

# Photon statistics in single molecule orientational imaging

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**Abstract:** Optical techniques in single molecule imaging rely heavily on photon counting for data acquisition. Extraction of information from the recorded readings is often done by means of statistical signal processing, however this requires a full knowledge of the photoelectron statistics. In addition to counting statistics we include a specific form of random signal variations namely reorientational dynamics, or wobble to derive the general probability density function of the number of detected photons. The relative importance of the two factors is dependent upon the total number of photons in the system and results are given in all regimes.

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## 1. Introduction

Single molecule detection (SMD) has become an important technique in recent years for studying dynamic processes such as chemical reactions and molecular motions at a fundamental level [1]. Historically these processes are usually studied using methods based on ensemble averaging of a sample of molecules, however these are based on the fundamental assumption that the sample is homogeneous. This ergodic hypothesis is invalid for many biological and chemical systems. Studies on single molecules are thus advantageous as information, such as statistical distributions of particular quantities, is not lost by averaging.

Single molecule imaging techniques, such as fluorescence microscopy, can also be used to track bio-molecular motions. This has applications in the pharmaceutical industry where a good understanding of processes such as protein folding [2] and molecule motions [3] is vital to new drug development. Relevant tracking techniques often employ fixed-site fluorescent probe molecules [4] whose properties, such as their orientation and position, vary according to their local environment. These changes can be detected with a suitable experimental setup [5, 6].

Optical techniques in single molecule imaging almost always require the use of photon counting since individual fluorescent molecules are very weak light sources. Under these conditions the accuracy of measurements are limited by random variations in the measured signal and statistical processing must thus often be used to extract the desired information. This however requires a good understanding of the random processes present. In this work we thus determine the probability density function (PDF) of the number of photons detected during a measurement of finite length as a means of describing the full statistical nature of the detection process.

In the next section we consider the importance of different contributions to photon statistics and find that both noise and statistical signal variations must be considered. As such in Section 3 we derive a general expression for the PDF of photon numbers allowing for both influences. Furthermore in Section 4 we consider one form that signal fluctuations may take, namely those arising from random rotations which fluorophores may undergo; a phenomenon we refer to as wobbling. Wobble of fluorophores, which within the framework of classical electrodynamics can be considered as electric dipole emitters, can be either a continuous angular variation or discrete orientational jumps [7]. We use the Laplace transform to obtain the PDF of the time averaged intensity when changes occur discretely. Successive jumps may depend on previous dipole orientations and we consider the case when this is true and when it is not. Limiting forms for both slow and fast wobble are also given. Further results are then derived for continuous variation in dipole orientation, which is found to be of a differing functional form to the discrete case. A short discussion of the results is finally given in Section 5.

## 2. Signal-to-noise considerations

Statistical fluctuations in the number of detected photons can derive either from noise present in a system or from random variations in the signal itself. The relative importance of these sources is conveniently parameterised by the signal-to-noise ratio (SNR). In the context of single molecule imaging Basché [8] states that the practically obtainable SNR can be approximated by

$$SNR = \frac{Dq\sigma P t_0 / A E_p}{\sqrt{(Dq\sigma P t_0 / A E_p) + C_b P t_0 + N_d t_0}} \quad (1)$$

where  $D$  is an instrument dependent collection factor typically ranging from 1-8%,  $q$  is the fluorescence quantum yield,  $\sigma$  is the peak absorption crosssection,  $P$  is the laser power,  $t_0$  is the integration time,  $A$  is the beam area,  $E_p$  is the energy of a photon in the beam,  $C_b$  is the background count per watt of excitation power (typically around  $2 \times 10^8$  photons/Ws in confocal experiments) and  $N_d$  is the dark count of the detector. Figure 1 shows the behaviour of the SNR over a range of experimental conditions from which it can be seen that a value no better than around 15dB is to be expected. Consequently noise properties of the detection process play an important role in determining the statistical behaviour of the detected signal.

