Fluorescence anisotropy: from single molecules to live cells

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The polarization of light emitted by fluorescent probes is an easily accessible physical quantity that is related to a multitude of molecular parameters including conformation, orientation, size and the nanoscale environment conditions, such as dynamic viscosity and temperature. In analytical biochemistry and analytical chemistry applied to biological problems, fluorescence anisotropy is widely used for measuring the folding state of proteins and nucleic acids, and the affinity constant of ligands through titration experiments. The emphasis of this review is on new multi-parameter single-molecule detection schemes and their bioanalytical applications, and on the use of ensemble polarization assays to study binding and conformational dynamics of proteins and aptamers and for high-throughput discovery of small-molecule drugs.

Introduction

Optical microscopy and spectroscopy techniques have greatly expanded in the biological and medical sciences because they are non-invasive and relatively straightforward to implement for imaging of live cells and tissues. A wide range of fluorescent proteins (FPs) with superior fluorescence properties designed through mutations of the popular green-fluorescent protein can now be expressed in cells and in living organisms.[1] In addition, new organic dyes[2,3] and semiconductor quantum dots[4,5] with enhanced brightness and photostability provide, through chemical labelling, a way to tag proteins and other biomolecules with virtually any colour desired in the visible spectrum. Under optical excitation, the signal collected from a biological sample is most commonly fluorescence, although more advanced, label-free applications use nonlinear optical signals such as second-, third-harmonic or sum-frequency generation and coherent anti-Stokes Raman signals as imaging modalities.

Fluorescence is a relatively simple physical process that involves the relaxation of a molecule from its lowest singlet excited state to the ground state *via* photon emission (Fig. 1). The fluorescence emission is characterized by several parameters: intensity, wavelength, lifetime and polarization, all of which can sharply vary through electromagnetic interactions with the local environment. The intensity of fluorescence is the most widely used contrast mechanism for imaging applications because the emission rate is directly proportional to the local concentration

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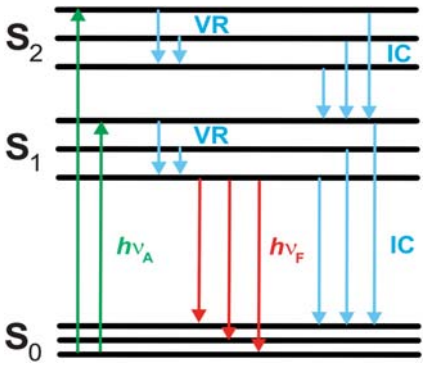

Fig. 1 A simplified energy level diagram of a molecule. The molecule is excited from the ground electronic state S_0 by absorption of a photon $h\nu_A$. It then rapidly relaxes by means of internal conversion (IC) and vibrational relaxation (VR) to the lowest vibrational level of the first electronic excited state S_1, from which it relaxes to the vibronic levels of the ground state by IC or by emitting a photon $h\nu_F$ (fluorescence).

of emitters. A spectrally sensitive detection is an elegant method to discriminate amongst several emitting species with overlapping contributions. This is particularly helpful against the background of intrinsic cell fluorescence and it enables multiplex imaging using the ever-growing palette of colours offered by modern fluorophores. FLIM (fluorescence lifetime imaging) uses spatial variations of the lifetime as the imaging contrast mechanism. The technique is largely insensitive to the concentration of probes but very susceptible to changes in their environment, including viscosity, pH, polarity and quenching *via* Förster resonance energy transfer (FRET). The polarization anisotropy can yield information about the properties of the sample that cannot be inferred from intensity, lifetime or emission spectrum data. These include molecular orientation, aggregation, rotational diffusion and energy migration among chemically identical molecules (homo-FRET). Polarization-sensitive detection is also important for estimating nanoscale directionality in FRET experiments, as orientation of the molecular dipoles involved in energy transfer enters the Förster radius calculation.

