# A Comparison between Photon Counting Histogram and Fluorescence Intensity Distribution Analysis

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Photon counting histogram (PCH) and fluorescence intensity distribution analysis (FIDA) are two methods that were developed independently but reported almost simultaneously. Both of them have been successfully applied to fluorescence fluctuation spectroscopy (FFS). Though publications have indicated that they are theoretically equivalent, they are still commonly considered as different methods, especially in their ways to treat the point spread functions (PSFs). In this paper, the two methods are examined in detail for a direct comparison. After a direct proof of the theoretical equivalence, the authors further point out that PCH and FIDA are completely equivalent in the way of modeling PSFs; that is, any modeling approach developed from one of them can always be applied to the other. It is also demonstrated that simplified FIDA and PCH formulas in the form of power series can be applied for fast and precise numerical calculations. The two methods are also compared for their merits in the calculation efficiency.

## 1. Introduction

In fluorescence fluctuation spectroscopy (FFS),[1,2] one detects the temporal fluctuation of the fluorescence photon counts emitted from a femtoliter sized detection zone, which typically contains less than a few sample particles at a time. Using proper statistical methods, one can obtain information on the fluorescent particles, such as their size, weight, brightness, and concentration. It is an ultrasensitive tool for probing a wide variety of biochemical and biophysical processes in different systems. The most attractive application of FFS is probing protein−protein interactions within living cells. The most popular analysis methods for FFS include fluorescence correlation spectroscopy (FCS)[3−7] and photon counting histogram (PCH),[8,9] or its equivalent, fluorescence intensity distribution analysis (FIDA).[10] The former focuses on the correlation function of the fluorescence photon counts and retrieves the average number, $N$, and average diffusion time, $\tau_D$, of the fluorophores in the detection zone. The latter analyzes the histogram, that is, the probability, $P(k)$, of detecting $k$ fluorescence photons in a time bin, and retrieves $N$ and the brightness, $\epsilon$, of the fluorophores.

PCH and FIDA were developed independently and reported in 1999 by two groups.[8,10] Both methods use the same statistics and retrieve the same set of parameters. Their theoretical frameworks, however, are different. They are commonly regarded in the literature as “similar”[9,11] or “mathematically equivalent”.[12,13] They are, however, considered different in the treatments of point spread functions (PSFs). For example, PCH treats the PSF analytically and needs its exact form, while FIDA treats the PSF empirically and needs calibration experiments.[1,12−15]

Recently, their equivalence has been indicated indirectly by several publications addressing extended photon counting approaches for finite sampling time[16,17] or for optically biased diffusion.[18] However, their equivalence in the modeling of PSFs still remains unclarified. In this paper, we will compare the two methods and clarify to which extent they are equivalent or differ. From the common physical and mathematical basis of the two, we give a direct proof that they are theoretically equivalent; thus, the advantages in one method equally apply to the other. Furthermore, we show that they can model PSFs in the same manner. Simplified FIDA and PCH formulas in the form of power series are presented for fast and precise numerical calculations. The differences in the numerical efficiencies of the two methods are discussed.

## 2. FIDA and PCH Theories

In this part, we summarize briefly the basic principles of FIDA and PCH according to the original publications,[8,10] with small modifications in notions of physical quantities for the purpose of direct comparison of the two methods.

The main idea of FIDA is that[10] the overall photon count spectrum is the convolution of the photon count spectra from all of the spatial volume elements, $dV_i$, and the spectrum from any $dV_i$ is determined by the number distribution of the sample molecules and the shot noise. Both the number distribution and the shot noise can be described by Poisson distributions:[10]

$$P_i(k) = \sum_{n=0}^{\infty} \mathrm{Poi}(n, C_0\, dV_i)\, \mathrm{Poi}(k, n\epsilon W_i) \qquad (1)$$

where $C_0$ and $\epsilon$ are the equilibrium concentration and brightness of the sample particles, $k$ is the number of photons emitted from $dV_i$ in a sampling time, and $n$ is the particle number in $dV_i$. $\mathrm{Poi}(x, \bar{x}) = e^{-\bar{x}}\bar{x}^x/x!$, where $x = 0, 1, 2, ...$, is the Poisson distribution with an average value of $\bar{x}$. $W(\vec{r})$ is the normalized PSF which satisfies $W(0) = 1$. (In the original FIDA paper, the PSF was described by the “spatial brightness function, $B(\vec{r})$”,

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and a different normalization was used. We use here the same normalization as that used in PCH publications.)

