parameters, such as the order and rates of reaction steps. Mechanical stresses and strains in infrared optical traps and distances between two fluorescent probes at nearby labeling sites by fluorescence resonance energy transfer (FRET) have become the most widely adopted single molecule techniques [7]. These techniques have their own limitations. For example, single-molecule FRET requires introduction of two fluorescent probes at specific locations, and reports quantitative distance changes only upon correction for many potential artifacts. FRET is sensitive to the relative orientation between the two probes and their mobility, often leading to the concern that changes of energy transfer efficiency may be due to changes in angle rather than the intended distance.

Another powerful method in single molecule structural dynamics has garnered much less attention. This technique measures real-time angular changes detected using the polarized excitation and emission from fluorescent dipole probes or light scattering from elongated metal nanorods. The power of this approach becomes clear when considering the mechanism of the F₁ ATP synthase complex. Although a rotational catalytic mechanism was proposed based on earlier data and the x-ray crystal structure [8], angular motions within the complex could not be directly measured. A subunit was attached to a non-symmetric probe to firmly establish that it rotates within a relatively stable shell of 6 AAA subunits (figure 1(C)) [9].

Here, we present the optical methods used to measure the orientation of polarized reporter probes bound to individual macromolecular domains. The macromolecule is labeled with a polarized fluorescent or light scattering probe with limited rotational mobility relative to the macromolecular domain and, ideally, with known local orientation relative to the structure. The orientation of the probe affects the absorption and emission of polarized light, and the spatial distribution of scattered light, physical characteristics that enable determination of the orientation of the target molecule. The rotations and dynamic fluctuations of a domain can then be determined for individual molecules in real time, during the normal enzymatic cycle, at physiological temperature, *in vitro* or *in vivo*. In some cases, the angles are measured relative to a known reference direction in the sample such as a membrane plane or the travel direction of a motor protein. Planning such an experiment involves choosing the probe and method of labeling, determining the required angular and temporal resolution, deciding whether the angular fluctuations (wobble) of the target macromolecule are of interest, and

accounting for the effects of various geometric symmetries and ambiguities that arise in the interpretation of the results.

Studying the angles and rotational fluctuations provides unique mechanistic insights in biological systems with special functions that intrinsically involve tilting or turning, such as transport motors, F_1 ATP synthase, and their homologs. More broadly, orientation studies provide advantages of high time resolution structural dynamics and elucidation of mechanistically relevant thermal fluctuations. The recent dramatic improvements in super-resolution optical microscopy [10–12] have focused renewed attention onto probe angles because fluorescent markers fixed at out-of-plane orientations can lead to errors of localization, substantially degrading image quality.

We briefly review the underlying principles of these methods, and address some practical considerations for conducting single molecule orientation experiments. We compare the advantages and trade-offs among the various methods for determining molecular orientation. Single molecule orientation and rotational techniques have been reviewed before with emphasis on fluorescent techniques [13–16] and light scattering [17, 18].

Photophysics of polarized probes

Interactions between probes with dipole moments and polarized light depend on their orientation. Absorption of light is most likely when the polarization, i.e. the transverse direction of its oscillating electric field, is oriented parallel to the absorption dipole. The probability of photon absorption, P_a , is proportional to the square of the projection of the electric field onto the dipole orientation, $P_a \propto \cos^2(\theta_a)$, where θ_a is the angle between the polarization of the exciting light and the absorption dipole (for instance, θ_{ax} in figure 2(A) when the light is polarized along the x axis, $\mathbf{e}_x$). As fluorescence is only produced when the molecule absorbs light, by varying the input polarization and detecting the fluorescence for each polarization, information about the probe angle is obtained. An early experiment making use of this angular dependence of fluorescence excitation was measurement of the angle of an individual fluorescent probe shown in figure 3(A). The excitation polarization was varied around the microscope axis and the emission changed as $\cos^2(\theta_a)$ (figure 3(A), inset), enabling precise measurement of the probe angle.

Similarly, the emitted photons are polarized along the emission dipole axis, so polarized detectors, often called analyzers, can also be used to determine the probe orientation. The relative probability of detecting an emitted photon that is captured by the collection optics and projected onto the detector is given by $P_e \propto \cos^2(\theta_e)$, where θ_e is the analyzer polarization relative to the emission dipole.

We have used the angular properties of fluorescence excitation and emission dipoles to detect rotational motions in myosin molecular motors and elongation factor Tu involved in protein synthesis [19–22]. An example recording showing changes in polarized fluorescence intensities of a probe on myosin V (figure 3(B)) demonstrate tilting of the probe angle as the protein steps along actin *in vitro* (figure 3(C)). This provides some of the strongest evidence that this molecule walks ‘hand-over-hand’ and tilts its lever arm back and forth while

translocating along actin. Advantages of this type of polarized dipole absorption and emission recording are discussed in several sections below.

The likelihood of capturing a photon is related to the emission dipole orientation relative to the propagation axis of the detector by $P_c \propto \sin^2(\omega_e)$, where ω_e is the angle between the dipole and the direction of observation (ω_{ey} for the observational eye on the y axis in figure 2(B)). No photons are emitted along the dipole axis and the probe looks brightest when it is perpendicular to the analyzer axis (figure 2(B)).

For rhodamine and other xanthene derivative fluorophores (fluorescein, eosin) and Cy-dyes, the absorption and emission dipoles are parallel to each other and to the long axis of the chromophore [23, 24]. Green fluorescent protein (GFP) and its variants also have aligned dipoles [24], whereas elongated quantum dots (QDs), termed quantum rods (QRs), have well polarized absorption and emission dipoles, but they are not necessarily aligned with each other [25].

In contrast to the excitation and emission of fluorescent probes, light absorption and elastic re-emission from metal nanorods is the result of coupling between the oscillating electromagnetic field and surface electrons in the particle, termed local surface plasmon resonance (LSPR). Metal nanorods are not fluorescent, but their interactions with light depend on the difference in the LSPR between the long and short axes of the rod [17]. These axes have similar relationships to input and output light as those described for fluorescent dipoles.

The properties of dipolar and elongated metal probes can be exploited to measure their spatial orientations. Changing the polarization of input light to detect the angle of the excitation dipole, detecting the polarization of the output light to determine the angle of the emission dipole, detection of the propagation distribution of emitted light, directional scattering and optical phase retardation from nanorods have all been used, separately or sometimes in tandem, to determine the angles of probes attached to biologically-relevant macromolecules.

Choice of probes

Many small organic fluorescent probes, variants of GFP, semiconductor nanoparticles (such as QDs), and light-scattering gold nanoparticles are available for site-specifically labeling macromolecules. Desirable traits for a single molecule probe include high brightness, which includes absorption cross-section and quantum yield, low probability of blinking, resistance to photobleaching, and facility for incorporation into the biological system without disturbing function. The latter characteristic depends on physical size, ligation chemistry, and the specificity of the labeling site. For angular studies, restricted rotational mobility is also important. Available fluorescent and light scattering probes vary greatly in these properties.

Fluorescent proteins

The minimally perturbing approach for interrogating macromolecular orientation is to use the native optical properties of a fluorescent protein, such as the light-harvesting complexes from a purple bacterium probed at the single-molecule level by Bopp *et al* [26]. Most biological materials, however, are not inherently fluorescent. Recent technological advances in the incorporation of unnatural amino acids have greatly increased the potential for expressing intrinsically fluorescent proteins that can be monitored for site-specific changes in orientation [27]. Promisingly, a 3-hydroxyflavone dye, which exhibits dual color fluorescence depending on the polarity of its environment, and acridon-2-ylalanine, a visible FRET acceptor, have been incorporated into unnatural amino acids for ensemble microscopic analysis [28, 29].