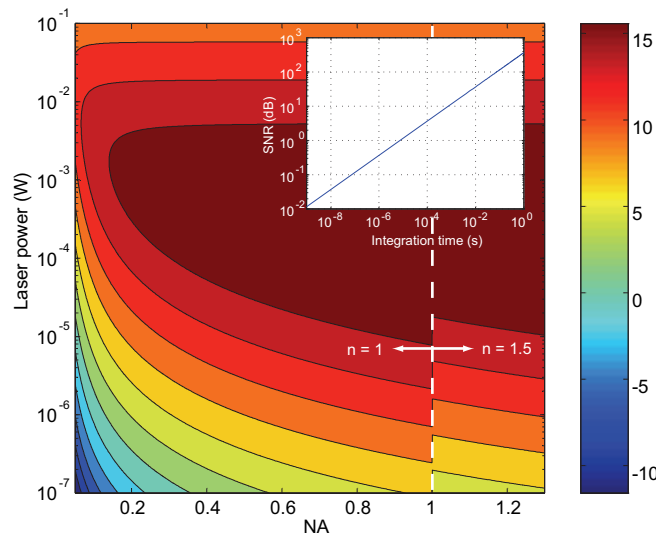


Fig. 1. Contour plot of SNR (dB) vs laser power and focused spot size (as parameterised by the NA of a focusing lens) assuming a wavelength of 395nm and the following parameter values based on use of GFP:  $D = 7\%$ ,  $q = 0.79$  [9],  $t_0 = 0.01$ s,  $C_b = 2 \times 10^8$  photons/Ws,  $N_d = 50$ . For numerical apertures greater than unity we have assumed an oil immersion lens of refractive index 1.5. Saturation effects are included such that  $\sigma = \sigma_0 / (1 + I/I_s)$ , where  $\sigma_0$  was taken to be  $4 \times 10^{-16}$  cm<sup>2</sup>,  $I = P/A$  and the saturation intensity  $I_s$  was assumed to be  $10^3$  W/cm<sup>2</sup> [10]. The inset shows the variation of the SNR versus integration time for a 5mW laser focused through a 0.95 NA lens.

## 3. Probability density function of the number of detected photons

Data acquisition in single molecule experiments is invariably done by means of photon counting in which the predominant source of noise is quantisation noise. Denoting the number of photons

arriving at the detector during a measurement of duration  $t_0$  by  $N(t_0)$ , the output reading is of the form  $I_{out} = GN$ , where  $G$  is some gain factor. The arrival of photons at the detector is a Poisson random process [11] and we can hence write the probability mass function of  $N$

$$p_N(n) = \frac{(I(t_0))^n}{n!} \exp(-I(t_0)) \quad (2)$$

where  $p_N(n)$  denotes the probability that  $N(t_0) = n$ . We note here that we use the convention whereby an upper case letter denotes a random process and/or variable, whilst the lower case equivalent denotes a particular outcome.  $I(t_0)$  is the average rate of arrival of photons (intensity) or equivalently the time average of  $\mathcal{I}(t)$  where  $\mathcal{I}(t)$  is the instantaneous rate of arrival of photons at the detector at time  $t$  i.e.

$$I(t_0) = \int_0^{t_0} \mathcal{I}(t) dt \quad (3)$$

As an example many experimental setups use polarisation sensitive methods e.g. [12] whereby the intensity of the detected signal is proportional to the square of the dot product of the illuminating field and the electric dipole moment giving

$$\mathcal{I}(t) = A \cos^2(\theta(t) - \beta) \quad (4)$$

where  $\theta(t)$  is the transverse orientation of the dipole at time  $t$ ,  $\beta$  is the transverse angle of the plane of polarisation of incident light and  $A$  is a constant.