In FIDA, $P(k)$ is expressed by the generating function $G(\xi) = \sum P(k)\xi^k$. $\xi$ is selected as $e^{i\phi}$, so $G(\xi)$ and $P(k)$ are connected by the Fourier transformation. According to the convolution theorem, $G(\xi)$ is the product of all $G_i(\xi)$: $G(\xi) = \prod G_i(\xi)$. This finally leads to[10]

$$G(\xi) = \exp(\int C_0\{\exp[(\xi - 1)\epsilon W(\vec{r})] - 1\}\mathrm{d}\vec{r}) \qquad (2)$$

PCH calculates the photon count spectra, $P(k)$, in quite a different manner.[8] First, the photon count spectrum, $p^{(1)}(k)$, of a single particle in a reference volume, $V_0$, is calculated. $p^{(1)}(k)$ is determined by the PSF and the shot noise:[8]

$$p^{(1)}(k) = \frac{1}{V_0}\int \mathrm{Poi}[k, \epsilon W(\vec{r})]\,\mathrm{d}\vec{r} \qquad (k > 0) \qquad (3)$$

$p^{(1)}(0)$ is determined by $1 - \sum_{k=1}^{\infty} p^{(1)}(k)$. If there are no particles in the reference volume, no photons will be detected, so $p^{(0)}(k) = \delta_{k0}$. When there are $n$ particles in the reference volume ($n > 1$), the photon count distribution is the convolution of $n$ $p^{(1)}(k)$:[8]

$$p^{(n)}(k) = \underbrace{p^{(1)} \otimes \cdots \otimes p^{(1)}}_{n \text{ times}}(k). \qquad (4)$$

Since the number of the particles in the reference volume follows Poisson distributions, the finial distribution, $P(k)$, is the weighted summation of all $p^{(n)}(k)$:[8]

$$P(k) = \sum_{n=0}^{\infty} \mathrm{Poi}(n, C_0V_0)\, p^{(n)}(k) \qquad (5)$$

## 3. Equivalence between PCH and FIDA

FIDA is implemented as the generating function of the probability histogram of the photon counts, a form different from that of PCH. However, it is known that any probability distribution is mathematically equivalent to its generating function, so FIDA and PCH actually do the same statistics on FFS data.

Both PCH and FIDA are essentially based on three factors: the particle number distribution, the PSF, and the shot noise. The particle number in a spatial volume follows Poisson distributions when the system reaches equilibrium and the concentration becomes time invariant. The shot noise also follows Poisson distributions. Since the shot noise is known, theories of photon count spectra consider only two problems: different particle numbers and different spatial positions.

FIDA first calculates the histogram for a given position and all particle numbers (eq 1) and then convolutes it over the whole space (eq 2). In contrast, PCH first calculates the histogram for a given particle number in a spatial volume, $V_0$ (eqs 3 and 4) and then sums it up for all particle numbers (eq 5). The two methods describe the same physical process and are therefore physically the same, but they are derived in different ways. PCH is confined in a reference volume, while FIDA treats the whole space.

Given the equivalence between extended PCH and FIDA for finite sampling time[16,17] or for optically biased diffusion,[18] it is only an inference to say PCH and FIDA are equivalent. However, since the proofs given in the above publications are complicated and indirect, it is helpful to give a direct and concise proof here. We prove the equivalence between PCH and FIDA by proving in general that the generating function of PCH formula eq 5 is the same as FIDA formula eq 2. We can calculate the generating functions of $P(k)$ in PCH as follows:

$$G^{(1)}(\xi) = \sum_{k=0}^{\infty} p^{(1)}\xi^k = (1 - \sum_{k=1}^{\infty} p^{(1)}) + \sum_{k=1}^{\infty} p^{(1)}\xi^k = 1 + \sum_{k=1}^{\infty} p^{(1)}(k)(\xi^k - 1)$$

$$G(\xi) = \sum_{n=0}^{\infty} \mathrm{Poi}(n, C_0V_0)[G^{(1)}(\xi)]^n = \exp[C_0V_0(G^{(1)}(\xi) - 1)]$$

$$\begin{aligned}
\ln G(\xi) &= C_0 \int \sum_{k=1}^{\infty} \frac{(\epsilon W)^k e^{-\epsilon W}}{k!}(\xi^k - 1)\,\mathrm{d}\vec{r} \\
&= C_0 \int \left( \sum_{k=0}^{\infty} \frac{(\epsilon W \xi)^k e^{-\epsilon W}}{k!} - \sum_{k=0}^{\infty} \frac{(\epsilon W)^k e^{-\epsilon W}}{k!} \right) \mathrm{d}\vec{r} \\
&= C_0 \int (e^{\epsilon W(\xi - 1)} - 1)\,\mathrm{d}\vec{r} \qquad (6)
\end{aligned}$$

This formula is exactly the same as that for FIDA (eq 2). Note that no specific PSF has been assumed in the above process. The conclusion is a general one which applies to any PSF.