Alternately, fluorescent proteins can be genetically fused to the target protein through a short linker. This approach has been used successfully at the single-molecule level with green fluorescent protein (GFP) [30] fused to nuclear pore complexes to determine their orientation at the nuclear membrane, and with the photoswitchable GFP variant, Dendra2, fused to actin or hemagglutinin to observe changes in rotational rate of single molecules within cells [31]. The linker length between GFP and the protein under study is a crucial parameter in this approach. Linker length must be varied to find the species that gives the highest fluorescence anisotropy (lowest mobility), as linkers with several flexible bonds in series may not effectively couple protein rotational motions to those of the fluorescent reporter. In fact, FRET experiments usually rely upon the assumption that probe motion is completely isotropic. For orientation studies, though, the probe motion must follow the protein motions and so cannot be isotropic.

Other ways of attaching fluorophores to proteins have been recently introduced [32], including SNAP-, Halo- and other tags [33], which are then modified with organic dyes. These protein tags are also subject to concerns regarding flexibility of the linkers.

Organic probes

Organic fluorescent probes with useful spectral properties in the visible wavelength range have extended aromatic rings leading to oriented absorption and emission dipole moments. They are much smaller than GFP variants and the semiconductor nanorods described below. The ideal small molecule reporter should bind specifically and rigidly to the macromolecule in a fixed or known orientation. The DNA-intercalating fluorophores YOYO-1 [34] and SYTOX Orange [35] adopt fairly rigid angles perpendicular to the DNA axis and have been used for single-molecule studies of DNA orientation within cells. In addition, the membrane-binding DiI has been used at single-molecule sensitivity to detect membrane packing [36], and effects of gangliosides on the formation of lipid rafts [37].

In special cases an organic fluorophore can be introduced into the biological system by linking it to a ligand that recognizes a specific target. For example, the mushroom toxin phalloidin is a small, bicyclic peptide that binds to actin filaments with nanomolar affinity and is commercially available tagged with many fluorescent probes [38, 39]. As with fluorescent proteins, the linker length between the dye molecule and the recognition motif

strongly affects the probe's rotational mobility [34]. Fluorescent antibodies have not been successful in orientation studies due to variable probe angle [34, 40].

Linker flexibility can be minimized with a short reactive moiety such as maleimide or iodoacetamide to covalently attach rhodamine [41, 42], Cy3 [43], eosin-5 [44] or other fluorophores to either native or introduced cysteines in the target protein. However, when bound to a single Cys residue, the local orientation of the probe relative to the labeling site is not known [45, 46] and may vary considerably depending on the geometry of the fluorophore and the local environment at that residue, which may allow free rotation about the attachment point or hinder motion. For this reason, Adachi *et al* [43], considered several cysteine point mutations in the γ subunit of F₁-ATP synthase labeled with Cy3-maleimide and chose the one showing the highest fluorescence anisotropy. Fortunately, single-molecule studies of rhodamine bound the most reactive cysteine in actin (Cys³⁷⁴) [42, 47] show very low probe mobility because the fluorophore occupies a groove in the protein with known local orientation [41].

Probes with two separate reactive groups that bind to the macromolecule can achieve markedly reduced local motions [48], and fixed local orientation relative to the structure. Bifunctional rhodamine (BR) contains two iodoacetamide groups that flank the chromophore and can crosslink two cysteines which are 7 or 8 residues (~1.1 nm) apart on an α -helix. This configuration aligns the absorption and emission dipoles parallel to the helical axis. BR was originally synthesized for labeling myosin light chains in muscle fiber polarized fluorescence studies [49]. BRs have also been adopted for labeling troponin C in muscle fibers [50, 51], calmodulin for single molecule polarized total internal reflection fluorescence (polTIRF) experiments in non-muscle myosins [19, 20, 52], kinesin motor proteins [53], the β subunit in F₁-ATPase [54], and ribosomal elongation factor EF-G [22].

Attachment of fluorophores to one or two cysteine residues can only be used for *in vitro* applications, as they are not specific enough for in-cell labeling. The use of bi-arsenical probes that bind specifically to tetra-cysteine motifs, such as FIAsh [55] and AsCy3 [56] should be considered, but they have not yet been used in published single molecule orientation experiments.

When the probe orientation is fixed relative to the macromolecule it may be possible use probe angle changes (figure 2(C), β_p and α_p) to calculate the orientation of the protein itself. For example, in the case of a myosin V motor walking straight along actin, rather than tilting around the filament, the local angle of the probe relative to the lever arm can be estimated. Using that local angle, the actual axial (β_L) and azimuthal (α_L) lever arm tilt angles can be estimated (figure 2(C) (bottom)), the more functionally relevant quantities than probe motions [57]. These studies provide the strongest evidence that the lever arm of myosin tilts by ~60° during stepping.

Nanoparticles

It is not always possible to express a cysteine mutant capable of specifically binding an organic dye or to rigidly attach fluorescent proteins in the desired domain. Certain applications might also require a substantially brighter probe than an organic fluorophore or

fluorescent protein. In these instances, nanometer-sized inorganic particles provide an alternative. Fluorescent semiconductor nanoparticles, such as QDs, are composed of CdS, CdSe, or CdTe [58], and are often coated with a shell of CdS or ZnS to increase brightness and reduce blinking [59, 60]. The fluorescent properties of QDs are highly tunable: by changing the size and composition of the QDs, the excitation and emission spectra can be easily controlled [61–63] in the visible wavelength range compatible with laboratory microscopes. QDs are generally much brighter and more photo-stable than organic fluorophores and fluorescent proteins, so they can be imaged for long periods under high intensity illumination with minimal changes to their spectral properties [64]. They are commercially available with a number of different surface coatings that can be used for specifically targeting protein groups such as carboxyls, amines, and antibodies, and streptavidin. Thus, they have found many applications in fluorescence imaging [61, 63, 65]. QDs are often prolate ellipsoids with aspect ratios ranging from 1.1 to 1.2, rather than perfect spheres. These small deviations from symmetry result in polarization-dependent optical emission and absorption [66]. Compared to organic fluorophores and fluorescent proteins, however, QDs are much larger, 5–20 nm in typical applications, which can lead to limitations or steric constraints on activity and difficulty inserting them into live cells, and they can have cytotoxic effects [67, 68].

Under controlled conditions quantum nanoparticles can be synthesized in a wide range of shapes and sizes including elongated prolate ellipsoids, termed QRs [69, 70]. As with QDs, the emission wavelength of QRs is tunable and they confer the same brightness and photostability advantages [25, 71]. The core and shell materials, various dot-in-rod or rod-in-rod configurations, and the aspect ratio are adjusted during synthesis [72]. High aspect ratio 5:1–20:1 QRs exhibit well polarized absorption and fluorescence emission [73], providing high signal-to-background ratio and high angular resolution in orientation experiments. Commercially-available nanorods are not as common as QDs, and thus may require both in-house synthesis and surface modification to make them soluble in aqueous media and biologically compatible [74].

Semiconductor nanoparticles can be functionalized either by covalently modifying the shell [75, 76] or by coating the surface with an amphiphilic polymer [77, 78]. These surfaces can then be modified with a targeting molecule such as streptavidin [79], NeutrAvidin [76], or halo ligand [71, 75]. In either case, the large surface area of nanoparticles accommodates the attachment of many ligands to each particle. This allows rigid multi-site attachment to the biological macromolecule if multiple target sequences are inserted with appropriate spacing [71, 76].

Unlike many of the organic dyes discussed earlier, the excitation and emission dipoles of nanoparticles are not necessarily aligned, and the offset between them may depend on the excitation wavelength and probe dimensions [25, 72]. This trait reduces the facility of measuring nanoparticle orientation using both the absorption and emission dipoles. Thus, polarized fluorescence microscopy with QDs typically uses the emission dipole only. Polarized emission TIRF microscopy with QRs has been used to observe the rotations of the myosin tail domain [71].