For a stationary dipole Eq. (2) fully describes the photon statistics at the detector, however a change in dipole orientation will cause a change in  $\mathcal{I}$ . If this change is random the arrival of photons at the detector and hence their subsequent detection is termed a doubly stochastic process. Possible sources of such randomness include fluctuations in the illuminating light source and/or movement of the molecule. It is this latter factor that we concentrate on here since for tracking applications the molecule's environment is unlikely to be static. Furthermore we consider only orientational changes since probe molecules are normally rigidly fixed to targets. Under these circumstances  $I(t_0)$  is a random variable and the probabilities as given by Eq. (2) differ for each possible value. As such we recast Eq. (2) by conditioning the probabilities on a particular outcome  $i(t_0)$  i.e.

$$p_N(n|i) = \frac{i^n}{n!} e^{-i} \quad (5)$$

where  $p_N$  is now a conditional probability and we have dropped the functional dependence on  $t_0$  for clarity.

Assuming knowledge of the random nature of the time average, as characterised by its PDF  $f_I(i)$  (see Section 4), we can use the identity [11]

$$f_{N,I}(n, i) = p_N(n|i) f_I(i) \quad (6)$$

to find the joint PDF of  $N$  and  $I$  i.e. the probability that  $N = n$  and  $I = i$ . Integrating over the joint PDF gives the PDF of the number of detected photons

$$p_N(n) = \int_0^\infty f_I(i) \frac{(\eta i)^n}{n!} e^{-\eta i} di. \quad (7)$$

where we have also included the non-ideal nature of the detector by introduction of the quantum efficiency  $\eta$ . Eq. (7) is equivalent to averaging the conditional probability with respect to the average intensity and requires knowledge of  $f_I(i)$  which is discussed in the following section.

## 4. Probability density function of time averaged intensity

### 4.1. Discrete reorientational jumps

In this section we turn our attention to determining the PDF of the time averaged intensity  $f_I(i)$ . We first consider the case when changes in the orientation of a dipole occur discretely. This could for example be associated with the desorption and readsorption of fluorophores from and onto a glass surface [12]. In what follows we shall talk of an electric dipole in an orientational state, by which it is meant that the dipole makes an angle  $\theta$  to the  $x$ -axis in the  $x$ - $y$  plane as illustrated in Fig. 2. The dipole then remains fixed at this angle for a time  $\tau$  before moving to a new state. It is this transverse angle that the signal  $\mathcal{I}$  in many experimental techniques is dependent on (c.f. Eq. 4). Techniques based on structured illumination and total internal reflection [13, 14] do however exist in which the signal depends upon the full three dimensional orientation of a dipole, although these are often restricted to very specific circumstances. Here we restrict our discussion to a two dimensional system for simplicity. Conceptually the full three dimensional situation is identical and requires only minor mathematical modifications as is discussed in the appendix.

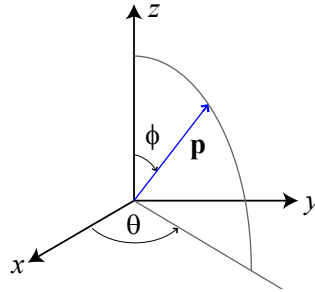


Fig. 2. An electric dipole  $\mathbf{p}$  has a transverse orientation described by the angle  $\theta$ . Here  $z$  describes the optical axis.

Assuming that  $M$  different orientational states are occupied during a single measurement the time averaged intensity is given by:

$$i = A (\cos^2(\theta_1 - \beta) \tau_1 + \cos^2(\theta_2 - \beta) \tau_2 + \dots + \cos^2(\theta_M - \beta) \tau_M) \quad (8)$$

where  $\theta_j$  and  $\tau_j$  are the parameters corresponding to the  $j^{\text{th}}$  occupied angular state. Without loss of generality the dipole is assumed to be initially orientated parallel to the  $x$ -axis. It should be further noted that changes in the dipole angle are assumed to occur instantaneously.

From the law of rare events [15] we can assert that  $M$  be a Poisson random variable or equivalently that the length of time a dipole remains in each state is distributed according to an exponential law i.e.

$$f_\tau(\tau) = \nu \exp(-\nu\tau) \quad (9)$$

where  $f_\tau(\tau)$  denotes the PDF of  $\tau$  and  $\nu$  is the average rate at which dipole jump events occur.