Upon exposure to linearly polarized light, the chance that a molecule will be excited is maximal when its absorption transition dipole moment is aligned parallel to the electric field vector of the excitation beam. The absorption and emission dipole are in most cases collinear, so the fluorescence will be polarized along the same direction unless the molecule rotates before relaxation or the excitation energy is transferred to a different molecule. The fluorescence anisotropy (FA) is defined in terms of the parallel and the orthogonal components of the emission intensity:[6]

$$r(t) = \frac{I_{\parallel}(t) - GI_{\perp}(t)}{I_{\parallel}(t) + 2GI_{\perp}(t)} \quad (1)$$

where G is a sensitivity correction factor for the two detection modes. This quantity is frequently measured in a fluorimeter as the time-averaged or the steady-state anisotropy $\bar{r}$. The conventional eqn (1) is only applicable for fluorimeters, while for polarized measurements made with high numerical aperture (NA) objective some corrections must be applied. The light cone formed by the microscope objective lens has a large solid angle and refraction by the lens changes the direction of polarization. Therefore, a molecule will be excited not only by original x- (parallel) but also by y- and z- (perpendicular) polarized light. The corrected FA in a microscope setup is defined as:

$$r(t) = \frac{D_x(t) - D_y(t)}{(1 - 3k_2)D_x(t) + (2 - 3k_1)D_y(t)} \quad (2)$$

where details about eqn (2) can be found in the work of Koshioka *et al.*[7] The use of a standard molecule, with known fluorescence and rotational parameters, is important for calibration of the system, especially for determining the depolarization due to the objective. Indeed, the high NA objective leads to decrease of the initial (limiting) anisotropy[8] without, however, modifying the FA decay.[9]

If the parallel and the orthogonal polarization components are resolved on the (sub)nanosecond scale, a time-resolved

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anisotropy curve $r(t)$ can be constructed. For a freely rotating fluorophore, FA decays exponentially:[10]

$$r(t) = r_0\exp(-t/\varphi) + r_\infty \quad (3)$$

where the time-zero (limiting) value r_0 is 0.4, φ is the rotational diffusion (or correlation) time and a non-zero residual value r_∞ is caused by sterical hindrance. For the common case of a dye attached to a larger, freely rotating particle FA decays as:[6]

$$r(t) = r_0[\alpha\exp(-t/\varphi_1) + (1 - \alpha)]\cdot\exp(-t/\varphi_2) \quad (4)$$

where φ_1 is the timescale of the locally hindered rotation of the dye, and φ_2 is the timescale of the free rotation of the large particle. The anisotropy at any time t depends on the extent of depolarization due to the internal motion of the dye with amplitude $r_0\alpha$ and the extent of depolarization due to overall rotation of the large particle with amplitude $r_0(1 - \alpha)$.

The Stokes–Einstein equation:

$$\varphi = \frac{\eta V_h}{k_B T} \quad (5)$$

relates the rotational diffusion time to molecular size (hydrodynamic volume V_h), viscosity η and temperature T of the surrounding medium. This dependency forms the foundation of using FA decay to measure the size of macromolecules and the spatio-temporal gradients of viscosity in lipid membranes or live cells. However, time-resolved anisotropy measurements are relatively difficult to implement due to the requirement for more sophisticated instrumentation and data analysis routines.[11,12] Another limitation is that the rotation of large molecular aggregates can be orders of magnitude slower than the excited-state relaxation of the probe. In these cases, the rotational diffusion time φ can be estimated either directly by fluorescence correlation experiments[13] or indirectly from steady-state FA and the fluorescence lifetime τ using Perrin's equation:

$$\frac{1}{\bar{r}} = \frac{1}{r_0}\left(1 + \frac{\tau}{\varphi}\right) \quad (6)$$

Two-photon absorption (2PA) involves the concerted interaction of two infrared photons that combine their energies to produce an electronic excitation analogous to that conventionally caused by a single visible photon of a corresponding wavelength. Unlike one-photon absorption, whose probability is linearly proportional to the incident intensity, the 2PA process depends on both spatial and temporal overlap of the incident photons and takes on a quadratic (nonlinear) dependence on the incident intensity. This results in highly localized photoexcitation within a focused beam and a value of 0.57 for the limiting anisotropy r_0.[14] For theoretical and experimental details as well as the benefits and applications of 2PA-FA we recommend several recent studies.[3,15,16]

In either static or dynamic, single-point or imaging mode, polarization-sensitive detection of fluorescence is a sensitive and versatile tool for the molecular scientist. The key outcome is the derivation of information about the orientation and conformation of macromolecular structures as they evolve during simple binding reactions and more complex biological events. In this review we discuss recent results derived by fluorescence polarization techniques, with emphasis on live-cell imaging and single-molecule detection, as well as bioanalytical applications involving aptamers, membrane protein complexes and small-molecule synthetic drugs.