Light scattering probes

Similar in size and shape to QRs, gold nanorods (AuNRs) can also be used for determining orientation of macromolecules [80–83]. As with QRs, they produce higher signal-to-background ratio than organic and protein probes, and they do not photo-bleach. AuNPs are less cytotoxic than cadmium-based QRs and are easily surface-modified for interactions with biomolecules [84]. AuNRs are not fluorescent but instead are imaged using dark-field scattering [85, 86] or LSPR, an interaction between the oscillating electromagnetic field and the surface electrons in the metal. The peak wavelength of the LSPR depends on the size and shape of the nanoparticle, and in the case of an elongated particle, is different for polarizations parallel and perpendicular to the long axis [87, 88]. For instance, with gold particles 25 nm in diameter and 75 nm long, the parallel and perpendicular absorption peaks are conveniently located at ~700 nm and ~500 nm wavelengths [89].

Ambiguities arising from optical symmetry

Dipoles exhibit two-fold rotational symmetry, so that any single measurement of a probe orientation will introduce at least the ambiguity that for an orientation described by the azimuthal angle ϕ_p (figure 4(A i)) and axial angle θ_p (figure 4(B i)), there exists a second angle, $(\phi_p + 180^\circ, 180^\circ - \theta_p)$, in the opposite hemisphere that absorbs and emits light in the same manner. These two orientations cannot be distinguished.

Figure 4 shows various additional symmetries that can arise in optical measurements of probe orientation, and the requirements for breaking those symmetries to remove the associated ambiguities. For this example molecule (pink double-headed arrow) oriented with the polar coordinates θ_p, ϕ_p , the absorption and emission dipoles are co-linear, and the microscope optical axis corresponds to z . The probability of absorption using a polarized excitation source is proportional to $\cos^2(\theta_a)$, and identically, the likelihood of detection of a polarized emitted photon is proportional to $\cos^2(\theta_e)$, where $\theta_a = \theta_e$ is the angle between the probe and either the polarized excitation or detection. Practical methods for making the required measurements will be discussed later.

Consider the case when the probe angle is located in the x - y plane (figure 4(A i)). Measuring orthogonally polarized intensities in the plane, I_x and I_y , gives $\phi_p = \tan^{-1} \left(\sqrt{I_y} / \sqrt{I_x} \right)$. Three other angles in the other quadrants of the plane produce the same intensities and are indistinguishable (figure 4(A ii)). If the probe is not actually in the x - y plane, then the projection of the probe into the x - y plane is measured. Although the magnitudes of I_x and I_y change, their ratio does not, and thus θ_p (the out-of-plane angle) is not determined.

Adding a z -polarized excitation or absorption signal (figure 4(B i)) enables measurement of the polar angle, θ_p , between 0° and 90° , but in this case positions in the upper and lower hemisphere cannot be distinguished, leading to an 8-fold ambiguity in probe orientation (figure 4(B ii)).

Detecting a third polarized signal within one of the sample planes, shown here at $\phi = 45^\circ$ within the x - y plane ($\sqrt{I_{45xy}}$, figure 4(C i)), breaks the symmetry between probes at $+\phi_p$ and $-\phi_p$, reducing the ambiguity to 4 angles (figure 4(C ii)) [90].

A fourth symmetry-breaking polarization is, for instance, a 45° excitation or emission in the y - z plane ($\sqrt{I_{45yz}}$, figure 4(D i)), or in the x - z plane. Strong z -axis excitation or detection can be achieved using TIRF microscopy, or annular [91] or radially [92, 93] polarized light. With these 4 intensities, I_x , I_y , I_{45xy} and I_{45yz} , the probe angle is determined uniquely in a hemisphere and the only remaining ambiguity is the oppositely pointing vector (figure 4(D ii)). As mentioned, this is the minimum ambiguity that can be attained for a single angle measurement, due to the photophysical symmetries of the probe and the light.

Adding additional polarizations, such as a 135° signal complementary to the 45° signals in figures 5(C) and (D), over-determines θ_p and ϕ_p , and does not remove any further symmetries or reduce ambiguity. However, many experimental setups still add these polarizations, as they may be advantageous for calibrating the instrument and enabling tests of the internal consistency of the intensity data.

These spherical ambiguities can be problematic because inappropriate choice of the region of the sphere used to quantify the probe angles may result in an incorrect interpretation of observed angle changes. Even though the probe is a dipole, its two ends are aligned or attached to different physical objects, e.g. two different cysteines on a helix. The same end of the probe must be tracked during structural changes. Otherwise, spurious 180° angular increments arise if the angle of one end is reported before the change and the other end, related by $(\phi_p + 180^\circ, 180^\circ - \theta_p)$, is reported after the change. Selection and choice of the relevant hemisphere specifying probe angles can thus be critical for properly relating signal changes to physical angles.

User input is required to appropriately select a quarter-sphere or hemisphere in which to mathematically define the scope of probe angles. The appropriate choice of hemisphere does not need to be aligned with Cartesian axes. As an example, consider the hypothetical molecule in figure 5, in which the probe tilts from State 1, with subpopulations of probes at $\phi=100^\circ$ and $\phi=70^\circ$, to state 2, with probes at $\phi=25^\circ$ and $\phi=-10^\circ$. In our hypothetical case, the largest angle changes are expected to be less than 180° , based on high resolution structures. Confining angles to the upper (+Y) or right (+X) hemisphere incorrectly reports some of the probe angles (red arrows, figure 5 bottom). Instead, a tilted hemisphere (figure 5, lower right) is the suitable choice resulting in correct reporting of the probe angles.

Finding the appropriate hemisphere to ensure that only one end of the probe is tracked in a particular experiment (rather than its $(\phi_p + 180^\circ, 180^\circ - \theta_p)$ alternate) requires further consideration. One method of defining an ideal confining hemisphere for a set of angles is to calculate a director which represents the average orientation of all probe angles within the set, irrespective of their 2-fold symmetry (figure 5, lower right, double-headed blue arrow) [21]. This approach is appropriate whenever angle changes are expected to be smaller than 180° and within 90° of the average angle for a dataset. The two ends of the director are

equivalent, so one is chosen and then the correct probe angle is assigned as the one within 90° of that end of the director (figure 5, lower right).

In general, we can improve upon spherical ambiguities if we have additional data or knowledge about the biological system. Knowing the local angle of the probe relative to the underlying target macromolecule can facilitate selection of the appropriate hemisphere. The plane of a biological membrane can define a single hemisphere based on the known position of a probe with respect to the membrane. For example, lipophilic DiI applied from the outside of a cell inserts into the outer leaflet of the plasma membrane, restricting the probe-accessible space to the hemisphere above the membrane [94].

In ensemble studies of muscle fibers, BR probes placed at different known angles relative to myosin regulatory light chain [49] or troponin C [50] enabled choice of the appropriate hemisphere. Mechanical stretches and releases that tilted the myosin lever arms in known directions also broke the hemisphere symmetry [95]. It should be possible to pull on an individual molecular motor in an optical trap to tilt its structure and similarly break the hemisphere symmetry.

Hemisphere selection may be assisted by other geometric characteristics of the system. For example in two-headed ‘hand-over-hand’ molecular motors, such as myosin V, the leading and trailing positions of the probe can be assigned by the length of the preceding step because the labeled head moves farther when it takes a step than when its unlabeled partner head steps [96]. This asymmetry allowed probe angles to be assigned to the leading and trailing positions in the hand-over-hand mechanism [97]. Incorporating extra information to assist interpreting the angular data from polarized fluorescence experiments, as in these examples, may be applied to other systems.

Detecting timescales of motion

Probes can undergo two kinds of motion when tethered to proteins: tilting independent of protein motion enabled by linker flexibility, and motions that are coupled to changes in orientation of the protein itself. For small organic fluorophores, wobble of the probe relative to the protein take place on the nanosecond timescale, similar to or faster than the fluorescence lifetime (τ_f). Fluctuations of protein domains tend to be slower, on the order of μs or ms. This slower wobble can be of biological interest; for example, the microsecond motions of a myosin motor while it ‘searches’ for its next actin subunit during a step [21, 98] or fluctuations of a cytoskeletal filament pushing the plasma membrane at a cell protrusion [99]. A subset of the microscopy methods described here can assess wobble at these timescales, including the polTIRF microscope described in the next section and figure 7(B).