Since a measured intensity is always positive we may use the Laplace transform to find the PDF of  $I$ . The Laplace transform  $X^*(s)$  of a random variable  $X$  is defined as

$$X^*(s) = \mathcal{L}[f_X(x)] = \int_0^\infty f_X(x) e^{-sx} dx = E[e^{-sX}] \quad (10)$$

where  $\mathcal{L}[\dots]$  denotes the Laplace transform and  $E[\dots]$  denotes the expected value.

From [11] and Eq. (8) the Laplace transform for  $f_I(i)$  is given by

$$I_M^*(s) = Z_1^*(s)Z_2^*(s)\cdots Z_M^*(s) \quad (11)$$

where we have let  $Z_j = A \cos^2(\Theta_j - \beta)\tau_j$ . The PDF of the average intensity  $I$  is then given by:

$$f_I(i) = \sum_{m=0}^{\infty} p_M(m) f_I(i|m) \quad (12)$$

where  $f_I(i|m) = \mathcal{L}^{-1}(I_{M=m}^*(s))$  and the weighted summation over the possible values of  $M = m$  is required since the number of reorientations during a measurement is random.

With this knowledge in hand it remains to find an explicit expression for  $Z_j^*(s)$ . From Eqs. (4) and (10) we can write

$$Z_j^*(s) = \int_{-\pi}^{\pi} \int_0^{\infty} f_{\Theta,\tau}(\theta, \tau) \exp(-sA \cos^2(\theta - \beta)\tau) d\tau d\theta \quad (13)$$

where  $f_{\Theta,\tau}(\theta, \tau)$  is the joint probability distribution of  $\Theta$  and  $\tau$ . Since dipole angle and state occupancy time are independent this is given by the product of the marginal probability distributions  $f_{\Theta}(\theta)$  and  $f_{\tau}(\tau)$ . Using Eq. (9) we can then write

$$\begin{aligned} Z_j^*(s) &= \int_{-\pi}^{\pi} \int_0^{\infty} f_{\Theta}(\theta) v \exp(-v\tau - sA \cos^2(\theta - \beta)\tau) d\tau d\theta \\ &= \int_{-\pi}^{\pi} \frac{v f_{\Theta}(\theta)}{v + sA \cos^2(\theta - \beta)} d\theta \end{aligned} \quad (14)$$

The physical process governing the random wobble of the electric dipole will dictate the form of the probability distribution for  $\Theta$ . For example, rebinding of a fluorophore to a probe site may be modeled using a uniform PDF

$$f_{\Theta}^{uni}(\theta) = \begin{cases} 1/2\Delta & \text{for } -\Delta \leq \theta < \Delta \\ 0 & \text{otherwise} \end{cases} \quad (15)$$

Standard integration tables [16] then give the analytic result

$$Z_{uni}^*(s) = \frac{1}{2\Delta\sqrt{sAv + v^2}} \times \left[ \arctan\left(\sqrt{\frac{v}{sA+v}} \tan(\Delta - \beta)\right) + \arctan\left(\sqrt{\frac{v}{sA+v}} \tan(\Delta + \beta)\right) \right] \quad (16)$$

Finding a full analytical result for  $f_I(i)$  would be complicated however in the limits of small and large  $v$  we can find simpler results. These limits correspond to only a few, and to many events per measurement respectively. As the rate at which events occur decreases the contribution from later terms in Eq. (12) becomes negligible. In the limit of  $v \ll 1$  only the first term produces a significant contribution and we can consider the dipole as fixed during a single measurement and hence

$$f_I(i) = f_{\mathcal{I}}(i) \quad (17)$$

i.e. the PDF of the average intensity is the same as the PDF for the instantaneous intensity. Fortunately this agrees with our intuitive expectations.

