

Cross-Validated Confocal Volume Characterization Using FCS and PSF Analysis

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Received March 28, 2026; accepted October 16, 2026; published October 17, 2026

Abstract— Accurate confocal volume characterization is fundamental to quantitative Fluorescence Correlation Spectroscopy (FCS) and confocal microscopy measurements. Refractive index mismatches and localized thermal effects can systematically distort biophysical parameters (diffusion coefficients, molecular concentrations) if not properly calibrated. This study cross-validates two complementary approaches for confocal volume characterization: FCS (using fluorescent molecules of known or unknown concentration) and direct Point Spread Function (PSF) measurement using sub-diffraction fluorescent beads, to assess accuracy in determining the effective confocal volume V_{eff} , beam waist w_0 , structural parameter s , diffusion time τ_D , diffusion coefficient D , and correlation amplitude G_0 . FCS and PSF yielded closely matched effective volumes of 0.89 fL and 0.84 fL, respectively.

Confocal microscopy has become an indispensable tool in biology and biophysics, offering improved optical resolution and the ability to probe samples in three dimensions [1]. At the heart of this technique is the confocal volume, which is a small region of the sample from which fluorescence is collected. Precise knowledge of the size and shape of this volume is fundamental to any quantitative analysis, particularly in Fluorescence Correlation Spectroscopy (FCS), where fluctuations in light intensity are used to infer molecular dynamics, such as diffusion coefficients and concentrations [2–4].

FCS is a powerful technique that analyses fluorescence fluctuations resulting from the Brownian motion of fluorescent molecules through a defined excitation and detection volume. These fluctuations are correlated over time, generating an autocorrelation function whose analysis allows the extraction of key biophysical parameters. However, the accuracy of these parameters is directly dependent on the precise characterization of the confocal volume. External factors, such as localized heating induced by optical traps [5] or refractive index mismatches [6], can influence these measurements.

The confocal volume is generally modeled as a three-dimensional Gaussian ellipsoid, characterized by a lateral beam waist (w_0) and an axial half-length (z_0). The autocorrelation function $G(\tau)$ in FCS for 3D diffusion is given by:

$$G(\tau) = G_0 \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + \frac{\tau}{s^2 \tau_D}\right)^{-1/2}, \quad (1)$$

where G_0 is the correlation amplitude at $\tau = 0$, which is inversely proportional to the average number of molecules in the confocal volume. τ_D is the diffusion time, which represents the average time taking by a molecule takes to traverse the beam waist. s is the structural parameter, defined as the ratio z_0/w_0 , which describes the ellipticity of the confocal volume.

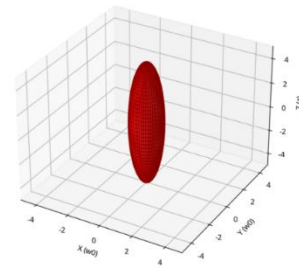


Fig. 1. Illustration of the 3D Gaussian confocal volume, defined by its lateral (w_0) and axial (z_0) dimensions.

The effective confocal volume V_{eff} represents the volume from which fluorescence is collected. For a 3D Gaussian volume, it is expressed as:

$$V_{eff} = \pi^{3/2} w_0^2 z_0 = \pi^{3/2} s w_0^3. \quad (2)$$

The diffusion time τ_D is directly related to the diffusion coefficient D and beam waist w_0 by the following relation:

$$\tau_D = \frac{w_0^2}{4D}. \quad (3)$$

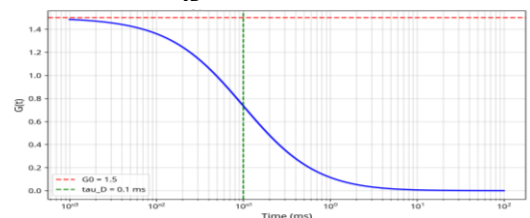


Fig. 2. A typical FCS autocorrelation curve showing the correlation amplitude (G_0) and the diffusion time (τ_D).