In single-molecule polarized fluorescence or scattering microscopy, the average angle of the probe is interrogated, and in addition, these other two time scales of motion, in the μs range and in the ns range, affect the polarized fluorescence intensities in different ways and therefore may be estimated. A qualitative explanation of these phenomena is given in figure 6.

Polarization ratios, which are independent of the excitation power and emission flux, are commonly used to record polarized fluorescence signals [23]. The vertical polarization ratio P_V is given by the ratio of intensities detected through horizontally and vertically polarized analyzers when exciting with vertically polarized light. $P_V = ({}_V I_V - {}_V I_H) / ({}_V I_V + {}_V I_H)$, where the prefix is the exciting polarization orientation and the suffix is the analyzer orientation. Similarly, the horizontal polarization ratio P_H is given by $P_H = ({}_H I_V - {}_H I_H) / ({}_H I_V + {}_H I_H)$.

In the case shown in figure 6(A), an ensemble of static molecules is excited by either vertically or horizontally polarized light. Intensity and polarization ratios for the two excitations are very different: ${}_V I_V / {}_V I_H \gg {}_H I_V / {}_H I_H$ and $P_V \gg P_H$ because there are more vertical molecules excited when the excitation is vertically polarized and vice versa. The excited population is anisotropic, a process termed photoselection. If these molecules can tumble under thermal agitation with a rotational time scale, τ_r , the two polarization ratios will decay until $P_V = P_H = 0$ and the excited sample becomes isotropic. In a typical ensemble anisotropy decay experiment, it is very difficult to detect tumbling, τ_r , much slower than the lifetime of the fluorescent excited state ($\tau_f = 4$ ns for rhodamine), because the fluorescence signals become too weak. The fluorescence lifetime puts a bound on the slowest rotational rate that can be measured.

For a single molecule, also static, the situation is quite different (figure 6(B)). Vertical and horizontal illumination may have different probabilities for excitation, but once the molecule is excited, the polarization of the emitted photon is independent of the polarization of the exciting photon. Then ${}_V I_V / {}_V I_H$ exactly equals ${}_H I_V / {}_H I_H$ and $P_V = P_H$. If, on the other hand, the single molecule has rotational mobility with rate τ_r , slower than τ_f and faster than the measurement time (τ_c), it will be excited several times during a measurement, but more often when it is aligned with the excitation than when it is perpendicular (figure 6(C)). In this series of excitation-emission events, the equivalent of photoselection takes place similarly to that in the static ensemble case of panel A. Thus, ${}_V I_V / {}_V I_H \neq {}_H I_V / {}_H I_H$ and $P_V \neq P_H$. The inequalities here are due to the rotational mobility, τ_r , in the microsecond range, $\tau_f \ll \tau_r \ll \tau_c$, allowing the molecule to be excited by the vertically polarized light at certain orientations and by the horizontally polarized light at others within a detection period.

Finally, if the probe motion is much faster than τ_f ($\tau_r \ll \tau_f$), then the effect of the motion on the polarization ratios is different (figure 6(D)). Regardless of the orientation of the probe at the moment of excitation, fast wobble of the probe will cause it to visit the entire accessible distribution of angles (within the limited range of probe attachment), on average, before it emits the fluorescent photon. In this case, the emission polarization ratio is again independent of the excitation polarization. The equalities ${}_V I_V / {}_V I_H = {}_H I_V / {}_H I_H$ and $P_V = P_H$ again apply, but the numerical values of the ratios themselves are smaller than in the static case (B) because the rapid wobble tends to depolarize the emission. In the limit where rapid wobble is completely unrestricted, ${}_V I_V = {}_V I_H = {}_H I_V = {}_H I_H$ and $P_V = P_H = 0$.

These qualitative arguments show how these two separate time scales of rotational motion affect single-molecule polarized fluorescence intensities differently. The motions can be quantified by calculating analytical expressions for the requisite polarized fluorescence

intensities that take into account the expected correlations between excitation and emission, and other characteristics of the instrument and optics [14, 19, 47, 100, 101].

The measurement time τ_c is related to switching times if the excitation is modulated and a function of the choice of detector. Two types of detectors are considered for orientation experiments with different time resolution, sensitivity, and data throughput. Avalanche photodiodes (APDs) are single-spot photon counting detectors with high quantum yield (photo-electrons per photon captured) in the visible range and $<1 \mu\text{s}$ time resolution. An APD can only count the photons projected onto its $\sim 200 \times 200 \mu\text{m}^2$ area (corresponding to $2 \times 2 \mu\text{m}^2$ at the sample for $100\times$ magnification), requiring the stage to be moved precisely into the correct location and limiting the number of recordings to one at a time. Concomitant measurement of position (e.g. molecular motor stepping) is lost while measuring intensity using APDs.

Electron multiplying CCD (EMCCD) cameras have sufficient sensitivity and low noise to quantify single molecule fluorescence emission from many molecules at a time. Trade-offs are their relatively low read-out rate, leading to framing intervals for full fields $>20\text{--}30$ ms, and excess noise introduced in the electron multiplying process. Fast CMOS cameras have recently been introduced into super-resolution imaging applications [102], but they are noisier and less uniform than EMC-CDs. Selection and tradeoff among these cameras has been evaluated [103, 104].

Methods for detecting polarized emission and excitation

Emission-only polarized detection

One way to obtain quarter-sphere resolution of orientation (figure 4(C)) is to detect the intensity of emission split into, at minimum, its x , y , and 45° - xy polarized components. We have built an objective-type TIRF microscope (figure 7(A)) in which emission from the probe is split into 4 polarized intensities (0° , 45° , 90° and 135°) which are spatially separated and projected onto the sensor of an EMCCD camera. To minimize differences in intensity due to the absorption dipole angle, the TIRF excitation beam is elliptically polarized by a quarter-wave plate (QWP) placed in the excitation path. Software combines the four images to simultaneously localize particles and quantify their individual polarized intensities for angle calculation. The θ_p and ϕ_p angles are over-determined and can be calculated by fitting the following equations to measured intensities [90, 105]:

$$\begin{aligned} I_0(\theta_p, \phi_p) &= I_{\text{tot}}(A + B\sin^2\theta_p + C\sin^2\theta_p\cos 2\phi_p) \\ I_{45}(\theta_p, \phi_p) &= I_{\text{tot}}(A + B\sin^2\theta_p + C\sin^2\theta_p\sin 2\phi_p) \\ I_{90}(\theta_p, \phi_p) &= I_{\text{tot}}(A + B\sin^2\theta_p - C\sin^2\theta_p\cos 2\phi_p) \\ I_{135}(\theta_p, \phi_p) &= I_{\text{tot}}(A + B\sin^2\theta_p - C\sin^2\theta_p\sin 2\phi_p) \end{aligned}$$

With a sufficiently bright probe, camera frame rates become the limiting factor for time resolution. As a result, EMCCD detection with frame rate >30 ms is best-suited for very bright fluorophores, such as QRs. Emission-only detection is preferred for QRs, because, as mentioned, their absorption and emission dipoles are not aligned [25]. Wobbling motions on

time scales faster than the camera exposure time (typically >30 ms) cannot be estimated. Myosin V tail domain rotations were measured using a similar system [71].