When dipole wobble is on a time scale much shorter than the duration of a measurement we must consider many terms in our summation i.e. large  $v$ . Since each value of  $\tau_j$  is independent each  $Z_j$  term is also independent. There are then two cases to consider; that when each

subsequent value of  $\theta$  is independent and that when they are not. In the former case we can invoke the Central Limit Theorem which states the PDF of a sum of independent, identically distributed random variables tends to a Gaussian distribution as the number of terms increases. As such the PDF of the average intensity in the limit of large  $\nu$  is given by

$$f_I(i) = \frac{1}{\sqrt{2\pi\sigma_I^2}} \exp\left(-\frac{i^2}{2\sigma_I^2}\right). \quad (18)$$

Assuming dependence of consecutive terms means the PDF of the dipole angle  $\theta_j$  is centered on its previous outcome,  $\theta_{j-1}$ . For a particular realisation of  $\theta$ , that is to say one possible outcome of the sequence of dipole orientations, we can write

$$f_{\theta_j}(\theta_j) = f_{\theta}(\theta - \theta_{j-1}) \quad (19)$$

When averaged over all possible realisations the result is similar to Eq. (15) except now the width of the distribution increases with each subsequent jump. Consequentially the condition of identical distributions required for validity of the Central Limit Theorem is not satisfied. If, however, the Lyapunov condition [17] is satisfied then the Central Limit Theorem still applies. Numerical simulations show that this is the case.

#### 4.2. Continuous angular variation

Changes in dipole orientation may occur continuously and it is here that we consider how this affects the PDF of the time averaged intensity. It can be shown [18] that the probability distribution function of the orientation of the dipole at a time  $t$  satisfies the differential equation:

$$\frac{\partial f}{\partial t} = \alpha \frac{\partial^2 f}{\partial \theta^2} \quad (20)$$

subject to the initial condition  $f(t=0) = \delta(\theta - \theta_0)$ , where  $\delta$  represents the Dirac delta function. This diffusion equation holds when subsequent orientations are dependent on the previous orientation. A solution to Eq. (20) is

$$f_{\theta}(\theta, t) = \frac{1}{\sqrt{4\pi\alpha t}} \exp\left(-\frac{(\theta - \theta_0)^2}{4\alpha t}\right). \quad (21)$$

This result applies to all time scales over which  $\theta$  may vary as is set by the diffusion coefficient  $\alpha$ .

To find the PDF of the average intensity we first use a transformation of variables to find the PDF of the instantaneous intensity  $f_{\mathcal{I}}(\mathcal{I})$  which we then need only integrate over the length of a measurement to give our desired result. Thus

$$f_I(i) = \frac{1}{t_0} \sum_k \int_0^{t_0} \frac{f_{\theta}(\theta_k, t)}{\sqrt{i(A-i)}} dt. \quad (22)$$

where  $\theta_k$  are the solutions to the equation  $i = A \cos^2(\theta - \beta)$  and the  $1/t_0$  factor is to ensure correct normalisation of the PDF. The integral can be evaluated using the substitution  $x^2 = t^{-1}$  and integration by parts which yields:

$$f_I(i) = \frac{1}{\sqrt{\pi\alpha t_0 i(A-i)}} \sum_k \left[ \exp\left(-\frac{\theta_k^2}{4\alpha t_0}\right) - \frac{|\theta_k|}{2\alpha t_0} \operatorname{erfc}\left(\sqrt{\frac{\theta_k^2}{4\alpha t_0}}\right) \right] \quad (23)$$