Single-molecule applications

Several groups have implemented polarization-sensitive detection of fluorescence on confocal, wide-field or total internal reflection fluorescence (TIRF) microscopes either to provide additional imaging contrast or for single-molecule spectroscopy experiments.[17–20] To illustrate the basics of instrument design, we describe a setup that was custom-built in our laboratory.[21] This is a multi-modal scanning confocal microscope capable of detecting simultaneously several fluorescence parameters, including lifetime and anisotropy, from individual fluorophores (Fig. 2). In brief, a pulsed, wavelength-tunable, linearly polarized laser beam excites tethered or freely diffusing fluorophores through a high NA objective, which is also used for collecting emitted light. The emission is spatially and spectrally filtered from out-of-focus fluorescence and scattering, and then is divided by a cube beamsplitter into two components with parallel and orthogonal polarization to that of the excitation laser. Each beam is focused onto an avalanche photodiode (APD) that features low dark noise, high photon detection efficiency (PDE) and ultrafast timing resolution. Each time the APD detects a photon it outputs a square pulse that is registered by a time-correlated counting module (PicoHarp300, PicoQuant GmbH). The multi-tagged photon data are analyzed using statistical methods to extract parameters such as brightness, lifetime and anisotropy and correlations between their fluctuations.

Single-molecule experiments uniquely address the heterogeneity of structure and kinetics that are characteristic of biological systems by avoiding ensemble and temporal averaging. Moreover, this type of investigation affords a straightforward assessment of theoretical models, most of which are developed within a single-molecule framework. The strength of multi-parameter single-molecule spectroscopy, including anisotropy measurements, is illustrated by several recent studies.[12,18,21–23]

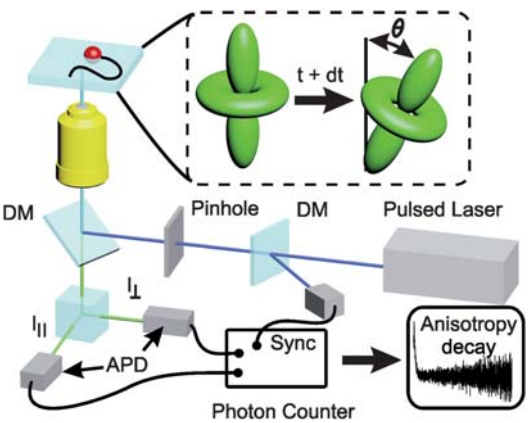

Fig. 2 Time-resolved fluorescence polarization microscopy on a home-built confocal setup. DM = dichroic mirror, APD = avalanche photodiode, Photon Counter = time-correlated single photon counting module (PicoHarp300, PicoQuant GmbH).

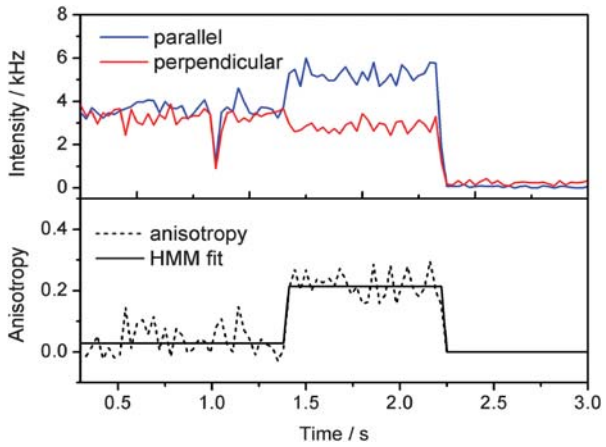

Fig. 3 Fluorescence intensity time-trajectories (parallel and perpendicular components) and the corresponding anisotropy trace constructed using eqn (1) for an individual single-stranded DNA labelled with a TAMRA (tetramethylrhodamine) dye and encapsulated in a 100 nm-diameter liposome. HMM = Hidden Markov Model.

