1. INTRODUCTION

The use of femtosecond pulsed excitation in microscopy has been recognized as an extremely powerful extension of the capabilities of advanced optical imaging.\(^1,2\) The major benefit of subpicosecond pulses is found in the capabilities of advanced optical imaging techniques applied to microscopic studies of biological samples.\(^1-6\) Therefore, whereas in the incoherent process the spatial intensity distribution of the signal field will ultimately dictate the optimal resolution in nonlinear coherent imaging.\(^1,2\)

The generation and propagation of coherent signals are governed by the well-known optical wave equation.\(^10\) To describe the evolution of nonlinearly generated coherent radiation in confined focal volumes, one must take into account explicitly the diffraction contribution to the wave equation. Moreover, because the longitudinal extent of the focal regions of high-numerical-aperture (NA) objectives is of the order of a wavelength, the validity of the slowly varying envelope approximation (SVEA) must be carefully considered. A similar situation occurs when the duration of the excitation pulses is so short that the spatial extent of the radiation burst compares with an optical wavelength.\(^11\) In both cases a straightforward application of the SVEA is no longer warranted, and a correct description of the development of coherent signals in either of these limits requires an evaluation of the wave equation beyond the SVEA.\(^1,2\)

In this paper we address the issue of coherent FWM signal generation in diffraction-limited focal volumes. A versatile model is presented that does not rely on the SVEA and is applicable to high-NA focusing conditions. In particular, we discuss the method of CARS microscopy as being a representative coherent FWM optical technique applied in a microscope configuration. Unlike in previous studies we explicitly calculate the focal intensity patterns.\(^12-15\) Differences between coherent (e.g., CARS) and incoherent (e.g., multiphoton excited fluorescence) signal generation are pointed out. Finally, we explore the limits of the SVEA compared with the exact analysis.

2. THEORY

Whenever a light field is incident upon a material, a polarization is induced. The polarization is generally modeled by a power series of the driving fields: \( P = \chi^{(1)} E_1 + \chi^{(2)} E_1 E_2 + \chi^{(3)} E_1 E_2 E_3 + \ldots \) \(1\)

where \( \chi^{(n)} \) is the nth-order nonlinear susceptibility of the medium and \( E \) is the electromagnetic field.
Here $\chi^{(n)}$ are the $n$th order susceptibility tensors, and the incident electric fields ($E_i$) are indexed with arbitrary numbers. The incident fields and the induced polarization are functions of both space and time. For high-NA focusing conditions this implies that the spatial width of the excitation pulses should largely exceed the focal interaction length. Note that this quasi-cw electric field criterion is readily fulfilled for pulses with durations as short as 100 fs. Under these conditions we can extract the time-dependent oscillating term, and the fields can be written as

$$E_i(r,t) = E_i(r)\exp(i\omega t) + c.c.,$$

$$P(r,t) = P(r)\exp(i\omega t) + c.c.,$$

where all the spatial variables $r = (x, y, z)$ are contained in the terms $E_i(r)$ and $P(r)$. Nonlinear FWM techniques rely on the polarization of the material that is proportional to $\chi^{(3)}$. The CARS signal-generation process is governed by the third-order susceptibility tensor, and its contribution to the nonlinear polarization is given by

$$P_{CARS}(r) = \chi_{CARS}^{(3)}(r)E_p^2(r)E_S^2(r)\exp[i(2k_p - k_S)z],$$

where we have assumed that the pump and the probe beams are degenerate. The subscripts $p$ and $S$ refer to the pump, probe, and Stokes fields, respectively, and $z$ corresponds to the direction of the optical axis. One usually obtains the optimal imaging qualities of high-NA objectives by overfilling the back aperture of the lens. We therefore assume that all beams are collinear and exhibit flat wave fronts on entrance of the microscope unit. A schematic layout of the collinear CARS microscope in the transmission mode is presented in Fig. 1. Because the excitation densities are highest near the focal spot, nonlinear processes are most likely to occur in this region. It is therefore sufficient to consider only the incident spatial field distribution near the focus, which can be written for diffraction-limited beams as

$$E_i(u,v) = 2\int_0^\infty J_0(v\rho)\exp(-iu\rho^2/2)\rho d\rho,$$

where $J_0$ is the zeroth-order Bessel function. The parameters $u$ and $v$ are the normalized axial and lateral optical coordinates, respectively; defined by

$$u_i = \frac{2\pi \sin \alpha}{\lambda_i} r,$$

$$u_i = \frac{8\pi \sin^3(\alpha/2)}{\lambda_i} z,$$

where $\sin \alpha$ is the numerical aperture of the objective lens and $\lambda_i$ is the wavelength of the incident light. The induced polarization acts as a source for the coherent emission field $E_c(r,t)$ via the wave equation that follows from Maxwell’s equations:

$$\nabla^2 E_c(r,t) - \frac{n^2}{c^2} \frac{\partial^2 E_c(r,t)}{\partial t^2} = \frac{4\pi}{c^2} \frac{\partial^2 P(r,t)}{\partial t^2}. $$

