Convective-diffusion-based fluorescence correlation spectroscopy for detection of a trace amount of E. coli in water

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Fluorescence correlation spectroscopy (FCS) is adapted for a new procedure to detect trace amounts of Escherichia coli in water. The present concept is based on convective diffusion rather than Brownian diffusion and employs confocal microscopy as in traditional FCS. With this system it is possible to detect concentrations as small as 1.5 × 10^5 E. coli per milliliter (2.5 × 10^-16 M). This concentration corresponds to an ~1.0-nM level of Rhodamine 6G dyes. A detailed analysis of the optical system is presented, and further improvements for the procedure are discussed. © 2003 Optical Society of America

1. Introduction

In modern food-processing applications, safety is of the utmost concern. Automation of food processing and increased concentration of plants stress the use of available water supplies. The water used for food processing must be free of undesirable biological agents, such as Escherichia coli bacteria. It is important to have on-line, real-time testing of water supplies. The current state-of-the-art procedures for determining bacterial contamination in water are relatively time consuming and not necessarily expandable to trace-amount detection. For example, the most frequently used method for the measurement of bacterial populations is the plate count, which takes 24 h or more to yield results.¹

Improvements in food safety can be assisted by fast and accurate methods for detecting low concentrations of microbial contaminants in water. Detection of E. coli is particularly important because it is associated with a broad range of diseases. This paper outlines a different approach for detection of trace amounts of E. coli in flowing water. The system used is based on fluorescence correlation spectroscopy (FCS) and a confocal microscopy system. Even though the current experimental setup resembles the traditional FCS systems, its optical arrangement as well as its setting of flow system are quite different. The current setup is based on convective diffusion; therefore, it is built around a capillary flow unit. The solution laden with a trace amount of stained bacteria is circulated several times through the detection volume to increase the sensitivity. On the other hand, the control volume defined by the focused laser beam is relatively larger than that of typical FCS systems, as our objective is to detect E. coli bacteria, which are much bigger than the traditional target dye molecules. Below, we first present a brief literature review, followed by experimental details and analysis of the optical system. Finally, we outline typical results and discuss possible improvements of the system.

FCS, which was first proposed by Magde et al.² in the early 1970s, is a special case of fluctuation correlation spectroscopy. FCS is based on the assumption that local spontaneous fluctuations of intrinsic thermodynamic properties of a physical or chemical system in equilibrium are potential sources of information rather than a mere noise. It is shown that the dispersion (mean square amplitude) of spontaneous fluctuations around the thermodynamic mean are proportional to the number of independent accessible degrees of freedom³; therefore, by analyzing and correlating these fluctuations, one can extract useful information from the noise in order to understand the underlying physical, chemical, or biological phenomena.

FCS is similar to photon correlation spectroscopy.
It is, however, based on detection of the laser-induced fluorescence emitted from a fluorescent molecule or particle rather than the light scattered by molecules or small particles going through Brownian motion. FCS is considered inherently more sensitive and is expected to have more precise spatial selectivity. In addition, FCS can be used with small, weakly scattering particles.

Over the years FCS has been widely applied to the investigation of processes related to particle concentration and particle diffusion in solutions and through membranes. With modern instrumentation, such as confocal detection optics\(^4\) and high-quantum-efficiency single-photon avalanche diode and two-photon excitation,\(^5\) FCS techniques saw a significant improvement in 1990s. These recent advancements have improved the applicability of FCS at the single-molecule level, which made it a likely candidate for rapid DNA sequencing and high-throughput screening assays for speeding the pharmaceutical drug discovery processes.\(^6\)

2. Theoretical Analysis

The parameter measured in traditional FCS is the laser-induced fluorescence signal, \(F(t)\), from particles or molecules suspended in water. The fluorophore absorbs energy from the incident laser beam. This causes electrons to be temporarily excited from their ground state. Upon their return to the ground state, electrons release a photon at a higher wavelength and lower energy than the absorption wavelength, producing the effect known as fluorescence. The fluorescence intensity is dependent on the number of fluorophores available in the measurement volume defined by the focus of the impinging laser beam. The fluorescence fluctuations are due to fluctuations in the concentration of fluorophores, which may be caused by Brownian diffusive motion of a fluorophore in and out of the observation volume. Alternatively, induced convective diffusion forces the fluorophores to go through the control volume sporadically, which is seen as fluctuations over background measurements. In addition, if within the control volume any chemical reaction or biological activity causes a fluorophore to move from a fluorescent state to a non-fluorescent state, the emitted fluorescence intensity fluctuates. Effectively, observing and analyzing these fluctuations allows the details of the diffusion process or the extent of the chemical or biological activity within the observation volume to be determined. A quantitative analysis is possible if these fluctuations are calibrated carefully.