## 4. Modeling of PSFs

Of the three factors mentioned in the last section, how to treat PSF $W(\vec{r})$ is the key problem when applying PCH and/or FIDA to analyze experimental data. In the current implementations, PCH and FIDA treat the PSFs in different ways. For example, FIDA treats the PSFs empirically and needs calibration experiments,[1,12−15] while PCH only applies when the PSF is known. In fact, the equivalence between PCH and FIDA is more general than the equivalence of theoretical histograms. They are equivalent in the treatment of the PSFs. Tricks and approximations developed for one of them can always be applied to the other.

**4.1. PSFs of Ideal Distributions.** If $W(\vec{r})$ can be measured or calculated, eqs 2 and 3 can be used directly. However, it is generally not easy to determine $W(\vec{r})$, and the numerical integrals are computation intensive. If $W(\vec{r})$ takes well-known distribution functions, the integrals can possibly be simplified.

We take the three-dimensional Gaussian (3DG) and the Gaussian−Lorentz squared (2GL) PSFs as examples. 3DG and 2GL are the most widely adopted PSFs in FFS experiments using single- and two-photon excitation.[8] The 3DG distribution is described by

$$W(\rho, z) = \exp\left(-\frac{2\rho^2}{{w_0}^2} - \frac{2z^2}{{z_0}^2}\right) \qquad (7)$$

where $\rho$ and $z$ are the cylindrical coordinates and $w_0$ and $z_0$ are the radial and axial beam waists, respectively. The 2GL distribution is described by

$$W(\rho, z) = \frac{{w_0}^4}{w^4(z)} \exp\left(-\frac{4\rho^2}{w^2(z)}\right) \qquad (8)$$

where $w^2(z) = w_0^2[1 + (z/z_R)^2]$ and $z_R = \pi w_0^2/\lambda$.

Before proceeding further, we have to discuss the choice of the reference volume, $V_0$, in PCH. $V_0$ is just an artificially introduced temporary quantity. One can see from eq 6 that $V_0$ is canceled out during the derivation and does not appear in $G(\xi)$. Therefore, the final histogram is independent of $V_0$. A precondition that $V_0$ should be large enough to ensure $p^{(1)}(0) > 0$ has been set by Chen et al.[8] and followed in later studies.[12] A quantity, $Q = V_0/V_g$, was introduced as the ratio between the reference volume and the focal volume. In fact, this requirement is physically but not mathematically necessary. One can choose a specific $V_0$ value to simplify the formula. We recommend that $V_0$ is chosen as the volume of the detection volume, $V_g = \pi^{3/2}w_0^2z_0$, that is, $Q = 1$, for the 3DG PSF, so $C_0V_0$ equals the average particle number, $N_g$, as is defined in the FCS literature. For the 2GL PSF, we can set $V_0 = V_{gl}$, where $V_{gl} = \pi^2 w_0^2 z_R$ and $N_{gl} = C_0V_{gl}$. For detailed discussions on the choice of $V_0$, please refer to Appendix A.

PCH and FIDA formulas in the form of single integrals were presented in the original papers and followed in later studies.[11,12] However, the efficiency and precision of numerical integrations are limited. One may accelerate the calculation by precalculating the integrals for different $k$ and $\epsilon$.[12] Since $\epsilon$ varies continuously, one cannot precalculate the integrals for all $\epsilon$. A power series of the 3DG PSF has been suggested previously[12] for PSF correction in PCH. Here, we show that such a power series can be used directly to calculate the PSF itself in both PCH and FIDA due to the equivalence between the two. By introducing $\omega_j = \int W^j(\vec{r})\,d\vec{r}$, PCH and FIDA can be expressed as

$$p^{(1)}(k) = \frac{1}{V_0 k!}\sum_{j=k}^{\infty}\frac{(-1)^{j-k}}{(j-k)!}\omega_j\epsilon^j \tag{9}$$

$$\ln G(\xi) = C_0\sum_{j=1}^{\infty}\frac{(\xi-1)^j}{j!}\omega_j\epsilon^j \tag{10}$$