Polarized excitation and emission detection

To obtain full hemisphere resolution of probe angles, at least four polarized components such as x , y , 45° - xz and 45° - yz are needed, as explained in conjunction with figure 4(D). As described with figure 6, combinations of excitation and emission polarization are needed to obtain ns and μ s wobble. This set of excitation directions and emission polarizations is obtained using a prism-type polarized TIRF microscope we assembled (figure 7(B)). Three electrically variable retardation plates (Pockels cells) control direction and polarization of an excitation beam. Two resulting excitation beams are alternately projected onto the sample through an optical coupling prism at a glancing incident angle along the x or y axis of the sample (figure 7(B), inset). Each of these 2 alternating paths is further modulated between horizontal (s -polarization) vertical (p -polarization) and $+45^\circ$ and -45° polarizations. These 8 combinations of path and polarization illuminate the sample for 100 μ s in a repeating sequence, thereby filling out all of the Cartesian excitation directions as well as symmetry-breaking intermediate directions. Fluorescence emission is then collected by a bright water immersion objective lens on the opposite surface of the sample chamber. Using a water-immersion lens with moderate numerical aperture (NA 1.2 here) is a trade-off between limiting the scrambling of polarized emissions at high collection angles versus efficient capture of the emitted photons. Fluorescence is then projected onto an EMCCD camera or else directed through a polarizing beam splitter onto two APDs detecting the x - and y -polarized emission. Combining the temporally modulated excitation and polarized emission detection in this instrument enables estimation of the mean angle approximately every 5 ms and the fast and slow wobble of the probe, by fitting analytical expressions of intensities as functions of θ_p , ϕ_p , fast wobble δ_f , and slow wobble δ_s . Time resolution for the mean angle is typically limited by the number of photons emitted by the fluorophore. For example, approximately 0–5 photons from a rhodamine dye are accumulated by the APDs during each 100 μ s interval. This system has been used to probe organic fluorophores attached to myosin motors and ribosomal elongation factors [19–22, 47, 52, 98].

Emission pattern imaging

As mentioned above, a dipole emits light perpendicularly to its axis, giving $\sin^2(\omega_e)$ dependence on the angle between the observation axis and the probe. Thus, the image of a dipole emitter displays asymmetry related to the distribution of emission intensity (figure 2(B)), especially when defocused or with purposefully introduced aberrations. This effect enables an alternative method for detecting the orientation of the dipole by making use of the angle-dependent formation of the image pattern instead of the polarization. Complicating this analysis is the fact that in many single molecule experiments the sample is close to a dielectric interface, such as the coverslip of a microscope slide, which collects rays of fluorescence emission at high angles and refracts them into the objective. The relationship between the orientation of the dipole and its image at the camera thus depends on complex calculations of the electric and magnetic field propagation across the interface. When the dipole is in focus at the detector, it appears as small, slightly irregular shape [106]. This

pattern represents the smallest achievable spot of light given the objective numerical aperture. Small deviations from the Airy disc approximation due to the $\sin^2(\omega_c)$ emission distribution are difficult to detect. Collecting slightly out-of-focus images, or the light distribution at the back focal plane (the exit pupil) of the objective, amplifies these deviations into patterns that can be used to determine probe orientation.

Defocused imaging

A defocused image of a field of single molecules is acquired by a slight shift (e.g. ~500 nm) of the sample away from the focal plane [107, 108]. The defocused image formed from a fluorescent dipole is the z component of the Poynting vector S [107]

$$S = \frac{c}{8\pi} e_z \cdot (\mathbf{E} \times \mathbf{B}^*),$$

where $\mathbf{E}$ and $\mathbf{B}^*$ are the electric field and the complex conjugate of the magnetic field, respectively, e_z is the unit vector normal to the camera, and c is the speed of light in a vacuum. $\mathbf{E}$ and $\mathbf{B}^*$ are related to Bessel functions of the coordinates and orientation of the dipole, the position of the imaging plane, the refractive indices of the aqueous medium and the glass, and the incident and refracted angles in the optical train.

Similarly, the defocused image formed by scattering from a gold nanorod is [109]

$$S = \frac{c \sqrt{\epsilon_m}}{8\pi} e_z |E_{(\text{scat})}|^2$$

where ϵ_m is the dielectric constant of the medium and $E_{(\text{scat})}$ denotes the electric field scattered by the AuNR. The electric field is a function of the angle between the excitation electric field and the dipole axis, the polarizability of the nanorod along each principal axis, the aspect ratio of the nanorod, the bulk dielectric function of the gold, and the relative strengths of the three orthogonal oscillation dipoles.

Qualitatively, a dipole with its axis aligned perpendicular to the optical axis emits directly into the objective, whereas dipoles oriented parallel to the optical axis mainly emit light at shallow angles relative to the sample plane. The defocused images of the probe in a theoretical image library (figure 8(A)) thus resemble either sections through a doughnut with slightly dimmer regions (probe parallel to sample plane, $\theta_p = 90^\circ$), multiple circular rings (probe perpendicular to sample plane, $\theta_p = 0^\circ$), or Pac-Man shapes for intermediate θ_p angles.

A library or grid of images is calculated from the equations above and recorded data (figure 8(B)) are compared to a best-fit model (figure 8(C)) to estimate the angles. Hemisphere symmetry (as in figure 4(D)) applies for this case of image detection. Fitting algorithms and metrics, including least-squares fitting [108], use of 3-D steerable filters [110], Pearson's correlation coefficient [111], artificial neural networks [112], geometric analysis [113], and

maximum likelihood estimation [114], have all been used to identify the closest simulated match for the azimuthal and axial angles of the probe. Estimation of orientation from defocused images is computationally intensive and can be quite slow compared to other methods that do not require fitting of complex shapes [113].

Dynamic fluorophores imaged using this method are precise to within 10–15° [97], while nanorods are brighter, leading to 1–5° estimated precision [111, 113]. However, Aguet *et al* [110] has suggested that with the appropriate fitting model, fluorophore angular precision could reach 2°. Wobble of the probe is not quantified, but extreme wobbling motions will blur the patterns. Caveats of this method include difficulty in defining and maintaining the ideal amount of defocus, deviations of the images from the theoretical ones, and varying suitability of commercial objectives for forming the defocused images [7, 115].

Although emission pattern detection is well-suited for orientation imaging, localizing a fluorophore within a defocused image is less accurate than for a focused image due to spreading of the light into an extended pattern. Toprak [97] and Dägel [116] approached this problem by taking alternating focused and defocused images of their samples. Although Toprak [97] reported better than ~30 nm *x-y* resolution from the defocused image alone, Aguet *et al* [110] suggest this could be improved to ~5 nm with better image analysis. Alternately, Xiao [111] took advantage of the ~700 nm longitudinal and 540 nm transverse LSPR scattering peaks of AuNRs to improve tracking resolution (figure 8(F)). At the same specimen distance, chromatic aberrations in the objective resulted in a well-focused spot at 540 nm (figure 8(D)), used for position tracking, and a defocused orientation-dependent image at 700 nm (figure 7(E)), that could be simultaneously imaged.

Defocused imaging has been applied to a number of biological systems, including the orientation tracking of bifunctional rhodamine attached to calmodulin on myosin V [97], observation of endocytosed vesicles within living cells [111], estimation of the torque produced during the 360° rotations of F₁-ATP synthase [113], recording the orientation of carbohydrate-binding modules on cellulose microfibrils [116], determination of lipid orientation during membrane packing [36, 117] and raft formation [37].

Back focal plane imaging

The primary method for generating an image of the objective's back focal plane is the insertion of a Bertrand lens [118] after the microscope tube lens. The fluorescence image is retrieved from a focused sample, eliminating the need to account for uncertainty in the focal position. Additionally, the calculations for the image *I* formed on the back focal plane are much-simplified:

$$I(\rho, \phi_{ba}, \theta_p, \phi_p) \propto \frac{1}{\sqrt{1 - \frac{\rho^2}{f^2}}} (E_p E_p^* + E_s E_s^*)$$

where E_p , E_p^* , E_s , and E_s^* are components of the electric field, *s*- and *p*-polarized relative to the plane containing the incident and refracted rays, and their complex conjugates, ρ is the

radial distance from the optical axis at the back-aperture plane, ϕ_{ba} is the azimuthal angle in the back-aperture, (θ_p, ϕ_p) is the polar angle of the probe, and f is the focal length.

Qualitatively, the back focal plane emission pattern contains 1 or 2 dark spots corresponding to the non-emitting distal axes of the probe, and a bright outer circle that corresponds to fluorescent emission acquired beyond a critical angle at the coverslip [118].