An example of single-molecule anisotropy measured on our setup is shown in Fig. 3. The data were recorded from an oligonucleotide chain labelled with a rhodamine dye and encapsulated in a lipid vesicle with a diameter of *ca.* 100 nm. The liposomes were tethered to the glass surface by biotin–streptavidin interaction and continuously exposed to laser excitation. The advantage of time-resolved photon counting detection is that any time bin value can be chosen when constructing the intensity/anisotropy trajectory. The data are shot-noise limited and therefore a time bin of 30 ms was selected for display purposes only. The minimum binning time is around 1 ms and is determined by the experimental signal-to-background ratio. In the data presented in Fig. 3, the signal is initially quasi-isotropic ($r = 0.028$), followed by a jump to a state of relatively high anisotropy, $r = 0.214$, before irreversible photobleaching occurs. Our studies suggest that this is due to non-specific interaction of the DNA with the lipid walls, which restricts the movement of the dye and is difficult to track and assign using intensity or lifetime variations.

A single-molecule approach was used to determine the orientation and the mobility of the two identical motor domains of the kinesin while moving along microtubules.[24] Kinesin is a processive motor that can walk for more than a micrometer, going through hundreds of ATP cycles without detaching from the microtubule. The bifunctional probe bis-((*N*-iodoacetyl)piperazinyl)sulfonerhodamine (BSR) was rigidly attached to one of the two kinesin heads so that the BSR transition dipole would align nearly parallel to the microtubule axis when the head was in the bound state. The laser light linear polarization was modulated in 4 different directions (0°, 45°, 95° and 135°) in sync with image acquisition so that order parameters and projected angles for single fluorophores were calculated with a resolution of 100 ms. The time traces obtained show periods of high order and zero angle alternating with dwells of low order and variable angles. A model is proposed for kinesin translocation that reconciles many experimental results, according to which during the ATP-waiting state one kinesin head is strongly bound to the microtubule, while the second head is unbound or weakly bound and shows high mobility.

Recent advances in single-molecule FRET measurements were aimed at deriving full 3D structures of DNA constructs based on absolute distances. In FRET the dyes are attached to fluorophores *via* flexible linkers and orientational freedom is usually assumed when calculating the Förster radius. However, this spatial freedom prevents a conversion of the data to a fixed geometric model. The multi-parameter fluorescence detection used by Seidel and co-workers makes use of correlations between lifetime, anisotropy and intensity to avoid the common pitfalls of calculating absolute distances from FRET efficiencies.[19,25] Two-dimensional histograms of correlated lifetime and FA values revealed slow positional and fast orientational fluorophore dynamics that result in an isotropic averaging of FRET efficiency. Coupled with molecular dynamics simulations this information can be used to determine the correct distance between the average donor and acceptor positions. Deviations from B-DNA helicity were observed in the distance dependence of the corrected FRET efficiency and were assigned to a kink in the DNA structure with an angle of 16°. Such bending, prominent around a distance of 15–20 bp, is sequence dependent and may play a biological role in regulating the assembly and function of DNA–protein complexes.

FA is uniquely sensitive to the presence of energy transfer between chemically identical fluorophores (homo-FRET). This often occurs inside the cell and in lipid membranes due to either high expression levels or receptor aggregation, or in large multimeric protein complexes, such as proteases or folding chaperones, that are assembled from several identical copies of the same structural unit. Steinmeyer and Harms describe a wide-field imaging setup in which the collected fluorescence light is split for polarization by a Wollaston prism and for colour by a dichroic wedge, which directs the light to a high-sensitivity CCD camera.[26] The authors use this microscope design to better determine protein–protein interactions in the pleckstrin homology domain and the conformational changes in the Parathyroid Hormone Receptor, a G-protein-coupled receptor, both fused to FPs for either inter- or intra-molecular FRET.