We calculated that

$$\omega_j = \begin{cases}(2j)^{-3/2}V_g & \text{for 3DG PSF}\\ \dfrac{(4j-4)!}{2^{4j-2}[(2j-2)!]^2 j}V_{gl} & \text{for 2GL PSF}\end{cases} \tag{11}$$

By inserting $\omega_j$ into eqs 9 and 10, we get the PCH and FIDA formulas in the form of power series. The coefficients of $\epsilon^j$ can be computed and stored in advance to save the fitting time. The detailed derivation of $\omega_j$ for 2GL is in Appendix B.

**4.2. PSFs of Nonideal Distributions.** In real experiments, instrumental PSFs are generally nonideal. It has been proved that 2GL is a good description for two-photon excitation, whereas the 3DG PCH model failed to fit one-photon excitation experimental data.[12,19,20]

A very clever approach has been exploited in FIDA to treat even completely unknown PSFs. If one defines $x(\vec{r}) = -\ln W(\vec{r})$ and $V(x_0)$ as the spatial volume in which $x(\vec{r}) < x_0$, eq 2 can be rewritten as[10]

$$G(\xi) = \exp\left(\int_0^{\infty} C_0\,\{\exp[(\xi-1)\epsilon e^{-x}] - 1\}\,\frac{dV(x)}{dx}\,dx\right) \tag{12}$$

$dV(x)/dx$ is described by an adjustable formula $\sum_k a_k x^k$, where $a_k$'s are to be determined by calibration experiments using known fluorescent dye solutions. Theoretically, any PSF can be approximated by such a polynomial. Although this approach has been regarded as a characteristic feature of FIDA, it can also be used in PCH if we rewrite eq 3 as

$$p^{(1)}(k) = \int_0^{\infty}\mathrm{Poi}(k, \epsilon e^{-x})\,\frac{dV(x)}{dx}\,dx \tag{13}$$

This approach is fast and versatile but is empirical and lacks physical insight. One can choose more physically meaningful $dV(x)/dx$. For example, we proved that the analytical form of $dV/dx$ for 3DG is $\sqrt{x/2\pi}V_g$ (see Appendix C). $dV/dx$ for the 2GL PSF can also be calculated numerically.

When the real PSF is not far from the ideal distributions, using correction parameters can be a good choice. One possibility is to introduce extra parameters to the analytical $dV/dx$, for example, $\sqrt{x/2\pi}V_g$ for 3DG. A successful approach was presented in a previous work,[12,20] which introduced the parameter $F_j$, the relative error in $\omega_j$, to correct the 3DG PSF in one-photon PCH experiments:

$$p^{(1)}(k) = \frac{1+F_2}{(1+F_1)^2}\left[p_g^{(1)}(k) + \frac{1}{k!}\sum_{j=k}^{\infty}\frac{(-1)^{j-k}}{(j-k)!(2j)^{3/2}}\epsilon^j F_j\right] \tag{14}$$

where the subscript g indicates the 3DG PSF. In this approach, $\omega_j$ was corrected as $(1 + F_j)\omega_j$ and $V_0$ as $V_0(1 + F_1)^2/(1 + F_2)$.

This approach can also be applied to FIDA. Furthermore, the correction is simpler and more concise for FIDA because the temporary quantity $V_0$ does not appear in the FIDA formula at all and only $\omega_j$ needs to be corrected:

$$\begin{aligned}\ln G(\xi) &= \ln G_g(\xi) + C_0\sum_{j=1}^{\infty}\frac{(\xi-1)^j}{j!(2j)^{3/2}}F_j\epsilon^j\\ &= C_0\sum_{j=1}^{\infty}\frac{(\xi-1)^j}{j!(2j)^{3/2}}(1+F_j)\epsilon^j\end{aligned} \tag{15}$$

When using this correction approach in FIDA or PCH, the simplified formulas eqs 9 and 10 can still be used for fast numerical calculations.

## 5. Differences between PCH and FIDA

We have demonstrated that PCH and FIDA are two equivalent data analysis approaches. Their theoretical histograms are the same, and analyzing experimental (or simulated) data by the two methods gives the same results. Thus, the differences between them, if any, lie in the numerical efficiency. We tested the performance of PCH and FIDA calculations based on both direct numerical integration (DNI) and power series expansion (PSE) by homemade c++ programs. FFTW[21] and GNU Scientific Library[22] are utilized in the programs.