Emission pattern detection is reportedly less sensitive to optical aberrations than defocused imaging, and has 1–2° accuracy in angular determination. However, the method is incompatible with wide-field microscopy, as multiple fluorophores in the sample field are not resolved and result in bright, homogenous rings. Whole fields of molecules can be only be acquired by raster-scanning across the sample stage using a confocal-type microscope with a pin-hole [119].

Differential interference contrast (DIC) microscopy

Re-emission by the LSPR of AuNRs produces a strong orientation-dependent signal which can be used in differential interference contrast (DIC), also termed Nomarski, microscopy, to determine the orientation of the probe. In DIC microscopy, the sample is illuminated with two orthogonally polarized beams offset by 100–200 nm along a ‘shear axis’. A bias is introduced so that the two beams are also out of phase, nominally by 90° (figure 9(A), left of sample). Shear distance and phase delay are introduced by a precisely aligned Nomarski prism (figure 9(B)) or de Sénarmont compensator. When the beams encounter the sample, differences in refractive index cause a relative change in the phase between the two offset beams. Beyond the sample, the shear and polarization differences are reversed by a second Nomarski prism, and the two beams optically interfere with each other.

Two rays that both traverse an identically-refracting region (figure 9(A), top red and blue rays), and thus retain their initial phase bias, are then circularly polarized when recombined. Regions where one ray encounters a different refractive index than the other introduce different phase shifts in the two rays (figure 9(B), middle and bottom rays). Thus, they become elliptically polarized when recombined. A final linear polarizer selects the component of light polarized at 0° which may have lighter or darker intensity than the grey, circularly polarized background (figure 9(A), right), depending on which edge of the object that part of the beam experienced. A sphere smaller than the diffraction limit produces an image with a bright lobe (generated by figure 9(A), center rays) and a dark lobe (figure 9(A), bottom rays) offset along the shear axis (figure 9(B), DIC image).

For AuNRs illuminated near either the transverse or longitudinal LSPR wavelength, the indices of refraction along the two axes are unequal, thereby providing an orientation dependent signal. The phase delay, and thus the DIC contrast at the longitudinal LSPR wavelength, is higher for the beam that is polarized parallel to the long axis. The contrast of either the light image lobe ($I/I_{\max}$) or the dark lobe ($\Delta I'/\Delta I'_{\max}$) depends on the rod angle in the sample plane (ϕ_p).

$$\frac{\Delta I}{\Delta I_{\max}} \approx \frac{\sin^4 \phi_p \sin \delta'}{\sin \delta} \text{ and } \frac{\Delta I'}{\Delta I'_{\max}} \approx \frac{\cos^4 \phi_p \sin \delta'}{\sin \delta},$$

Where δ' and δ are functions of the polar angle θ_p [120]. The azimuthal angle θ_p can be estimated by comparing the contrast between the bright and dark lobes. A rod oriented in the sample plane produces the most image contrast. As θ_p decreases toward the optical axis, the contrast decreases toward zero. A limitation of this technique is that the interference patterns become very complex for $\theta_p < 45^\circ$ [121], limiting the precision of determining θ_p .

As in defocused fluorescence imaging, the contrast image produced in DIC microscopy is not ideal for localizing position at high resolution because the image with bright and dark lobes is more difficult to fit than a centro-symmetric Gaussian or Airy point spread function (PSF). For better position tracking accuracy, DIC can be combined with bright-field imaging [89]. Here, the sample is illuminated at both the longitudinal and transverse plasmon wavelengths. The longitudinal wavelength is used for DIC and the transverse is used for bright-field imaging. Orienting the final linear polarizer in the transverse LSPR channel at $+45^\circ$ or -45° gives a single spot that can be fitted by a Gaussian peak to determine the position. DIC of AuNRs produces high contrast, so it is useful for orientational imaging inside live cells, such as motions of neuronal endocytic cargo moving along fluorescently labeled microtubules [122].

Applications to 3D microscopy

Optical section imaging

Confocal fluorescence microscopy creates ‘optical sections’ of biological samples [123] spatially resolved within ~ 200 nm in the x - and y -directions and ~ 500 nm in z . It has recently been modified by several groups to add in angular measurements, usually by alternating the polarization of the excitation. Laboratories have employed this method both for high-resolution ensemble studies of anisotropy and for single molecule orientation detection, depending on the extent of fluorescent labeling. This technique has been used to map the 3D orientation of membrane-bound di-8-ANEPPQ, to measure the fluidity of membranes under different biological conditions [124, 125], and to detect conformational changes in fluorescently-tagged membrane proteins [126]. Extracting angular information from the fluorescent emission in confocal microscopes is somewhat more challenging than in a wide-field microscope because central and peripheral rays have different relationships to θ and ϕ due to the confocal pinhole aperture. Measurements of the angles are distorted if these portions of the emission are not separated. Börner *et al* [127] separated the central rays into x - and y - components and detected those along with the peripheral rays using 3 APDs. This method yields θ_p and ϕ_p angles resolved within $1/8$ of the sphere [127, 128].

Two-photon microscopy [129, 130] also provides 3D capabilities, with improved depth and signal-to-background ratio over standard confocal microscopy. It has been used to measure GFP-actin anisotropy to distinguish between the monomeric pool (low actin-actin homo-

FRET, high anisotropy) and filamentous actin (high FRET, low anisotropy) [131], and to study membrane ruffling signaled by DiO and DiI anisotropy [132].

Selective plane imaging illumination microscopy (SPIM) enables high resolution imaging deep within samples by illuminating a thin slice of the sample using a light sheet from the side [133, 134]. Hedde *et al* [135] modified SPIM by adding polarized emission detection to visualize the position and orientation of EGFP-paxillin in live cells. The compaction of paxillins during formation of focal adhesions led to homo-FRET between chromophores, causing local decreases in anisotropy at the membrane.

Multifocus microscopy (MFM) simultaneously images up to 25 planes within a sample using a multifocus diffraction grating at a Fourier plane conjugate to the objective back focal plane and a chromatic correction grating and prism to form the optical sections [136]. A modification that alternates between several elliptically polarized excitations has been implemented to image a variety of biochemical processes, including reorientation of the GFP-septin ring during yeast budding, the orientation of the *E. coli* membrane, and the birefringence of the phytoplankton *P. angulatum* [137].

Probe orientation in super-resolution microscopy

Super-resolution techniques such as PALM, STORM and STED have dramatically broken the classical 200 nm resolution limit in optical microscopy by localizing fluorophores at 10–20 nm precision [10–12]. In PALM- or STORM-type experiments, probes that operate as photo-switches are converted to a dark state, then stochastically excited. Molecules are individually localized in three dimensions by fitting a PSF to the emission pattern, assuming either an isotropic point emitter, often approximated using the 2D Gaussian shape, or a fixed dipole emission source [138]. A structural map is built from many of these localizations.

The dipole characteristics of fluorophores have been very useful in single-molecule studies, as discussed so far. However, in the super-resolution microscopy field, those same characteristics can lead to concerns rather than benefits. As discussed regarding defocused imaging, the $\sin^2(\omega_e)$ distribution of emission intensity (figure 3(B)) produces asymmetric image patterns that are functions of the orientation of the fluorophore and its *z*-location relative to the focal plane. The resulting image pattern at the detector is neither Gaussian nor a simple analytical function to fit. When the probe is in focus, this deviation from an idealized Gaussian pattern is still minimal, and the *x-y* position of the probe can be readily determined. When the probe is out of focus, however, the dipole emission pattern is stretched asymmetrically, and deviations from a Gaussian shape become much more severe. The centroid of the image is displaced from its correct location at the detector by an amount that depends strongly on the *z*-axis position, with much higher displacements in PALM and STORM methods than for STED techniques [139]. Use of a 2D Gaussian function to fit the detected spot can thus result in artifacts in *x*- and *y*-positioning greater than 100 nm. For experiments attempting 10–20 nm localization accuracy, this deviation is unacceptable. If the fluorophore is freely rotating during the analysis period, the localization problem is minimized, as the dipole emission is then isotropic [140, 141]. Thus, labeling methods for super-resolution experiments when orientation is not of interest should ideally use fluorophores that wobble rapidly over a large angle. Within the accessible labeling strategies

for a given structure, though, this may not be achievable. In such cases, two options have been proposed: measure the probe orientation and use that information to correct the inaccuracies in x -, y -, and z - localization, or remove the orientation-induced distortion through optical processing in the microscope.

