Photon Counting Histogram for One-Photon Excitation

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Single-molecule fluorescence is a powerful tool with which to study features hidden by an ensemble average, for example, conformational changes.[1] In many cases, optimum signal to noise is attained through the study of a few molecules at one time, which allows molecular properties to be deduced by analyzing fluctuations in the fluorescence. With this goal in mind, in 1999 Chen et al.[2] introduced the photon-counting histogram (PCH) technique to account for the fluctuations in fluorescence amplitude for molecules diffusing through a confocal laser focus. This method was first applied to two-photon fluorescence excitation. PCH was able to determine two parameters for each fluorescent species present: the average number of particles in the observation volume, N, and the molecular brightness, ε. Chen et al.[2] suggested that the same analysis procedure could be applied to one-photon excitation by using a three-dimensional Gaussian profile to describe the observation volume; however, we have found that this profile is unable to adequately fit the data. We present an alternative procedure, which is a corrected form of the 3D Gaussian profile. This procedure is able to fit the data, is easy to implement, and appears to be quite robust.

Let W(0) describe the observation volume profile, which combines the excitation strength and detection efficiency as a function of the position of a molecule. According to the PCH model, the probability of detecting k photons (k > 0) from one fluorescent molecule in a sufficiently large reference volume V0 is:[2] [Equation (1)]

\[ p^{(r)}(k; V_0, \varepsilon) = \frac{1}{V_0} \int \text{Poisson}(k \cdot x, W(\hat{r})) d\hat{r} \]  

where ε is the molecular brightness, and [Equation (2)]

\[ \text{Poisson}\left(k, x\right) = \frac{x^k e^{-x}}{k!} \]

is the Poisson distribution that describes the probability of getting k photons when the average number of photons received is x. Following the standard convolution procedure described by Chen et al.[2] the final form for the photon counting histogram is obtained, which is determined by two parameters for each fluorescent species: the average number of molecules in the observation volume, N, and the molecular brightness, ε. In order to have the same N as in fluorescence correlation spectroscopy (FCS), V0 is set proportional to the observation volume defined in FCS:[4] [Equation (3)]

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\[ V_s = Q \cdot V_{\text{FCS}} = Q \left( \frac{\int W(\vec{r})d\vec{r}}{\int W^2(\vec{r})d\vec{r}} \right)^2 \]  

(3)

where \( Q \) is chosen to be large enough so that [Equation (4)]:

\[ \sum_{i=1}^{\infty} p_i(k; V_s, \varepsilon) < 1 \]  

(4)

Subsequent convolutions use a number of molecules equal to \( Q \cdot N \) from which the value of \( N \) can be deduced. In two-photon experiments, \( W(\vec{r}) \) is well approximated by the square of the Gaussian–Gaussian–Lorentzian profile. In the case of one-photon excitation experiments with confocal detection, Chen et al. proposed that one should use a 3D Gaussian approximative to describe the observation volume profile, which is the same as the one used in FCS [Equation (5)]:

\[ W_G(x, y, z) = \exp \left[ -\frac{(2x^2 + y^2)}{w_x^2} - \frac{2z^2}{z_0^2} \right] \]  

(5)

where \( w_x \) is the beam waist, and \( z_0 \) is the effective length of the confocal volume.

We have attempted to fit experimental one-photon excitation photon counting histograms with this 3D Gaussian PCH model for two separate samples: tetramethylrhodamine-5′-maleimide (TMR) (Figure 1a) and Cy3-maleimide (Cy3) (Figure 1b). We have found that at low concentration and high molecular brightness the single-species fitting fails (see Table 1). This fact is particularly distressing because these conditions are those for which PCH has the best resolution. A two-species model is able to fit the data (fitting not shown); however, the fitted parameters are unphysical and contradict the FCS results on the same sample. We conclude that the 3D Gaussian model can deviate significantly from the real process, although as the molecular brightness decreases and the average number of molecules increases the deviation becomes minor (Figure 1b).

Previous theoretical work has investigated the difference between the actual one-photon excitation observation volume profile \( W(\vec{r}) \) and a 3D Gaussian function \( W_G(\vec{r}) \). Hess and Webb point out that this deviation leads to appreciable artifacts in FCS. According to their results and our own calculations, \( W_G(\vec{r}) \) well describes the observation volume profile when the molecule is close to the focal point, but \( W_G(\vec{r}) \) drops much faster than \( W(\vec{r}) \) when the molecule is far away from the focus. We define the signal from the difference between \( W(\vec{r}) \) and \( W_G(\vec{r}) \) as the out-of-focus emission profile [Equation (6)].

\[ W_{\text{OOF}}(\vec{r}) = W(\vec{r}) - W_G(\vec{r}) \]  

(6)

Although both the excitation strength and the detection efficiency are low in the out-of-focus region, its vast spatial extent makes its contribution a significant fraction of the detected photons. According to our calculations, in some conditions signal from \( W_{\text{OOF}}(\vec{r}) \) can be as much as nearly half the total signal. This fact is confirmed by the calculations of Sandison and Webb under paraxial approximation, in which they find the signal to background ratio (which is roughly equal