Autocorrelation functions are used to identify the nature of fluctuations. More accurately, with an autocorrelation function, a fluctuation \(F(t)\) at time \(t\) is compared with another one shifted by a time lag \(\tau\), i.e., \(F(t + \tau)\). The normalized autocorrelation function \(G(\tau)\) is defined as

\[
G(\tau) = \frac{\langle F(t)F(t + \tau) \rangle - \langle F(t) \rangle^2}{\langle F(t) \rangle^2},
\]

where \(\langle \rangle\) denotes the time average. If \(\tau = 0\), \(F(t)\) and \(F(t + \tau)\) are completely in phase with each other and \(\langle F(t)F(t + \tau) \rangle\) is a relatively large quantity. As \(\tau\) increases, \(F(t)\) and \(F(t + \tau)\) begin to become out of phase with each other, the autocorrelation function \(\langle F(t)F(t + \tau) \rangle\) tends to approach \(\langle F(t) \rangle^2\), and \(G(\tau)\) approaches zero. This means that the autocorrelation function \(G(\tau)\) characterizes the time-dependent decay of the fluctuations according to their equilibrium value.

The value of the autocorrelation function when time lag is zero, \(G(0)\), is given as

\[
G(0) = \frac{\langle F^2(t) \rangle - \langle F(t) \rangle^2}{\langle F(t) \rangle^2} = \frac{\sigma^2F}{F^2},
\]

where the numerator \(\sigma^2F\) is the variance of \(F\), indicating deviations of \(F\) from its mean value. The denominator is simply the square of the mean value. The fluorescence intensity is directly related to the number of fluorophores in the measurement volume element. The number of fluorophores in the sample volume element obeys Poisson statistics. \(G(0)\) can eventually be expressed in terms of the number density of fluorophores:

\[
G(0) = \frac{\gamma}{N},
\]

where \(N\) is the mean value of the fluorophore number in the sample volume element and \(\gamma\) is the geometric shape factor of the illumination beam.\(^7\) For a Gaussian beam, the value of \(\gamma\) is \((1/2)^{3/2}\). Equation (3) indicates that the amplitude of \(G(\tau)\) depends on the number of fluorophores in the sample volume element. Theoretically, \(G(0)\) can be calculated directly from Eq. (2) without requiring any autocorrelation analysis; however, in reality \(F(t)\) contains shot noises from photon detectors, which can lead to significant errors. Thus autocorrelation functions are still necessary to filter out those shot noises. This is generally done by extrapolating the curve of \(G(\tau)\) to \(\tau = 0\).

The objective in this project is to detect very low concentrations of \(E.\ coli\) in flowing water. For the experiments very dilute suspensions are prepared, and sample solutions are circulated with a flow system and a pump. Even though the traditional FCS experiments are validated on stationary solutions, the analysis that leads to Eq. (3) should still hold for flowing samples if the sample is homogeneous and the flow is uniform. It should be noted that circulating the sample does not increase the spatial probability of a fluorophore’s appearing in the sample volume element. However, the fluctuation decay time is shortened, and the convergence of the statistical accuracy is accelerated; therefore the required experiment time can be significantly shorter. The use of convective diffusion is particularly beneficial for the detection of very low concentrations, which otherwise needs lengthy experiments to secure reasonable statistical accuracy.
3. Experimental Details

A. Flow System

The flow system consists of a 32-mm-long, 700 μm-inside-diameter, 850 μm-outside-diameter fused-silica capillary (Polymicro Technologies, Phoenix, Arizona). The polyimide coating of the capillary is removed by being burned away. The two ends of the capillary are connected with Teflon heat-shrinking tubes. The fused-silica capillary is put under an objective lens and mounted on an xyz stage, which allowed precise positioning of the capillary to the focal point of the laser beam. The circulation of the sample solution is established with the help of a variable-flow peristaltic pump (Fisher Scientific, Pennsylvania). The inlet of the flow system is put at the bottom of a 25-mL beaker, which serves as a reservoir. The outlet of the flow system is suspended over the beaker, above the level of sample solution so that droplets coming down from the outlet stir the sample solution and ensure that the flow is uniform. All experiments are conducted at room temperature. (It is possible that temperature of fluorophores increases during the experiment; however, because of rapid circulation of solution this effect is not considered important.)