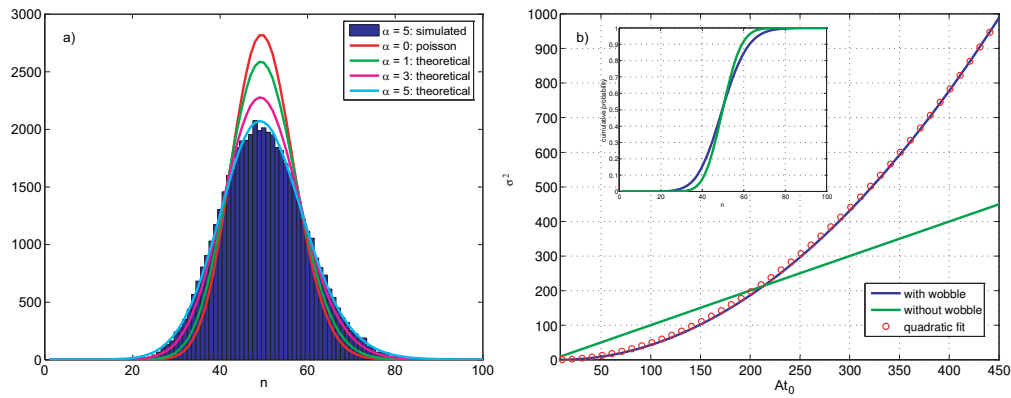


Fig. 3. a) Histogram of the time averaged intensity for a dipole undergoing continuous angular diffusion with  $\alpha = 5$ ,  $\beta = \pi/4$ ,  $t_0 = 10^{-3}$  s and  $A = 10^5$  photons/s shown with theoretical fits for differing diffusion coefficients. b) Variance of the number of detected photons as a function of the peak signal strength  $A$  and the cumulative probability functions for wobbly and stationary dipoles (inset) for the same parameter values as a).

where  $\text{erfc}(\dots)$  denotes the complimentary error function.

For the independent case  $f_{\Theta}(\theta, t)$  can not depend on time (assuming the physical cause of the wobble does not vary in time) and as such Eq. (22) reduces to  $f_I(i) = f_{\mathcal{I}}(\mathcal{I})$ .

Figure 3a) shows a histogram of the result of Monte-Carlo simulations with  $10^4$  realisations for continuous variation and a diffusion coefficient of  $\alpha = 5$ . Various theoretical fits, as based on Eq. (23), are also drawn from which it can be seen that for  $\alpha = 0$  (no dipole wobble) the PDF is identical to that of a Poisson distribution as would be expected. Good agreement can also be seen between the simulated and theoretical results.

Furthermore, using these PDFs it is possible to calculate the total cumulative probability of  $N$  taking any value below  $n$  as plotted in the inset of Fig. 3b) as a function of  $n$ . Confidence levels including or neglecting dipole wobble can then be calculated. Assuming the values  $\beta = \frac{\pi}{4}$ ,  $\theta_0 = 0$ ,  $A = 10^5$  photons/s,  $t_0 = 10^{-3}$  s and  $\alpha = 5$  we calculated that when neglecting dipole wobble an experimental measurement can determine the orientation of a dipole within a range of  $1.78^\circ$  with 90% confidence. Inclusion of dipole wobble causes this to increase to  $2.43^\circ$ . Such a discrepancy further highlights the need to include dipole wobble in statistical processing and error analysis.

## 5. Discussion

It has been shown that the variation of the orientation of a dipole over the course of a finite duration measurement can alter the statistical properties of the number of photoelectrons induced in a photon counting detector. Although analytic evaluation of Eqs. (7) and (12) will in general not be possible we can make some general observations regarding the probability functions involved.

Considering first the PDF of the time averaged intensity we can expect different forms and behaviour for differing dependence conditions and time scales. More specifically, although the distribution will always be peaked around the initial angle, when subsequent orientations of the dipole are dependent on earlier positions the distribution is narrower for slower changes, whilst the converse is true when independence holds. This can be understood since the dependent situation is essentially a diffusion problem and so the larger the ratio of spreading rate (as



given by the diffusion coefficient) to integration time the larger the range of angles the dipole can cover during a measurement. On the other hand the distribution focuses when successive orientations are independent since the central probability peak for each  $Z$  term is reinforced with each additional term in the average.

For wobbling on faster time scales the PDF of the average intensity has been shown to tend to that of a bell-shaped Gaussian distribution (for discrete variations). Slower time scales will exhibit a sharper more centralised distribution, since for small  $\nu$  i.e. slow variation only a few terms significantly contribute to the average performed by the detector. In this case the peaked nature of both the exponentially distributed state occupancy times and the Poisson PDF for the number of events per measurement dominate. For larger  $\nu$  the Poisson PDF becomes smoother and the position of the peak moves to larger  $m$ . Low  $m$  terms of Eq. (12) are then negligible and the peaked nature of the exponential PDF is less dominant. Eventually the Poisson PDF tends to a Gaussian itself whereby it acts as an envelope for the PDF of the average dipole angle.