![Figure 1. Experimental one-photon PCHs and their fittings: a) Tetramethylrhodamine-5′-maleimide (TMR) (Figure 1a) and Cy3-maleimide (Cy3) (Figure 1b).](image)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Model</th>
<th>( \bar{N} )</th>
<th>( \varepsilon )</th>
<th>( F )</th>
<th>Reduced ( \chi^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMR</td>
<td>3D Gaussian</td>
<td>2.36</td>
<td>0.77</td>
<td>N/A</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td>corrected</td>
<td>2.27</td>
<td>0.67</td>
<td>0.34</td>
<td>0.98</td>
</tr>
<tr>
<td>Cy3</td>
<td>3D Gaussian</td>
<td>16.2</td>
<td>0.147</td>
<td>N/A</td>
<td>1.73</td>
</tr>
<tr>
<td></td>
<td>corrected</td>
<td>16.1</td>
<td>0.213</td>
<td>0.44</td>
<td>0.75</td>
</tr>
<tr>
<td>TMR + Cy3</td>
<td>one species[a]</td>
<td>4.37</td>
<td>0.93</td>
<td>1.07</td>
<td>4.61</td>
</tr>
<tr>
<td></td>
<td>one species[a] (fixed ( F ))</td>
<td>4.51</td>
<td>0.60</td>
<td>0.38</td>
<td>259</td>
</tr>
<tr>
<td></td>
<td>two species[a]</td>
<td>1.1</td>
<td>1.12</td>
<td>0.38</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>two species[a]</td>
<td>7.0</td>
<td>0.21</td>
<td></td>
<td>0.21</td>
</tr>
</tbody>
</table>

(a) Using the corrected model.

Table 1. PCH analysis with different models for data in Figure 1.
to the ratio of in-focus and out-of-focus emission) is 2.6 in a
confocal microscope with a small pinhole. We believe this out-of-focus
correction to the total signal accounts for the failure of the
3D Gaussian PCH model to reproduce observations.

To correct for the deviation, we propose a semiempirical
model that introduces one additional fitting parameter, the
out-of-focus emission ratio, \( F \), which is defined by
Equation (7).

\[
F = \frac{\int W_{\text{OOF}}(r) \, dr}{\int W_{\text{G}}(r) \, dr}
\]  

(7)

Because Equation (1) is mainly connected to the integrations
of the observation volume profile, using the parameter \( F \), we

calculate its corrected form [Equations (8a) and (8b)]:

\[
p^{(1)}(k; V_0, r) = \frac{1}{1 + F} \, p_G^{(1)}(k; V_0, r) \quad (k > 1)
\]  

(8a)

and

\[
p^{(1)}(k; V_0, r) = \frac{1}{(1 + F)^2} \, p_G^{(1)}(k; V_0, r) + \frac{F}{(1 + F)^2} \, \frac{1}{2 \sqrt{2}} \, e^{k} \quad (k = 1)
\]  

(8b)

where \( p_G^{(1)}(k; V_0, r) \) is the integration using the 3D Gaussian
approximation. Note that our model is based on two approximations:
1) The absolute value of \( W_{\text{OOF}}(r) \) is small, so that when
\( n > 1 \), the integration of the \( n \)th power of \( W_{\text{OOF}}(r) \)
is negligible compared with that of \( W_{\text{G}}(r) \); 2) \( W_{\text{G}}(r) \) will fit the
observation volume profile when close to the focus; therefore, \( W_{\text{G}}(r) \)
and \( W_{\text{OOF}}(r) \) do not overlap. Details of the derivation will be presented
in a future publication.

We find that this corrected model is able to fit the two
experimental photon counting histograms shown in Figures 1a and 1b, as shown in Table 1. To test the validity and robustness of
the corrected model, we varied the dye concentration by a factor
of 20 and the laser power by a factor of 6. As Table 2 shows, as

<table>
<thead>
<tr>
<th>Relative Concentration</th>
<th>( \langle k \rangle ) [kHz]</th>
<th>( N_{\text{CC}} )</th>
<th>( N_{\text{OOF}} )</th>
<th>( \varepsilon )</th>
<th>( F )</th>
<th>( N_{\text{CC}}/0.50 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15.9</td>
<td>0.40</td>
<td>0.50</td>
<td>2.62</td>
<td>0.45</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>79.7</td>
<td>1.94</td>
<td>2.33</td>
<td>2.65</td>
<td>0.39</td>
<td>4.7</td>
</tr>
<tr>
<td>10</td>
<td>161.1</td>
<td>4.05</td>
<td>4.79</td>
<td>2.59</td>
<td>0.36</td>
<td>9.6</td>
</tr>
<tr>
<td>20</td>
<td>326.6</td>
<td>8.41</td>
<td>10.81</td>
<td>2.30</td>
<td>0.35</td>
<td>21.6</td>
</tr>
</tbody>
</table>

Table 2. PCH analysis of TMR at different concentrations. The first row corresponds to a concentration of about 1 nM. \( \langle k \rangle \) is the average fluorescence photon count rate. \( N_{\text{CC}} \) is the average number of molecules in the confocal volume determined by FCS. The bin time of PCH is 20 μs.

To confirm that this corrected PCH model is able to resolve
fluorescent species with different degrees of brightness, we
mixed equal amounts of the two samples in Figures 1a and 1b and measured its resulting PCH. The one-species PCH model can
fit the PCH only when \( F \) goes to an unreasonable value greater
than 1, whereas the two-species fitting gives the expected
results: molecular brightness unchanged and the number of
particles halved upon mixing (see Table 1).

In conclusion, we have presented a means of carrying out the
photon-counting histogram procedure of Chen et al. [2] for one-photon excitation. Because one-photon excitation is so common
in single-molecule studies, the method we have presented
should be of wide applicability.

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