The DNI PCH, DNI FIDA, PSE PCH, and PSE FIDA algorithms are based on eqs 13, 12, 9, and 10, respectively, with further transformations to optimize the calculation speed. In our tests, DNI FIDA costs much longer calculation time than DNI PCH due to the deficiency in the program algorithm available to us to calculate integrals of complex number functions, so the results are not listed below.

The computation time of the calculations is closely related to the required numerical precision. Therefore, we compare the efficiency of different approaches using a numerical precision of $10^{-8}$, which corresponds to a maximum number of $10^8$ total photon counts collected in an experiment. The length of the histograms is 100, which is more than enough for typical experiments. The precision of the integrations and power series summations are controlled to meet the requirement that the

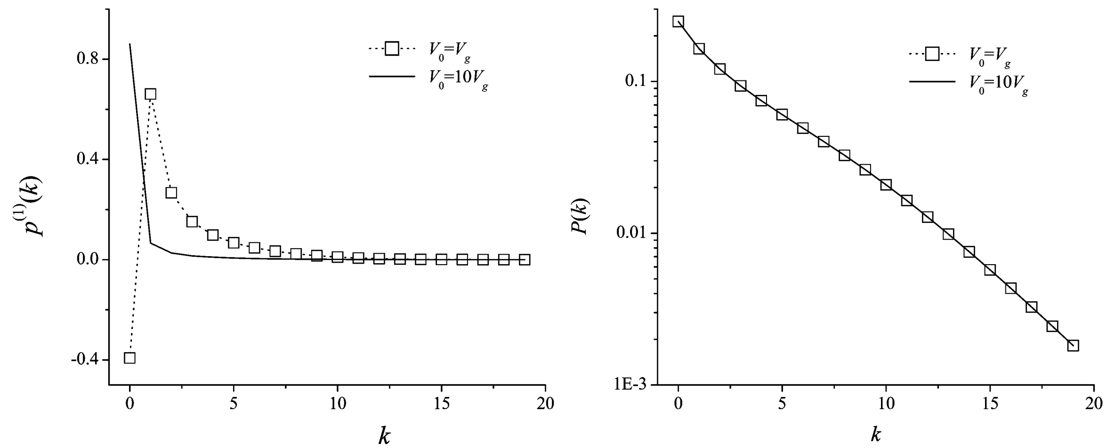


**Figure 1.** PCH calculated using $V_0 = V_g$ and $V_0 = 10V_g$, separately. $p^{(1)}$ is different with different choices of $V_0$, but the final histogram, $P(k)$, is identical.

**TABLE 1: Comparisons of the Computation Time (seconds) of Calculating Theoretical Histograms for PCH and FIDA[a]**

| method | $N = \epsilon = 0.08$ | $N = \epsilon = 0.8$ | $N = \epsilon = 8$ |
|---|---|---|---|
| PSE PCH | $3.07 \times 10^{-5}$ | $3.26 \times 10^{-5}$ | $4.27 \times 10^{-5}$ |
| PSE FIDA | $3.56 \times 10^{-5}$ | $4.95 \times 10^{-5}$ | $11.58 \times 10^{-5}$ |
| DNI PCH | $3.28 \times 10^{-3}$ | $3.38 \times 10^{-3}$ | $4.18 \times 10^{-3}$ |

[a] The maximum absolute error of the histograms is less than $10^{-8}$, and the length of the histograms is 100. The calculations are implemented under Windows XP and Microsoft Visual Studio 2005 on a typical personal computer.

maximum absolute error of $P(k)$ is smaller than $10^{-8}$. The parameters for the tests are chosen arbitrarily in the most possible range of their practical values.

The results (Table 1) show that computations by PSE are always 2 orders of magnitudes faster than those by DNI. Therefore, PSE should be preferred over DNI in data analysis. The computation times for PSE PCH and PSE FIDA are of the same order, but when the parameters ($N$, $\epsilon$) increase, the computation time of PSE FIDA grows faster than that of PSE PCH. The reason is as follows. When calculating histograms with larger parameters, more terms should be calculated to keep the precision of the power series summation. The main contribution to the computation time in PSE PCH is by the convolution calculation, which is independent of the number of terms, while that in PSE FIDA is by the power series summation.