Escherichia coli is purchased from Molecular Probes Inc. (Catalog E-13231, Bioparticle, Alexa Fluor 488 conjugate). This version of the bacteria is already stained with Alex Flur dye. A stock E. coli suspension is prepared by dispersing 0.5 mg of E. coli in 10 mL of deionized water. The concentration of bacteria in the original solution is approximately 1.5 × 10^7 cells/ml. The stock suspension is mixed for 20 min and vortexed for 2 min to yield a homogenous suspension. The stock suspension is then diluted to prepare samples with concentrations of 1.5 × 10^6, 1.5 × 10^5, 1.5 × 10^4, 1.5 × 10^3, 1.5 × 10^2, 1.5, and 0.15 ml^-1, respectively, for different experiments.

For each of these eight suspensions, a 5-mL quantity is transferred into the reservoir. For each sample, ten consecutive measurements are performed at a flow rate of 5 ml/min and autocorrelation duration of 2 min.

There are two reasons that we choose to perform ten shorter experiments rather than a single length one. First, we obtain a better statistical average if we have multiple experimental data, and having multiple results of long experiments is both impractical and potentially prone to errors because of photobleaching. The second reason arises from an agglomeration problem; we found out that once in a while a very bright fluorescence emission is received because of large agglomerates of stained bacteria. These agglomerates may severely distort the autocorrelation curves; however, if the experiments are repeated multiple times, these effects do not have a significant effect on the results. Preliminary results showed that these time intervals and repetitions yield reliable and replicable results. The experiments are performed in a random sample order. After each experiment, the system is purged with ethanol and water alternately until the fluorescence signal is reduced to that of deionized water. This procedure ensures the use of an accurate concentration for each experiment.

B. Optical System

In most recent FCS systems a Minsky-type or infinity-corrected confocal fluorescence microscope is employed. The advantages of confocal optics are twofold. (1) The same objective lens is used for illumination and collection of fluorescence signal. The objective lens has a high numerical aperture (N.A.), yielding diffraction-limited measurements. (2) A pinhole at the image plane is used to eliminate all background light coming from the out-focal planes.

Figure 1 shows that the optical system consists of a laser, diverging lens, dichromatic mirror, objective lens, and an optical fiber. A cw argon-ion laser (Laser Physics, Salt Lake City, Utah) is used to shine a 488-nm beam of light to excite prestained E. coli present in a sample solution flowing through a clear capillary tube. In this paper our objective is to detect a very small amount of prestained bacteria, which are much larger and much brighter than a single dye molecule. Actually the bacteria become so bright that we have to lower the laser intensity (to ~0.1 mW in sampling volume); otherwise the fluorescence saturates the photomultiplier tube and seriously distorts the autocorrelation curves. Therefore the light intensity we used is far less than that for conventional FCS experiments, where the required intensity is of the order of several microwatts. We also have to add that the dye used in these experiments is Alexa Fluor 488, which is quite stable. Because of the stability of the dye and the use of a very low laser intensity, we believe that photobleaching is not a major problem in our experiments.

The E. coli is stained with Alexa Fluor dye with a maximum absorption wavelength of 498 nm and an emission wavelength of 515 nm. The excitation light beam is diverged by a negative lens (f = −25 mm) and directly reflected by a dichromatic mirror (Model 505DRLP, Omega, Brattleboro, Vermont) into a standard microscope objective lens (40×, N.A. 0.55, Nikon) with an effective focal length of 4.48 mm. These components ensure that the light beam is expanded to just cover the back aperture of the objective lens, resulting in a tightly focused laser beam.