A similar instrument was used earlier to determine the rotational diffusion of single fluorophores embedded in different lipid phases.[20] In a lipid fluid phase a rotational diffusion constant D_{rot} on the order of 10^7 rad^2/s was estimated from measurements. In an L_β gel phase the rotation was several orders of magnitude slower, on the order of 1 rad^2/s. In the latter case, angular trajectories of individual molecules were visualized directly on the millisecond time scale. This can be instrumental in monitoring conformational dynamics of membrane proteins, such as G-coupled receptors.

An elegant solution for high-contrast intermolecular FRET imaging between genetically encoded fluorescent proteins (FPs) is the use of polarized light to detect the presence of FRET on the basis of FP anisotropy (AFRET).[27] Fluorescence from directly excited FPs is highly polarized, and is easily distinguished from FRET fluorescence, which is highly depolarized. Thus, AFRET can simply and unambiguously resolve FRET with 10-fold greater contrast than FLIM, especially in the range of efficiencies of 20% or less. Furthermore, accurate FA images of live cells can

be collected rapidly using a minimal set of images, and this method can be easily adapted to any imaging modality.

Temperature jumps of a few Kelvin induced by nanosecond to picosecond laser pulses are a well-established method to unfold nucleic acids and proteins at room temperature. FA microscopy was employed by Orrit's group for monitoring laser-driven microsecond temperature cycles of single dyes in a glycerol film.[28] The sample was maintained at low temperature in a custom-built cryostat and was imaged through optics designed to minimize aberrations under low-temperature conditions. The local heating by a focused infrared laser generated a micrometer-sized hot spot with temperature changes of several hundreds of Kelvin in a few microseconds, enough to supercool water. The hot spot was imaged through polarized fluorescence, including steady-state anisotropy and fluorescence anisotropy correlation spectroscopy (FACS). Based on the measured rotational correlation time, a temperature calibration between 200 and 350 K was achieved with an accuracy of ± 5 K. This method opens up interesting possibilities to thermally cycle large biomolecules like proteins and DNA without damage by expanding ice. At the same time, it promises to improve the temporal resolution and lengthen the observation time of the dynamics of single molecules at room temperature by using a series of freeze–thaw–measure cycles.

FA imaging can also be used to shed light on the conformations adopted by a DNA chain confined in a nanofluidic channel.[29] Depending on the dimensions of the channel, two different regimes are identified. When the channel diameter D is much larger than the persistence length of the DNA then the chain can be considered as a series of blobs of diameter D (De Gennes regime). When DNA is confined in a channel with dimensions much smaller than its persistence length, the chain will act as rigid segments deflecting off the channel walls (Odijk regime). Single DNA chains stained with an intercalator dye and stretched in a nanochannel grid were imaged with polarized light and, as expected, showed a decrease of polarization bias in larger 2D channels. The polarization ratio and total fluorescence intensity revealed non-uniform stretching for DNA molecules that had been partly introduced in a narrow channel, with the effect likely caused by entropic bias. This type of anisotropy imaging can be extended to studies of local structural and conformational changes of DNA in nanochannels as might be caused, for example, by binding of restriction enzymes or transcription factors.

Binding interactions and aptamers

The investigation of biomolecular binding interactions remains one of the most intensely studied topics using techniques that are based on emission of fluorescence. The development of small and large molecule agents that serve as therapeutics by virtue of binding to receptor sites represents one significant area of practical application. Even broader in scope are the areas of proteomics and systems biology, where understanding protein–protein interactions are of primary interest.

The application of polarization-based analytical methods is now widely adopted in the search for molecular interactions such as those associated with drug candidates. A typical example is the report of the identification of a peptide within a collection of peptides that inhibit the activity of human immunodeficiency virus type 1 integrase. In this case, the specific integrase target was separately titrated into each of the fluorescein-labelled peptides. The spectroscopic data clearly showed which compounds interacted to form a complex by virtue of the extent of change in anisotropy, and the titration data also permitted calculation of the dissociation constant, K_d, using the Hill equation.[30]