Several approaches to calculating single molecule orientation and position have been proposed. In the simplest case, polarized emission is split into x - and y - components and used to calculate the in-plane fluorophore angle. This technique was used in conjunction with PALM to measure the orientation of the photoswitchable probe Dendra2 attached to actin and hemagglutinin inside of cells [31]. As discussed earlier, symmetry-induced ambiguities can be decreased by adding multiple polarized excitations, and detection of polarized annular (marginal ray) or radially polarized components of the emitted light. Objective-rim ray collection was applied to measure both the orientation and slow wobble of actin bound to labeled phalloidin using STORM [34]. Dedecker *et al* [142] modeled the angular effects of excitation and stimulated depletion of fluorophores in a STED microscope, yielding an image library that could be used to calculate the polar angles of the probes. The orientation and depth information in these super-resolution approaches could then be applied to optimally determine the correct x - and y - positions using maximum likelihood estimation [114, 143].

The orientation and corrected 3D localization of a single fluorophore can be simultaneously determined using sophisticated PSFs, generated by optical Fourier processing in the microscope. Inserting a spatial light modulator (SLM) into the emission fluorescence path at a plane conjugate to the objective's back focal plane implements a phase mask to the image, resulting in filtering of the Fourier transform. The SLM is an electrically addressable array of pixels with independently adjustable phase retardation [144].

A 'quadrated pupil' phase mask splits the x - and y - polarized components of emission at the exit pupil into four spots [145]. For each fluorescent molecule, four spots arranged in an X appear in the two polarized channels in the camera image, giving a total of eight spots associated with each molecule. The 3D orientation of the probe, with quadrant ambiguity, can be inferred from the relative intensities of these eight spots, and the separation between the spots (i.e. the size of the X s) is related to the z -position. A simplification of the quadrated pupil technique, employing a 'bisected pupil' phase mask in the SLM, splits the polarized images into two pairs of spots. This mask provides improved spatial localization over the quadrated pupil at the expense of angular precision. It has been used to observe the 3D positions of microtubules in fixed cells [40].

With a more complex phase mask, a 3D PSF forms a double helix aligned with the optical axis of the microscope. Each probe appears at the camera as two spots offset from a central position at angles related to the z -position. The probe orientation is determined from their relative intensities. In successive images, the double-helical phase mask is rotated by 90° , leading to quarter-sphere angular resolution and x - y - z localization [146].

Finally, a metamaterial nanofabricated phase mask, termed y -phi, very effectively corrects for orientation-based localization errors by rejecting radially polarized components of the

image Fourier transform, which normally lead to the mis-localization [147]. A proof-of-concept of this method with a biological sample was supplied using a fluorogenic peptide cast onto a slide and shifted in focus. The mis-localization error due to out-of-plane orientation was reduced markedly using the γ -phi phase mask, and allowing fast calculation of the in-plane ϕ_p angle with a 180° range.

Conclusions

We have described a number of methods for determining the orientation of biological molecules through the attachment of anisotropic fluorescent or scattering probes. Labeling can be achieved with either fluorescent proteins, organic dyes, or nanoparticles, and each one conveys various advantages and disadvantages. Fluorescent proteins can be genetically encoded, but bleach quickly and may not be rigidly coupled to the target protein. Organic dyes can be specifically and rigidly attached to proteins or nucleic acids, but not all proteins can be modified with the necessary target sequences. Metal nanoparticles provide an alternative to fluorophores. They are brighter and photo-stable but larger and thus may not be suitable for some biological applications.

Many fluorescence and scattering imaging methods are available, tailored to probe types and experimental requirements. Polarized TIRF or TIRS techniques combine excitation and/or emission intensities to determine orientations. Defocused imaging compares the recorded image shapes to simulated sets of images at sets of calculated angles. And interference microscopy takes advantage of different indices of refraction along the longitudinal and transverse axes of polarized nanoparticles to measure orientation. The accessible time and angular resolution and the spherical ambiguity depend on the selected method. Probe angles affect precision in super-resolution microscopy, and methods to measure or remove orientation-dependent artifacts have emerged. Brighter fluorescent probes, resistance to photobleaching, faster switchable probes and smaller nanorods would advance the field. With new microscopy and nanoparticle functionalization methods continuously being developed, this field will continue to grow and find applications in more biological systems both *in vitro* and *in vivo*.

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Figure 1. Rotational motions of domains around flexible hinges are common in macromolecules. (A) Topoisomerase II DNA binding and cleavage core from *Saccharomyces cerevisiae*. In addition to conformational changes in the DNA-opening platform, a dramatic reorganization of accessory domains may occur during catalysis. O, M and C correspond to open, intermediate and closed conformations during DNA cleavage and passage through a gap formed in another strand. (B) Elongation Factor EF-Tu from *Thermus aquaticus* in the GTP and GDP forms. The GTP binding domain rotates relative to the tRNA binding cleft, opening the tRNA groove markedly to release it. (C) F₁-ATP synthase, showing the shaft and δ subunit (yellow) which rotates to phosphorylate ADP and produce ATP in the α and β subunits, cutaway blue and cyan. Space-filling atoms are shown for ADP and SO_4^- bound on $\beta 1$. From [148] with permission.

Figure 2. Relative probabilities for fluorophore absorption (panel (A), $\cos^2(\theta_{ax})$ and $\cos^2(\theta_{ay})$) and collection of its emission (panel (B), $\sin^2(\omega_{ex})$ and $\sin^2(\omega_{ey})$). In the microscope coordinate frame (x, y, z), the optical axis is z. For axial illumination (heavy arrow in panel (A)), ϵ_x and ϵ_y are excitation polarizations. The probe absorption and emission dipole moments (considered to be parallel) in the (x, y, z) frame are defined by θ_p (axial angle) and ϕ_p (azimuth, the angle between the projection of the dipole onto the x-y plane (grey triangle) and the positive x axis). θ_{ax} and θ_{ay} are angles between the probe dipole and excitation polarizations along the x- and y-axes. ω_{ex} and ω_{ey} are angles between the probe dipole and detector optical paths in the y- and z-axes. Adapted from [15] with permission.

Figure 3.

(A) Fluorescence time trace of a single Texas red molecule (solid line) as the polarization angle of the incident light was varied (dotted line). The inset shows the averaged signal of the first four periods (filled triangles), and a fit to $a \cos^2(x - b) + c$ (solid gray line). The in-plane orientation of the molecule was determined to within $<1^\circ$ in this experiment. From [13] with permission. (B) Recording of polarized fluorescence intensities from a translocating myosin V molecule, labeled with bifunctional rhodamine (BR) on a calmodulin subunit in the motor lever arm, using the experimental setup shown in figure 7(B). The colored intensity traces are for various combinations of optical path and polarization as described later. Prefixes indicate s , p , L and R polarizations and path 1 or 2 (figure 7(B)). Suffixes indicate the x - or y -polarized emission. The total fluorescence intensity (top black trace) is relatively constant as the other intensities vary, showing that the probe molecule is tilting abruptly as the motor protein steps along actin. (C) Calculated probe angles of bifunctional rhodamine (β_P and α_P) and lever arm (β_L and α_L) axial and azimuthal angles relative to the axis of the actin filament. Probe angles return to their initial values after two steps, indicating that the molecule is walking quite straight along actin. That fact enables the local angle of the probe relative to the calmodulin subunit to be calculated and thereafter the lever arm angles β_L and α_L , to be determined. β_L alternates each step between the leading and trailing positions in the hand-over-hand mechanism of walking. α_L

is constant, indicating that the molecule is not spiraling around the actin filament appreciably during the 8 steps observed. Adapted from [57] with permission.

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Figure 4.