Given the low conversion efficiency as well as the short interaction lengths, a perturbative description, which ignores the effect of depletion of the incident radiation fields, seems plausible. Because of the rotational symmetry of the problem it is convenient to express the Laplacian in terms of cylindrical coordinates $(r, \phi, z)$. Substituting Eqs. (2) into the wave equation and evaluating the time derivatives yield

$$\frac{\partial^2 E_c(r, \phi, z)}{\partial t^2} + \frac{1}{r} \frac{\partial}{\partial r} \left(r \frac{\partial E_c(r, \phi, z)}{\partial r} \right) + \frac{1}{r^2} \frac{\partial^2 E_c(r, \phi, z)}{\partial \phi^2} E_c(r, \phi, z)$$

$$+ k_c^2 E_c(r, \phi, z) = \frac{4\pi \omega^2}{c^2} P(r, \phi, z).$$

If it is assumed that the fields have no functional dependence on $\phi$, the equation can be expressed in the spatial-frequency ($\rho$) domain by means of a Hankel transformation, defined by

$$E_c(\rho, z) = 2\pi \int_0^\infty E_c(r, z)J_0(2\pi r\rho) r dr$$

and yielding

$$\left(\frac{\partial^2}{\partial z^2} - 4\pi^2 \rho^2 + k_c^2\right)E_c(\rho, z) = \frac{4\pi \omega^2}{c^2} P(\rho, z).$$

This is a second-order nonhomogeneous differential equation that describes the generation and propagation of a coherent field in the presence of a driving polarization. In the spatial-frequency domain, diffraction effects are explicitly taken into account by the term $4\pi^2 \rho^2$ on the right hand side of Eq. (9). The formal solution of Eq. (9) can be written as

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![Fig 1. Experimental layout of the CARS microscope in transmission mode. A combined pump–probe beam is aligned collinearly with the Stokes beam by a dichroic beam splitter and focused by an excitation objective in the sample. After collection by a secondary objective, the spectrally filter CARS signal is detected by a large-area detector.](image-url)
\[ E_c(\rho, z) = E_c^+(\rho, z) \exp(ik_c z) + E_c^-(\rho, z) \exp(-ik_c z), \tag{10} \]

with

\[
E_c^+(\rho, z) = \left( E_0^+ \frac{-1}{2ik'} \int_{-\infty}^{\infty} 4\pi k_c^2 P(\rho', z') \exp(i\Delta k^z z') \right.
\times \exp(-i(k' - k_c) z') \bigg) \nonumber \\
\times \exp(i(k' - k_c) z) \nonumber \\
\times \exp(-i(k' - k_c) z')dz'. 
\]

\[
E_c^-(\rho, z) = \left( E_0^- + \frac{1}{2ik'} \int_{-\infty}^{\infty} 4\pi k_c^2 P(\rho', z') \exp(i\Delta k^z z') \right.
\times \exp(i(k' - k_c) z') \bigg) \nonumber \\
\times \exp(i(k' - k_c) z) \nonumber \\
\times \exp(-i(k' - k_c) z')dz'. 
\]

where \( E_0^+ \) and \( E_0^- \) are determined by the far-field boundary conditions; \( k' \) is an effective wave vector, given as \( k' = (k_c^2 - 4\pi^2 \rho^2)^{1/2} \).

The solution is composed of two waves, \( E_c^+(\rho, z) \) and \( E_c^-(\rho, z) \), that propagate with wave vectors \( k_c \) and \( -k_c \), respectively, along the optical axis. Both waves depend on wave-vector-mismatch terms, which are given as \( \Delta k^- = 2k_p - k_S - k_c \) and \( \Delta k^+ = 2k_p - k_S + k_c \). Whereas the forward-propagating beam is not affected by the wave-vector mismatch in the absence of any dispersion, the backward-propagating beam is seriously altered by the mismatch \( \Delta k^+ \). We shall consider only the signal beam that travels in the direction of the excitation beams (transmission mode) because the intensity of the forward-propagating wave by far exceeds the intensity of the backward-propagating field. Assuming a zero initial far-field contribution for the signal amplitude \( (E_0^+ = 0) \), the envelope of the signal can be rewritten as

\[
E_c^+(\rho, z) = \exp(i(k' - k_c) z) \frac{2i\pi k_c^2}{k'} \int_{-\infty}^{z} P(\rho', z') \exp(i\Delta k^z z') \exp(-i(k' - k_c) z') \bigg) \nonumber \\
\times \exp(i(k' - k_c) z) \nonumber \\
\times \exp(-i(k' - k_c) z')dz'. 
\]