FA works particularly well for binding interactions that lead to significant change in the size of the molecular system that is associated with a reporter fluorophore. Labelling can make use of conventional and readily obtained organic dyes such as fluorescein, Texas Red, and TAMRA. There is no need for separation steps to purify complexes from unbound interactants, and this offers opportunity to determine parameters such as the dissociation constant by titration. FA has the added advantage of being able to operate in a ratiometric mode, with an interesting example being an application that studied cellular activation using cytometry.[31] Examples of the use of FA in the areas of receptor research and high-throughput drug discovery are provided in reviews by Leopoldo *et al.*,[32] and Wu and Doberstein.[33]

Various classes of binding agents can be identified, with a common distinction being categorization as either a protein/peptide, or a nucleic acid. Aptamers are nucleic acid and peptide sequences that spontaneously fold due to the specific primary sequence of monomeric units, resulting in the formation of active sites that can bind targets such as small molecules, peptides and proteins. The binding affinity of nucleic acid aptamers to specific targets typically lies in the micromolar to picomolar range. Nucleic acid aptamers can be prepared by a number of approaches. The best known method involves repeated rounds of *in vitro* selection, commonly known as SELEX, systematic evolution of ligands by exponential enrichment. A newer approach involves use of kinetic capillary electrophoresis for the selection aptamers by using chromatography to select aptamer–target constructs with defined equilibrium constants (K_d), rate constants (k_{off}, k_{on}) and free energy values. The chromatography method has the advantage of collecting aptamers of defined binding activity in only a few rounds of selection. The SELEX method often requires 15 cycles to refine a mixture to identify a specific aptamer of high binding activity, and even then often fails to provide selection of an aptamer of high binding constant. The molecular size of an aptamer, the availability of functional groups for fluorescence tagging, and the ability to serve as a binding agent or a displacement agent, combine to offer opportunities when considering use of FA to study binding, and to develop diagnostic methods and biosensors.

Nucleic acid aptamers find use in a number of areas including selective capture and in therapeutics.[34,35] Aptamers composed of nucleic acids have inherent advantages when compared to antibodies, even though the general principles of selective binding are similar. Unlike antibodies, aptamers can be created for small molecules such as drugs and toxins. Aptamer preparations that contain a single unique sequence of nucleic acid (DNA or RNA) tend to have a reproducible binding activity regardless of the source of synthesis. In many cases there is opportunity to minimize the sequence length, thereby improving binding affinity and kinetics. Aptamers tend to be structurally stable and can

renature after heating. They have intrinsically long shelf life because there is no unfolding. A comprehensive review comparing properties of aptamers and antibodies has been presented by Nimjee *et al*.[36]

Noteworthy to the analytical community is that aptamers can also directly serve as transducers of selective binding by virtue of changes in structure that can occur upon binding, which creates opportunity in the area of biosensor design.[37,38]

Numerous aptamer-based FA methods targeting small molecules, peptides and proteins have been developed in the past few years. An exciting development has been in the area of analysis of small molecule binding. A fluorescence polarization-based displacement assay for aptamer–target quantification was developed by Cruz-Aguado and Penner.[39] An aptamer complex was formed that used a fluorescently-labelled oligonucleotide as the initial target. The design was based on the oligonucleotide being selected to have a much smaller mass than the aptamer. The small organic molecule ochratoxin was then used to displace the fluorescently-labelled oligonucleotide from the aptamer-binding site. The subsequent change in fluorescence polarization was then used for the determination of analyte concentration.

Use of aptamers to detect protein–protein interactions first began to appear in the literature about a decade ago. One example of such earlier work included the use of a DNA aptamer for detection by FA of the antibody IgE. Both fluorescein and Texas Red were investigated as labels in these experiments, and it was determined that Texas Red was the better choice due to its greater sensitivity to motion. Detection limits of 350 pM were reported.[40] A selective aptamer has similarly been used to quantitatively determine angiotensin, which was done in real time in homogeneous solution with a detection limit of 1 nM. The authors went on to indicate that the assay permitted investigation of the binding affinity of other potential ligands for angiotensin.[41]