The reason we use an objective lens with relatively low N.A. is twofold. First, we do not need to focus the laser beam to a very small volume, as the E. coli bacteria we attempt to detect is much larger than the fluorophores used in traditional FCS settings. In addition, if the N.A. of the objective lens is larger than 0.8, one has to be careful about the shape of the focusing laser beam. Under these conditions, filling the back aperture may lead to a non-Gaussian sampling volume; one has to underfill the back aperture to reduce the effective N.A. in order to get a Gaussian sampling volume. The second reason for our choice of lower N.A. is to have longer working distances. A
high-N.A. setup requires a short working distance, which is not desirable in our case, as a capillary needs to be used under the objective lens. Our system is optimized around an objective lens with N.A. 0.55. In addition, a 10-μm-diameter pinhole in the image plane of the microscope is used to eliminate the out-of-focus light. It is important to add here that Hess and Webb⁹ include more details of FCS optics and design for microscopy applications.

The fluorescence light is detected by a photon counter based on a photomultiplier tube (Model HC120-08, Hamamatsu). The electrical signal from the photon-counter unit is sent to a multitau digital correlator (Model Flex999R-480) connected to a processor. An image of the laser beam focused on the capillary tube is shown in Fig. 2.

C. Analysis

A detailed analysis of the optical system is performed to optimize the light detection from fluorophores. Below, the analysis for a fixed set of distances is outlined first (see Fig. 1 for definitions, and Table 1 for the values used).

The laser beam ($J_0$) with a waist of 0.65 mm enters neutral-density filter 2 of transmissivity $\tau_1$. The transmitted laser beam then has the intensity $H_1$:

$$H_1 = \tau_1 J_0.$$  (4)

The laser beam then enters laser bandpass filter 3 of transmissivity $\tau_2$, and the transmitted beam's intensity is reduced to $H_2$:

$$H_2 = \tau_2 \tau_1 J_0.$$  (5)

Fig. 1. Detailed schematic of the optical system composed of (1) argon-ion laser, (2) neutral-density filter, (3) bandpass filter, (4) diverging lens, (5) dichromatic mirror, (6) microscope objective lens, (7) stage, (8) filter, (9) converging lens, (10) pin hole, (11) optical fiber, (12) photon-counting unit, (13) digital correlator, (14) processor, (15) capillary tube or flow system, (16) reservoir, (17) pump.

Fig. 2. Image of the focused laser beam. The beam waist is $\sim$20 μm.
As the laser beam travels through the path, it is reflected by the dichromatic mirror and transmitted through the objective lens and the capillary tube; therefore the excitation irradiance, \( H_6 \), on the *E. coli* suspension becomes

\[
H_6 = \frac{\tau_6 \tau_3 \tau_2 \tau_1 J_0}{\rho \left( d_5 + d_6 \right)} \kappa A J_0,
\]

where \( \tau_6, \tau_3, \tau_2, \) and \( \tau_1 \) are the transmissivities of the capillary tube, objective lens, diverging lens, bandpass filter, and neutral-density filter, respectively, and \( \rho \) is the reflectivity of the dichromatic mirror. The intensity of the laser beam, \( J_0 \), is assumed to be constant over the small solid angle subtended.

The intensity of the emitted fluorescence \( F_6 \) is proportional to the excitation irradiance \( H_6 \):

\[
F_6 = \kappa H_6,
\]

where the constant \( \kappa \) is the proportionality ratio between the excitation irradiation and the emitted fluorescence. The same analysis can be expanded to the path of emitted fluorescence. This time, however, the emitted fluorescence is treated as a point source. Then one can obtain the following expression for the irradiance \( F_6 \) of the fluorescence transmitted through the optical fiber:

\[
F_6 = \frac{\tau_6 \tau_5 \tau_4 \tau_3 \tau_2 \tau_1 J_0 \rho}{\left( d_5 + d_6 \right) A^2} \kappa A J_0,
\]

where \( \tau_5, \tau_6, \tau_7, \tau_8, \) \( \tau_6(488), \tau_6(515), \tau_5(488), \) \( \tau_5(488), \) \( \tau_5(515), \) \( \tau_6(488), \) and \( \tau_6(515) \) are the transmissivities of the optical fiber, converging lens, filter, dichromatic mirror, objective lens at 488 and 515 nm, and the fused silica capillary tube at the wavelengths of 488 and 515 nm, respectively. \( CA \) is the clear aperture of the objective lens. \( A_1, A_2, A_3, A_4 \) are the fluorescence-intensity-covered areas of the mirror, filter, converging lens, and optical fiber, respectively. The parameters \( A_1, A_2, A_3 \) are dependent on \( d_{10} \), the distance between the objective lens and the stage. Note that Eq. (9) is derived assuming that the emitted fluorescence fills the clear aperture of the objective lens.