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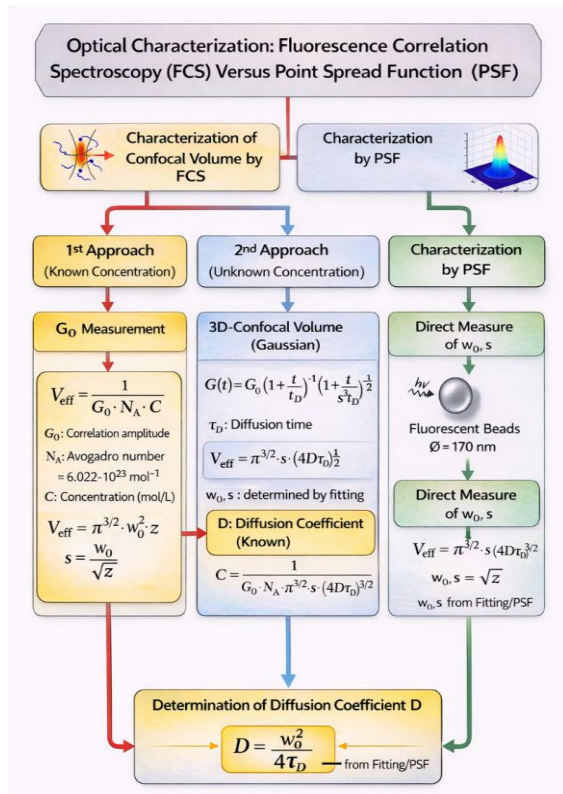


Fig. 3. Synoptic diagram illustrating the two complementary strategies for optical characterization of a confocal detection volume.

FCS Setup (Approaches 1 and 2): FCS measurements were conducted using an inverted microscope (Nikon) equipped with an Olympus UPlanAPO NA 1.2/60x water immersion objective. The excitation source was a 532 nm laser (Optoelectronics Company, UK) that operated at a maximum power of 2 mW. The laser beam was expanded through an afocal system to ensure complete filling of the back aperture of the objective. A dichroic mirror reflected the excitation beam to the objective, which focused the beam onto a fluorescent solution (Alexa Fluor 532; Life Technologies, USA). The fluorescent light emitted by the solution was collected with the same objective, transmitted through a dichroic mirror, and focused onto a 30 μ m pinhole by a lens. Subsequently, a 50/50 beam splitter divided the light and directed it to two avalanche photodiodes (APDs) with single-photon sensitivity (SPCM-AQRH, Excelitas Technologies, Canada).

PSF Measurements Setup (Approach 3): Fluorescent polystyrene latex microsphere samples were prepared for PSF characterization. These microspheres had a diameter of $\Phi = 170 \pm 5$ nm (PS-Speck™ Kit, Invitrogen), a refractive index of 1.6, and exhibited maximum excitation absorption and emission at 540 and 560 nm, respectively. A droplet of a highly diluted solution (10^6 stock dilution) of these microspheres was dried on a coverslip. The scan plane of the sample was precisely moved in different directions using a XYZ piezo flexure stage (Physik Instrumente, Germany). A scan distance of 200 nm was

chosen between consecutive XY sections and 20 nm for the z-sections to obtain detailed PSF profiles.

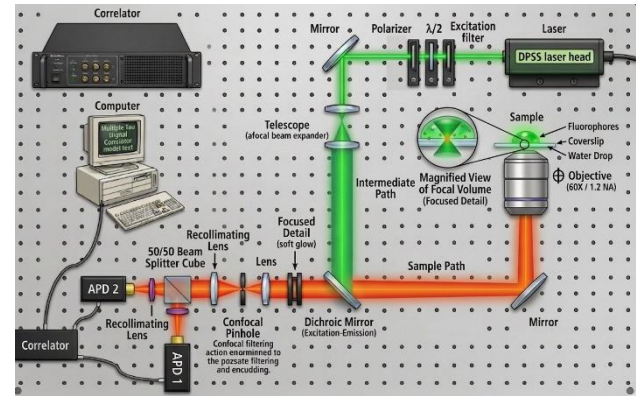


Fig. 4. Schematic of the experimental FCS setup.

Confocal Volume Measurements: Numerical Calculation Using the experimental conditions ($\lambda_{ex}=532$ nm, NA=1.2, and $n=1.33$), the theoretical lateral and axial resolutions were determined using:

$$FWHM_{xy} = \frac{0.51\lambda_{ex}}{NA}, \quad (4)$$

$$FWHM_z = \frac{0.51\lambda_{ex}}{NA}. \quad (5)$$

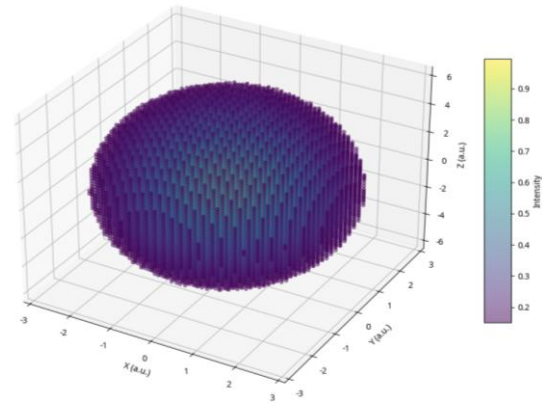


Fig. 5. A representative numerical 3D Point Spread Function (PSF).