Note that Eq. (11) is derived directly from the exact wave equation and circumvents the need to use the SVEA. In Appendix A an alternative derivation of the signal is given, in which SVEA is imposed. Within the SVEA the signal field can be expressed as

\[
E_{\text{SVEA}}(\rho, z) = \exp\left(\frac{-2i\pi^2 \rho^2 z}{k_c}\right) \frac{2i\pi k_c^2}{k'} \int_{-\infty}^{z} P(\rho', z') \exp(i\Delta k^z z') \exp(-i(k' - k_c) z') \bigg) \nonumber \\
\times \exp(i(k' - k_c) z) \nonumber \\
\times \exp(-i(k' - k_c) z')dz'. 
\]

It is instructive to compare Eq. (11) with Eq. (12). In the limit of low NA the field derived in the SVEA approaches the solution given in Eq. (11), as one can easily verify by realizing that for small values of \( \rho \) the following relations hold: \( k_c^2/k' = k_c \) and \( k' - k_c = -2\pi^2 \rho^2/k_c \).

Under the condition that any diffusion effects can be ignored, only the lowest spatial frequencies contribute to the solution. In this case \( (\rho = 0) \), both equations reduce to a solution that depends only on the position along the optical axis:

\[
E_{\rho=0}(z) = 2i\pi k_c \int_{-\infty}^{z} P(z') \exp(i\Delta k^z z') dz'. \tag{13} \]

Equation (13) corresponds to the general solution of the one-dimensional wave equation in the SVEA that can be found in many textbooks.\(^6\) Clearly, if lenses with high NAs are considered, contributions at higher spatial frequencies cannot be neglected, and Eq. (13) fails to describe the effects of diffraction. A correct description of the signal field, therefore, requires the evaluation of Eq. (11).

The procedure for calculating the coherent field is organized as follows: For a particular plane perpendicular to the optical axis, the induced polarization is calculated according to Eqs. (3) and (4). The result is transformed to the frequency domain by use of a discrete Hankel transform and substituted as a source term into Eq. (11). One performs the integration numerically by repeating the procedure for successive planes at discrete steps along the optical axis. The signal field is finally found by transformation of the complex amplitude back to the spatial domain through the inverse Hankel transform given by

\[
E_c(r, z) = 2\pi \int_{0}^{\infty} E_c(\rho, z) J_0(2\pi\rho r) \rho d\rho. \tag{14} \]

Whereas the exact three-dimensional distribution of the coherent signal is rather difficult to reach experimentally, one can easily obtain a measure for the axial resolution by scanning a thick (semi-infinite) slab of optically active material axially through focus. For coherent signals the edge response is given as

\[
I(u) = 2\pi \int_{0}^{\infty} v dz |E_c(v, u)|^2 = 2\pi \int_{0}^{a} \rho d\rho |E_c(\rho, u)|^2. \tag{15} \]

Note that Eq. (15) is valid when an infinitely large detector monitors the signal. If the signal is collimated by a second objective that is collinearly positioned, we may calculate the total amount of signal generated at plane \( u \) that is captured by the collimating lens as

\[
S(u) = \frac{2\pi}{I(u)} \int_{0}^{a} |E_c(\rho, u)|^2 \rho d\rho, \tag{16} \]

where \( a = NA_{\text{col}}/\lambda_c \) corresponds to the maximum spatial frequency that is monitored by the detection objective. In the derivation of Eq. (16) we assumed that all signal is registered by a photodetector without introducing any pinhole.

3. RESULTS

In general, the nonlinear emission intensity depends on wave-vector mismatch \( \Delta k \). In many nonlinear spectroscopic methods the minimization of \( \Delta k \) is an important factor. However, in the condition that \( \Delta k \) is significantly different from zero, the exact three-dimensional distribution of the total coherent function is impractical to obtain. The signal is most intense in the region within 0.1 mm of the excitation beam, whereas it is less than 0.3 mm of the excitation beam, no signal is contributed.

In Figs. 4(a) and 4(b) the signal distribution is shown for a homogenized medium. It is clear that the emission distribution is symmetric around the plane of the incident beam. However, the intensity of the signal is much higher in the direction of the excitation beam. CARS microscopy features a similar signal intensity distribution in inhomogeneous media as can be seen in Figs. 3(b). The intensity distribution is dissymmetric along the optical axis and exhibits a finite value in colormaps.