An interesting approach to study protein–protein interactions was presented by Cao and Tan.[42] The strategy was to investigate protein–protein interactions by development of a displacement assay. Thrombin was used as a model, and the work developed both FRET and FA methods as complementary strategies. TAMRA dye was attached to the 3′ end of aptamers that were designed to bind thrombin. The resulting binding of targets that showed affinity resulted in anisotropy changes of more than 30%. Displacement of the aptamer from thrombin was achieved using a sulfonated hirudin fragment with rapid kinetics, and the process was observed to occur in real time using anisotropy measurements. The FRET method that was developed indicated that displacement occurred, but the added information from the anisotropy data was fundamental in defining which of two potential displacement mechanisms was at play. The thermodynamic properties of the aptamer–thrombin complex provided the basis for determination of K_d. The authors presented a detailed theoretical development about how a single change in anisotropy signal that occurred on adding displacer to the thrombin–aptamer complex could lead to derivation of the value of K_d. A review of progress in the area of use of aptamers to study protein–protein interactions was recently published by Tan's team, with particular reference to systems biology.[43]

Extension of applications of aptamer binding to the development of biosensors has resulted in a number of reports that cover concepts such as structure–switch signalling, use of allosteric chimeras, various staining procedures to detect specific structures, and various amplification methods to improve signal development. The sensitivity of anisotropy measurements to molecular size and motion suggests that careful attention must be given to the selection of structure, and in the design of the rigidity of linkers. Determination of anisotropy can be useful to assist the design of DNA-based biosensors. An example of biosensor design being guided by anisotropy measurements is in the examination of the impact of extending the length of aptamers.[44]

The implementation of FA for the development of optical biosensing that is based on nucleic acid probes is shown for example by work that developed alteration of the degree of fluorescence quenching in a system where fluorescently-labelled probes could approach carbon nanotubes.[45] An important advance in the application of aptamers for biosensor development has been to extend molecular beacon technology to produce molecular beacon aptamers (MBAs). Such stem–loop systems that incorporate a donor and acceptor pair can ideally operate to provide concurrent FRET and FA data. MBAs have, for example, been demonstrated for targets such as thrombin and platelet-derived growth factor.[46] Further insights about applications of aptamers and molecular beacon probes can be found in a review from Le's team.[47]

Molecular aggregates in cells

In cells, FA is a versatile tool to study density distributions of membrane proteins, monitor ligand receptor binding and probing rotation dynamics and interactions of biomolecules. It has been demonstrated that FA microscopy is capable of probing the rotational mobility of fluorescent molecules and their interaction with the surroundings, but the method offers lower axial resolution than fluorescence intensity images.[14] The fluidity of cell membranes is an important parameter in physiopathology because it is related to tumour cell proliferation and metastasis processes. Images of a fluorescein-labelled antibody in human pulmonary giant cell carcinoma showed an increase in anisotropy upon treatment with plant origin ammonium salt berberine, indicating a higher cell membrane rigidity.[14]

Several cell-surface lipid-tethered proteins exhibit a concentration-independent, cholesterol-sensitive organization of nanoscale clusters and monomers. The spatial distribution and steady-state dynamics of fluorescently tagged glycosylphosphatidylinisotol (GPI) anchored protein nanoclusters in living cells was studied using high-spatial and temporal resolution fluorescence microscopy. The dynamics of recovery of intensity and anisotropy, the dynamics of interconversion between nanoclusters and monomers and the unique mechanism of complexation of cell-surface molecules were regulated by cortical actin activity.[48]

Bacterial chemoreceptors form mixed trimers of homodimers that cluster further in the presence of other cytoplasmatic components. Ligand-induced orientational dynamics of homodimers of serine or aspartate chemoreceptors was detected *via* the anisotropy of fused yellow fluorescence protein (YFP).[1] The physical proximity between receptors is thought to promote conformational coupling that enhance sensitivity, dynamic range

and collaboration between receptors of different types. Two types of *Escherichia coli* receptors, one of which was fused with YFP, were stimulated independently. The changes in the relative orientation of the labelled receptors were monitored by anisotropy variations that were assigned to changes in homo-FRET induced by ligand binding. It was shown that binding of ligand to one type of receptor affected the conformation of the other type of receptor but not in the same way as occurred upon binding of ligand to that receptor.[49]