## 6. Conclusion

We demonstrated that FIDA and PCH are equivalent not only in the statistical method and the theoretical formulations but also in the manner of modeling experimental PSFs. The two methods can be drawn into the same unified theoretical framework. Approaches developed from any one of the methods can be applied to the other. For example, PCH can model the experimental PSF using an adjustable formula as FIDA,[10] FIDA can model the PSF analytically as PCH, the correction approach of the PSF developed for PCH[12,20] can be used in FIDA, and both PCH and FIDA formulas can be expanded into power series. As far as a sufficiently high precision is set in the calculations, one method can completely replace the other. More specifically, PCH and FIDA share the same modeling approaches as follows.

Known PSFs can be modeled by (a) a full three-dimensional description, $W(\vec{r})$, (b) a reduced one-dimensional description, $\mathrm{d}V(x)/\mathrm{d}x$, or (c) moments, $\omega_j$. Unknown PSFs can be modeled by (d) introducing empirical parameters to the ideal $\mathrm{d}V(x)/\mathrm{d}x$ formula, (e) $\mathrm{d}V(x)/\mathrm{d}x$ in power series form, or (f) introducing correction parameters to $\omega_j$.

The differences between the two methods only lie in their numerical efficiency. Though the calculation time may vary due to different hardware and software and may be subject to further optimization, our results show that PSE formulas are of much higher efficiency than the DNI formula. When a power series expansion is used for the calculations of PSFs, we recommend PCH over FIDA in data fittings, because PCH calculations require less computation time than FIDA for large $N$ and $\epsilon$.

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## Appendices

**A. Choice of $V_0$ in PCH.** As can be seen from eq 6, $V_0$ will be canceled out and does not affect the final histogram. Thus, it is just an artificially introduced temporary quantity. Chen et al.[8] required that $V_0$ should be large enough to ensure $p^{(1)}(0) > 0$. In fact, this requirement is physically but not mathematically necessary. We calculated $P(k)$ with the 3DG PSF using $V_0 = V_g$ and $V_0 = 10V_g$, separately. The results are shown in Figure 1.

One can see that different choices of $V_0$ lead to distinct $p^{(1)}(k)$ but identical $P(k)$. Even if $p^{(1)}(0)$ is negative, the final result is correct. Therefore, one can arbitrarily choose any $V_0$ value in the calculations. We recommend that $V_0$ is chosen as the volume of the detection volume, $V_g = \pi^{3/2}w_0^2z_0$, for the 3DG PSF. In this case, $C_0V_0$ equals the average particle number, $N_g$, as is defined in the FFS literature. Thus, the results of PCH and FCS can be compared directly. For the 2GL PSF, we can set $V_0 = V_{gl}$, where $V_{gl} = \pi^2w_0^2z_R$ and $N_{gl} = C_0V_{gl}$.

**B. Derivation of $\omega_j$ for the 2GL PSF.**

$$W(\vec{r}) = \frac{{w_0}^4}{w^4(z)} e^{-\frac{4\rho^2}{w^2(z)}}$$

$$\begin{aligned}\omega_j &= \int W^j(\vec{r})\, d\vec{r} \\ &= \int_{-\infty}^{\infty} dz\, \frac{{w_0}^{4j}}{w^{4j}(z)} \int_0^{\infty} 2\pi\rho\, d\rho\, e^{-\frac{4j\rho^2}{w^2(z)}} \\ &= \int_{-\infty}^{\infty} dz\, \frac{{w_0}^{4j}}{w^{4j}(z)} \frac{\pi w^2(z)}{4j} \\ &= \frac{\pi {w_0}^2}{4j} \int_{-\infty}^{\infty} \frac{1}{(1 + (z/z_0)^2)^{4j-2}}\, dz \\ &= \frac{\pi {w_0}^2 z_0}{4j} \frac{\sqrt{\pi}\,\Gamma(2j - 3/2)}{\Gamma(2j - 1)} \\ &= \frac{(4j - 4)!}{2^{4j-2}[(2j - 2)!]^2 j} V_{gl}\end{aligned} \tag{16}$$

**C. Derivation of d$V(x)$/d$x$ for the 3DG PSF.** For the 3DG PSF, a $W(\vec{r}) = w$ surface is described by

$$\frac{2\rho^2}{{w_0}^2} + \frac{2z^2}{{z_0}^2} = -\ln w = x \tag{17}$$

The volume it encloses is

$$V(x) = \frac{4}{3}\pi {w_0}^2 z_0 \left(\frac{x}{2}\right)^{3/2} \tag{18}$$

Therefore,

$$\frac{dV(x)}{dx} = \sqrt{\frac{x}{2\pi}} V_g \tag{19}$$

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