In terms of the photoelectron statistics we can say that for smaller angular ranges of dipole oscillation one would expect less deviation from conventional Poissonian behaviour. Furthermore if the variations are on a timescale much longer than the integration time then the additional random behaviour will be unobservable. On the other hand if fluctuations are much faster than the detector response the effects are likely to again go unnoticed. That said dipole wobble has been seen at many different time scales ranging from the subnanosecond level [19], through the millisecond regime [5] and higher [7, 20]. In conjunction with the varying time resolution of different experimental setups [21] and the large angular ranges over which fluorophores can oscillate e.g.  $26^\circ$  has been observed [22], it is likely that non-Poisson behaviour will be frequently encountered.

Finally we draw attention to Fig. 3b) which shows a plot of the expected variance of experimental data as a function of the number of photons in the system (as parameterised by  $A$ ), when signal variations from photon counting and dipole wobble are considered separately. Quadratic behaviour can be seen for the case of dipole wobble only, whilst for photon counting the linear behaviour expected from a pure Poisson random variable is evident. The relative importance of the two factors can be seen. At very low light intensities, where it is likely to be impractical to conduct experiments, photon counting dominates. For the intermediate regime both influences are comparable until eventually at higher intensities the molecular wobble dominates.

Our work has detailed the statistical behaviour in each of these regimes (Eqs. (2), (7) and (12) or (23) respectively) and allows further development of suitable signal processing algorithms and estimation procedures so as to maximise the precision of angular information extracted from experimental data and thus help to fulfil the potential of single molecule techniques.

### Appendix - Three dimensional dipole wobble

Earlier discussion has been restricted to the case of two dimensional dipole wobble, however we here explicitly state the mathematical modifications required to accommodate rotation in all three dimensions. Obviously such three dimensional variation is only of significance if the measured signal is sensitive to the full three dimensional orientation as described by the two angles  $\theta$  and  $\phi$  as shown in Fig. 2 i.e.

$$\mathcal{I} = \mathcal{I}(\theta, \phi) \quad (24)$$

Random variation in the orientation of the dipole is described by the joint PDF of  $\Theta$  and  $\Phi$ . Since in most physical situations  $\Theta$  and  $\Phi$  will be independent we can write the PDF in the form

$$f_{\Theta, \Phi}(\theta, \phi) = f_{\Theta}(\theta)f_{\Phi}(\phi) \quad (25)$$

When considering the discrete case this means Eq. (13) becomes a triple integral

$$Z_j^*(s) = \int_{-\pi}^{\pi} \int_0^{\pi} \int_0^{\infty} f_{\Theta}(\theta) f_{\Phi}(\phi) f_{\tau}(\tau) \exp(-s \mathcal{J}(\theta, \phi) \tau) d\tau d\phi d\theta \quad (26)$$

however all of the subsequent working remains unchanged. In the continuous case we must solve the two dimensional diffusion equation

$$\frac{\partial f}{\partial t} = \alpha \left( \frac{\partial^2 f}{\partial \theta^2} + \frac{\partial^2 f}{\partial \phi^2} \right) \quad (27)$$

to give the joint PDF

$$f_{\Theta, \Phi}(\theta, \phi) = \frac{1}{4\pi\alpha t} \exp\left(-\frac{(\theta - \theta_0)^2}{4\alpha t}\right) \exp\left(-\frac{(\phi - \phi_0)^2}{4\alpha t}\right) \quad (28)$$

which when integrated according to the three dimensional analogue of Eq. (22) yields

$$f_I(i) = \frac{1}{8\pi\alpha t_0 \sqrt{i(A-i)}} \sum_k \left[ \Gamma\left(0, \frac{(\theta_k - \theta_0)^2 + (\phi_k - \phi_0)^2}{4t_0\alpha}\right) \right] \quad (29)$$

where  $\Gamma(a, z) = \int_z^{\infty} x^{a-1} e^{-x} dx$  is the incomplete Gamma function and  $\theta_k$  and  $\phi_k$  are the solutions to the equation  $i = \mathcal{J}(\theta, \phi)$ .