Assuming that the transmissivities of the objective lens and the capillary tube at the wavelengths of 488 and 515 nm are similar, Eq. (6), relations (7), and Eqs. (8) and (9) can be combined to yield an expression relating the irradiance of the fluorescence at the optical fiber and the intensity of the laser beam:

\[
F_6 = \frac{T}{D} \kappa A J_0,
\]

where

\[
T = \frac{\tau_6 \tau_5 \tau_4 \tau_3 \tau_2 \tau_1 J_0 \rho}{\left( d_5 + d_6 \right) A^2} \kappa A J_0, \\
D = \left[ d_6 \right]^2 \left[ d_5 \right]^2 \left[ d_10 \right]^2.
\]

This final equation is derived assuming that \( d_5 \) is negligible compared with \( d_6 \). It is shown that as \( d_{10} \) increases, i.e., as the stage moves away from the objective lens, the irradiance of the fluorescence at the optical fiber decreases significantly. The parameters \( T, CA, \) and \( \kappa \) are the properties of the components and the system. To increase the irradiance \( F_6 \), the distances between the components need to be minimized. For example, moving the filter closer to the dichromatic mirror while using the parameter values listed in Table 1 increases the fluorescence irradiance \( F_6 \). This relationship is depicted graphically in Fig. 3.

### 4. Results and Discussion

Experiments are conducted as outlined above ten times for a 2-min test period for each sample. The data within the first two shortest time delays are always discarded to eliminate the well-known after-pulsing problem of the photodetectors. The best way to avoid this after-pulsing problem requires using two photodetectors and performing cross correla-

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**Table 1. Examples of Parameter Values**

**Fig. 3.** Final incident energy \( F_6 \) as a function of various \( d_6 \) and \( d_{10} \) values.
tion. On the other hand, we can also simply overcome this problem by fitting measured autocorrelation curves with the following model and by assuming that the diffusion of E. coli in water is negligible:

$$G(\tau) = G(0)\exp\left[-\left(\frac{\tau V}{\omega}\right)^2\right], \quad (12)$$

where $\omega$ is the beam waist in the lateral direction and $V$ is the flow velocity. Equation (12) is used to fit all experimental data.

Figure 4 depicts the $G(\tau)$ profiles obtained from nine separate tests carried out for a sample with concentration of $1.5 \times 10^7$ ml$^{-1}$ E. coli. Experiments are conducted consecutively, and the average of all results is plotted along with the individual runs. The averaged $G(0)$ value is found to be 4.6. The drift of $G(0)$ in the experiment is due to the instability of the measured fluorescence intensity, which might be caused by sample degradation. The averaged $G(0)$ profiles are obtained in the same manner for all samples.

Fluorescence fluctuations for all samples, including water, are shown in Fig. 5. Naturally, the fluorescence decreases with decreasing concentration, and the amplitude of fluctuations becomes larger as the E. coli concentration in sample volume decreases. The signal from deionized water is considered background, which includes the Raman scattering from water and the fluorescence from the optical system, including the capillary.

For lower concentrations (i.e., $1.5 \times 10^4$ to $1.5 \times 10^5$ ml$^{-1}$), the fluorescence counts are too close to that of the background fluorescence. Thus, when the signal is comparable with the background, the measured $G(0)$ is deemed unreliable (although the decay time of fluctuations can still be precisely detected for very low concentrations, all the way down to $1.5 \times 10^3$ ml$^{-1}$). For these cases, the $G(0)$ expression is corrected, following the suggestions given in Ref. 11, as:

$$G(0) = \frac{G(0)_{\text{Meas}}}{1 - \frac{F_{\text{Total}} - F_{\text{Backg}}}{\bar{F}_{\text{Total}}}}. \quad (13)$$

The usefulness of this relationship is demonstrated in a series of auxiliary experiments, conducted either with $1.5 \times 10^5$ ml$^{-1}$ E. coli or 1 nM Rhodamine 6G, or a mixture of $1.5 \times 10^5$ ml$^{-1}$ E. coli and 1 nM Rhodamine 6G. These results are depicted in Fig. 6.