FA in combination with computer modelling can yield structural data of molecular aggregates beyond the diffraction limit. A method combining a genetic algorithm with Brownian dynamics and Monte Carlo simulations was applied to studies of BODIPY-labelled filamentous actin (F-actin) and yielded a resolution approaching several ångströms.[11] The technique registered the local order and reorienting motions of the fluorophores, which were covalently coupled to cysteine 374 (C374) in each actin monomer and interacted by homo-FRET within the actin polymers. Analyses of F-actin samples composed of different fractions of labelled actin molecules revealed the known helical organization of F-actin, demonstrating the usefulness of this technique for structure determination of complex protein polymers. The distance from the filament axis to the fluorophore was found to be considerably less (10.5 ± 0.5 Å)[11] than expected from the previously proposed position of C374 at a high filament radius (13.5 Å). The method could be widely applied for structural studies of such protein aggregates as occurs for prions, or for toxins in solution that cannot be crystallized or that are too large to be studied by NMR. A unified theoretical description for one- and two-photon excited fluorescence depolarization and electronic energy migration within pairs of chromophores was recently presented by Opanasyuk *et al.*[15]

Inhibition of cancer-promoting, constitutive protein–protein complexes *via* disruption of binding interfaces offers significant value as a molecular-targeted therapy. Stat3, a member of the STAT family, is frequently over-activated in cancer cells, and is well-recognized as a master regulator of the underlying events in malignant transformation. An effective approach to silence aberrant Stat3 transcriptional activity may involve the disruption of the transcriptionally active Stat3–Stat3 homodimer complex.

Efforts to disrupt the Stat3 dimerization using SH2 domain proteomimetics and novel synthetic organometallic complexes were assessed by a competitive FA assay in a recent report.[50] The method is based upon the displacement of a 5-carboxyfluorescein-labelled (F*) phosphopeptide, *i.e.* F*-pYLPQTV, which binds with high affinity to the Stat3-SH2 domain. Low molecular weight organometallic proteomimetic inhibitors compete with the much larger Stat3 protein (90 kDa) for binding the fluorescent peptide. Unbinding F*-pYLPQTV from Stat3 results in a significantly lower anisotropy of the emitted fluorescence reflecting the higher mobility of the fluorophore[50] (Fig. 4). The high inhibitory activity (K_i values in the micromolar range) was confirmed by experiments on whole cells and on human tumour lines such as prostate and breast cancers and thus lends credence to a molecular approach focused on organometallic-mediated disruption of oncogenic protein–protein interactions.

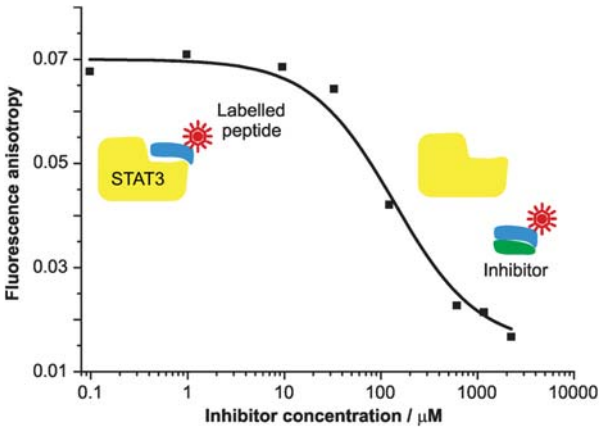

Fig. 4 Titration of inhibition activity using a competitive fluorescence assay. A sharp decrease of anisotropy is caused by a synthetic organometallic drug that disrupts binding of a fluorescently-labelled peptide to the Stat3 protein.

Conclusions

Fluorescence anisotropy yields information about the structure, photophysics and the environment of molecules. The method is applied in diagnostic and screening technologies for circumstances where a substantial change in mass or molecular cross-sectional size is anticipated, most often by virtue of binding interactions. A wide variety of sophisticated methods that hinge on anisotropy measurements have been developed for analyzing specific structural motifs and processes in biological systems. Anisotropy measurements in cellular applications are perhaps most powerful when combined with other fluorescence parameters to understand structure–function relationships.

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