Symmetries and ambiguity of angular detection with various combinations of polarized excitation or emission. If the probe absorption and emission dipoles are parallel, then either can be quantified to resolve probe angles. (A i) In-plane quadrant ambiguity. Upper: the magenta dipole transition is excited or detected using x - (red) and y -polarized (cyan) light. (A ii) Fluorophores reflected across the axes into the other three quadrants would give the same intensities, I_x and I_y , and therefore these intensities could come from any of the 4 angles shown. (B) Eighth-sphere ambiguity. (B i) Adding z -polarized excitation or detection provides an elevation angle, θ_p , but does not resolve the quadrant ambiguity. (B ii) A probe in any of the 8 quadrants (thin magenta vectors) would give the same intensities I_x , I_y , and I_z . (C) Quarter-sphere ambiguity (C ii) x -, y -, and 45° (brown, I_{45xy})-polarizations in the x - y plane halves the 8-fold ambiguity. An elevation angle also emerges from the contrast among the three intensities, leaving 4 possible angles for any one measurement. (D) Hemisphere ambiguity, adding 45° absorption or emission in the y - z plane (teal, I_{45yz}), or in the x - z plane, to I_x , I_y , and I_{45xy} reduces the symmetry-related ambiguity of angular detection to the minimum of two oppositely directed angles.

Figure 5.

Choice of hemisphere in polarized fluorescence experiments. Top row: a hypothetical molecule experiences two states (1 and 2) with some angular variation or uncertainty in each state (top row, green arrows). The molecules occupy three quadrants in this example. The probe at each individual angle is indistinguishable from an oppositely directed vector (thin red arrows). Bottom row: In order to contain the same end of the probe vectors in states 1 and 2, a hemisphere must be chosen that contains all of them. Choice of any hemisphere with equator parallel to the coordinate axes, here either the $+Y$ or $+X$ hemisphere, results in reporting the wrong end of one of the probe vectors (red dashed arrows), possibly erroneously increasing the angular transition reported between states 1 and 2. A tilted hemisphere chosen based on outside knowledge, in this example to minimize the angular range, contains all of the correct probe vectors. Other ways of choosing the appropriate hemisphere are mentioned in the text.

Figure 6.

Effect of fluorophore rotational mobility on polarized emission fluorescence ratios: (A) when an ensemble of static probes with random orientations is excited at vertical and horizontal polarizations and the fluorescence is detected through vertical and horizontal analyzers, photoselection causes unequal emission polarization ratios (P_V and P_H); (B) for a single static molecule, once it is excited, the orientation of the illumination is irrelevant to its emission, and emission polarization ratios are equal ($P_V = P_H$); (C) if the single molecule wobbles with rotational correlation time, τ_r , much greater than the fluorescence lifetime, τ_f , but much smaller than the measurement time, τ_c , then $P_V \neq P_H$, as in the ensemble case; (D) if the probe motion is much faster, $\tau_r \ll \tau_f$, then P_V and P_H are equal but smaller than in panel (B). These qualitative effects enable fitting of an analytical expression to polarized fluorescence intensities to estimate the amplitude of μs and ns wobbling motions. From [15] with permission.

Figure 7.

Optical arrangements for polTIRF microscopy. (A) Schematic of an objective-type polarized TIRF microscope. This setup enables tracking the position of a fluorescent probe at super-resolution and uses four polarized emission intensities to over-determine the 3D orientation of a fluorescent probe within an unambiguous quarter sphere (as in figure 4(C)). 532 nm laser illumination (green rays) passes through a quarter-wave plate (QWP) to produce circularly polarized light projected onto the sample (SAM) at a glancing angle via a high numerical aperture objective (OBJ), producing an evanescent wave by total internal reflection. The emission from fluorescent probes (red rays) is directed toward the detection optics by a dichroic mirror (DC). Fluorescence is separated into four polarized intensities components (0° , 45° , 90° and 135°) by an unpolarized 50:50 pellicle beamsplitter (BS), a polarizing beamsplitting cube (PBS) and a wire grid polarizer (WGP) with its wire array set at 45° to the horizontal. Each light path is projected, spatially separated, onto an EMCCD camera detector. A slit narrows the image field so that the four polarized component images fit onto a single detector chip. (B) Schematic of a prism-type polarized TIRF microscope. A 532 nm laser beam is passed through a Pockels cell (PC0) and split by a polarizing beam splitting cube (PBS0) into two paths. Each of these beams pass through a 2nd Pockels cell (PC1 and PC2) and a Berek compensator (BC1 and BC2), and is then directed by mirrors into a coupling prism at a glancing angle for TIRF illumination. Path 1 is in the plane of the drawing; path 2 is in a vertical plane perpendicular to the page. Rapidly cycling the high voltages on the Pockels cells results in four linear input laser polarizations per beam oriented at 0° , $\pm 45^\circ$, and 90° for p , L/R , and s polarizations (inset) giving 8 combinations of path and polarization. Fluorescence emission from single fluorophores is collected by a water immersion objective (OBJ), passed through a long pass blocking filter (BF) and directed onto an EMCCD camera. A fluorescent molecule is selected from the camera images and centered in the image plane by a piezoelectric stage (not shown), and a removable mirror (RM) is positioned to direct the polarized fluorescence emission through a beam splitting cube (PBS1) and onto two photon counting avalanche photodiodes (APDs). This leads to 16 unique combinations of the eight time-multiplexed input paths and polarizations and two simultaneously detected polarized emission intensities. By modulating the polarization of the illumination while detecting at two orthogonal polarizations, 3D orientation resolved to a hemisphere (figure 4(D)) plus probe wobble longer than the fluorescence lifetime (figure 6) are determined. Modified from [98], and [57] with permission.

Figure 8. Defocused imaging of gold nanorods combined with super-resolution tracking. (A) Simulated image library of the plasmonic scattering of AuNRs with an aspect ratio of 2.5. Modified from [109] with permission. (B) and (E) Defocused dark-field image of gold nanorods at 700 nm. (C) Simulated image corresponding to the best-fit solution for the image in (B). (D) Focused image of particle in (E) at the transverse plasmonic wavelength, 540 nm, used for super-resolution positional tracking. (F) Microscope configuration for two-wavelength imaging. Light scattered from a gold nanorod is divided into two beams and imaged at 540 and 700 nm onto a CCD camera. Chromatic aberrations inherent in the microscope objective cause defocusing of the 700 nm image. Modified from [111] with permission.

Figure 9.

Differential interference contrast microscopy for determining orientation of plasmonic nanorods. A. Phase bias is introduced into orthogonally polarized ($\pm 45^\circ$), sheared illumination beams (red and blue). After passing through the sample, the relative phase of the beams changes in areas of graded refractive index, such as the edges of a nanoparticle. The polarized beams are recombined by reversing the shear distance, and the recombined beams interfere to produce circularly or elliptically polarized light dependent on their phases. A final linear polarizer selects horizontally polarized light, resulting in different intensities when interfering rays pass through a nanoparticle. (B) Bottom: illumination light at the plasmonic wavelength passes through a Nomarski prism (NP1) to divide the beam into two components that are offset by ~ 100 nm (the shear distance) and orthogonally polarized at 45° (blue) and -45° (red) relative to the microscope coordinate frame. The offset beams scatter off the sample (SAM), causing differential phase shifts. A second Nomarski prism (NP2) recombines the two polarized beams and reverses the shear. Detecting at this point in the beam path would produce a brightfield image appearing as two offset images of the object or point spread function (upper brightfield oval). Passing the beam through a 0° polarizer (LP) results in an image containing bright and dark lobes (DIC Images) which depend on the shape and orientation of the particle. A spherical particle produces equivalent phase shifts in both beams (beam phase), resulting in light and dark lobes of equal intensity in the DIC image. Rod-shaped particles produce a larger phase shift in light polarized parallel to the plasmonic resonance (PR) axis (e.g. longitudinal polarization at 700 nm) than polarized perpendicular to the PR axis. Thus the contrast of the bright and dark lobes in the DIC image are functions of the particle orientation.