The sensitivity of the current FCS system is affected by the low speed of the correlator, which is only 480 ns for the first channel. Preliminary experiments showed that the Rhodamine 6G solution results can be considered the background and do not adversely affect the fluctuations. The $G(0)$ of pure E. coli is measured as 21, which can also be obtained by correcting the $G(0)$ of the mixture with Eq. (13). In this fashion the measured $G(0)$ values are corrected for different E. coli concentrations by taking water as the background fluorescence source. Both the measured and corrected $G(0)$ profiles are plotted in Fig. 7.

To allow us to estimate the bacteria concentration, the volume of the sample excited by the laser is to be
determined. Based on the Gaussian optics and the parameters of the optical system, the smallest theoretical beam waist at the focus point of the objective lens is found to be 0.4 μm. However, owing to the curvature of the capillary, the effective beam waist is deemed to be as large as 20 μm (see Fig. 2). This figure is obtained by use of the decay time of $G(\tau)$ ($1 \times 10^{-7}$ s) and the flow velocity (200 mm/s). The focus depth is limited by the pinhole and is set to ~2 μm. Thus the measurement volume element is estimated to be $2.5 \times 10^{-9}$ ml. For the concentration of $1.5 \times 10^5$ ml$^{-1}$, using the $G(0)$ value, it is found that there is 0.0766 unit volume of bacteria in $2.5 \times 10^{-9}$ ml, which corresponds to a concentration of $3.06 \times 10^7$ ml$^{-1}$. Although this value is not exactly equal to the input value, it is a remarkably good estimate for such a low concentration and shows that this relatively simple approach can be used for detection of E. coli in water. The other concentrations are also analyzed by following the same procedure, and similar values are found.

The variations in the experiments are caused mainly by two factors. The nonlinearity of the fluorescence emission from the sample affects the results depending on the laser input power, which is altered as a function of concentration. The second problem is due to the error in estimating the sample volume, which can be reduced by considering the capillary tube shape and structure. This will be the focus of future studies.

The results show that the lower limit of the current system is approximately $1.5 \times 10^5$ ml$^{-1}$; below that concentration level, the results are not very accurate. Note that $1.5 \times 10^5$ ml$^{-1}$ is equal to $2.5 \times 10^{-16}$ M. In the experiments, an E. coli concentration of $1.5 \times 10^5$ ml$^{-1}$ yields a fluorescence emission equivalent to a $1 \times 10^{-9}$ nM Rhodamine solution. After E. coli is stained, the fluorescence brightness of the bacteria is determined to be $4 \times 10^8$ times that of a single Rhodamine 6G molecule.

5. Conclusion
A FCS technique based on the principle of convective diffusion is designed and built to detect very small concentrations of E. coli in water. With this system, concentrations as low as $1.5 \times 10^5$ ml$^{-1}$ or $2.5 \times 10^{-16}$ M of E. coli in water are detected. As bacteria are generally much larger than fluorescing dye molecules, a stained bacteria may have several thousand dye molecules attached to itself. Therefore the brightness of stained bacteria may be much greater than that of the dye molecules themselves. In addition, as the size of bacteria is of the order of micrometers, one does not need to focus the incident laser beam onto a submicrometer region, and the observation volume can be relatively larger than that in the conventional FCS experiments. These two factors ensure that measured fluorescence fluctuations are from the stained bacteria rather than the free-flowing dye molecules in the solution. The results of our experiments clearly show that measurement of sparse bacteria concentrations is possible if the bacteria are properly stained or conjugated. By conjugating E. coli cells with fluorescence protein (for example EYFP or EGFP$^{13}$), it is possible to detect trace E. coli in water in its natural form.

This approach can be used in laboratory experiments very effectively. The sensitivity of the current setup can be further improved by use of a more sensitive detector, such as a single-photon avalanche diode. In addition, adopting a two-photon excitation or time-gated excitation$^{14}$ can reduce the background noise significantly. The optical system may also be improved further to yield improved sensitivity by the procedure discussed here. Note that it is also possible to expand this approach to applications for process control; for this additional modifications of the current setup are needed, which are in progress.

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