Accurate characterization of the confocal volume is essential for obtaining reliable quantitative measurements. The experimental data obtained from FCS and PSF measurements are summarized in Table 1.

Tab.1. Dimensions of the effective volume determined by theoretical calculation, FCS, and PSF measurements.

Method	$FWHM_{xy}$ (nm)	$FWHM_z$ (nm)	V_{eff} (fL)
Theoretical	217 ± 10	530 ± 10	0.11 ± 0.012
FCS	400 ± 30	1000 ± 10	0.89 ± 0.013
PSF	680 ± 10	1120 ± 10	0.84 ± 0.026

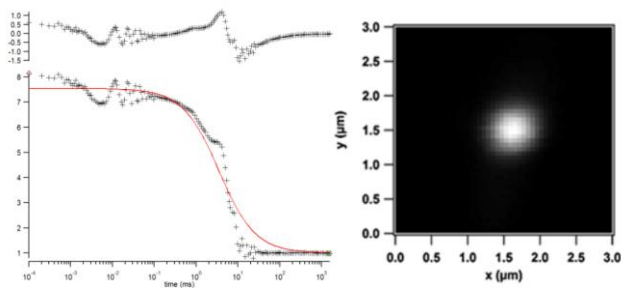


Fig. 6. (left) Fluorescence correlation curve of Alexa Fluor 532 and the fitting curve. (right) Lateral PSF image of a fluorescent bead.

The effective volumes obtained by FCS (0.89 ± 0.013 fL) and PSF (0.84 ± 0.026 fL) are in close agreement, although both exceed the theoretical value (0.11 ± 0.012 fL). This discrepancy is attributed to optical aberrations inherent in the system. The lateral and axial *FWHM* also show differences between the methods, reflecting the specificities of each technique.

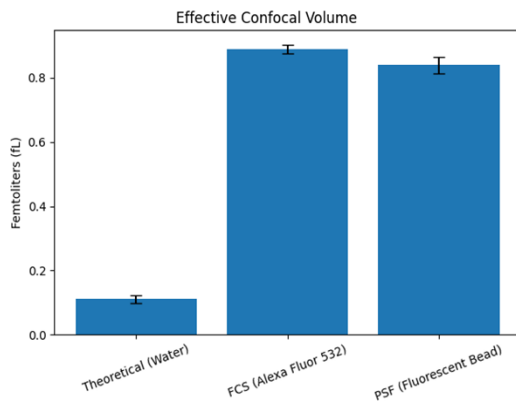


Fig. 7. Comparative analysis of effective confocal volume (V_{eff}) obtained through theoretical calculation, FCS, and PSF measurements.

A comparison between FCS and PSF for confocal volume characterization revealed their complementarity. While the PSF provides a direct measurement of the volume's geometric dimensions, FCS enables extraction of dynamic and concentration information. The use of fluorescent beads for PSF calibration is a standard method; however, it is important to note that the beads' optical properties may differ from those of the molecules being studied [11]. Furthermore, environmental factors, such as localized heating in the focus of an optical trap, can affect diffusion measurements and require special attention [5].

Refractive index mismatches between the sample and immersion medium can also distort the Gaussian beam, affecting volume confinement and leading to systematic errors in FCS measurements [6]. Therefore, accurate PSF modeling is critical, and recent studies have focused on applying effective medium approximations to better describe the axial PSF, which is particularly sensitive to optical aberrations [13]. The use of adaptive optics to correct such aberrations in real time represents a promising

frontier for improving the accuracy of quantitative microscopy [14].

The accuracy of diffusion coefficients is essential for many applications, including studies on protein-protein interactions, membrane dynamics, and intracellular transport [4, 15]. Compilations of reference data [7] are invaluable for calibrating and validating FCS measurements. The integration of these methods into advanced systems enables quantitative, reliable characterization of the confocal volume, paving the way for deeper studies of molecular processes in complex environments.

In conclusion, rigorous characterization of the confocal volume is a cornerstone of quantitative confocal microscopy and Fluorescence Correlation Spectroscopy. Approaches based on FCS, whether using known or unknown concentrations, as well as direct PSF measurements with fluorescent beads, offer powerful and complementary tools for this task. Understanding and correctly applying the theoretical frameworks governing V_{eff} , τ_D , w_0 , s , D , and G_0 are crucial for obtaining reliable data on diffusion coefficients and molecular concentrations. By combining these techniques and accounting for potential artifacts, such as refractive effects and thermal loading, researchers can overcome calibration challenges and conduct biophysical studies with increased precision and confidence.

The authors would like to thank the Algerian Ministry of Higher Education and Scientific Research MESRS (CNEPRU Research Project 14MDU921) for their financial aid and technical support.

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