Diffractive effects are clearly visible in Figs. 4(a). They are caused by the fact that the excitation and the CARS beams are not collinear. The intensity distribution is calculated in the plane of the excitation source and is shown in an inhomogeneous medium. In Figs. 4(b) the generated function is integrated over the respective wavelength region. The signal is clearly visible as the green spot.
factor in the optimization of the signal. Under high-NA conditions, however, the effective interaction length $L_{\text{int}}$ is significantly reduced to the micrometer range. In the visible spectral range with normally dispersive materials, the wave-vector mismatch product $\Delta k L_{\text{int}}$ does not significantly affect the strength of the emission field, as is illustrated in Fig. 2, where the effect of wave mismatch on the total CARS signal in an aqueous medium is plotted as a function of NA. For NA's of 0.3 and higher the total signal is not altered considerably by the mismatch factor, even if the pump and Stokes wavelengths differ by more than 300 nm. Therefore, assuming high-NA (larger than 0.3) conditions, we can safely neglect the wave-mismatch contribution.

In Fig. 3(a) the spatial organization of the CARS signal is shown in the focal region where it is focused into a homogeneous bulk material by an objective with a NA of 0.9. It is obvious that the intensity pattern of the coherent emission differs significantly from the corresponding excitation intensity ($\propto I_p^2 I_s$) that is shown in Fig. 3(b). The symmetry of the excitation profile is broken in the coherent buildup process of the CARS signal. At a given plane perpendicular to the optical axis, both the accumulation of signal along the optical axis and the diffraction-induced flow of energy in the lateral direction shape the final contours of the signal intensity. The distinct pattern of the CARS signal is a direct consequence of the coherent nature of the process. Nonlinear signals that exhibit a similar power dependence on the incident fields but rely on incoherent addition of signal waves show emission characteristics similar to the intensity distribution in Fig. 3(b). This situation pertains, for instance, to the focal intensity of multiphoton-excited fluorescence. The striking dissimilarities between the focal fields of coherent and incoherent signals emphasize the distinctive imaging mode in coherent FWM microscopy.

Differences between the excitation and the coherent signal intensity along the axial axis are illustrated in Fig. 4(a). The rise of illumination intensity is accompanied by the generation of coherent signal. Although the excitation energy peaks at the focal plane, the maximum of the CARS signal is slightly offset from this plane, indicating that growth of the signal persists just beyond the focal point. Away from the focal plane the illumination intensity drops and the growth in the signal decreases. In this region, the profile of the signal is dominated by diffraction of coherent radiation away from the optical axis, resulting in an asymmetric tail. The signal properties in the lateral direction are given in Fig. 4(b). The coherently generated CARS signal shows a higher intensity in the wings relative to the excitation profile. This property is ascribed to the diffraction of accumulated signal into this region. The resultant focal intensity has broadened with respect to the spatial confinement of the incident radiation.

The flow of energy in the lateral dimension strongly depends on the size of the excitation volume. In Fig. 5 the distribution of the signal intensity in the focal plane is shown for various values of $\lambda_p/\lambda_S$. For increasing values of $\lambda_p/\lambda_S$ the illumination spot contracts in both dimensions. A more-confined illumination intensity corresponds to a more-pronounced contribution of energy that is diffracted into the wings. The significance of diffraction thus grows when the size of the excitation volume is reduced. This implies that in CARS microscopy the
file of the emission field differs for different shifts in wavelength between the pump and the Stokes beams.

The discussion above underlines the influence of diffraction on the final signal. Although the initial signal accumulation occurs mainly in the direction of the optical axis, a significant amount of energy is diffracted at certain angles while the signal is traversing the focal volume. A glance at the CARS signal profile [Fig. 3(a)] reveals that part of the signal is diffracted into regions where the excitation density is almost negligible [Fig. 3(b)]. This feeding mechanism permits growth of signal in regions with excitation intensities of only 10^{-4}. Accordingly, the volume responsible for the final signal is significantly larger than its corresponding excitation volume. Because of this strong signal dependence on the details of the excitation field we suspect that the FWM signal is particularly sensitive to aberrations.

In general, the imaging properties of a coherent FWM microscope critically depend on the spatial dimension and optical characteristics of the object in focus. Under the condition that the optical activity is homogeneously distributed throughout the object, the size of the object will still affect the spatial characteristics of the coherent emission. The profile in Fig. 3(a) was calculated for an infinite material. This limit relates to objects that scale well beyond the dimensions of the focal excitation volume. The other extreme occurs if the object can be considered to be a point object, i.e., if the dimensions of the object are much smaller than the volume of the focal excitation intensity. In this case the nonlinear signal is generated at a single point in space. The integration of Eq. (11) over the spatial coordinates can be omitted, and the signal is just proportional to the induced polarization. In this situation the microscope behaves as an incoherent imaging system. Consequently, the image of a pointlike object that is scanned through focus mimics the excitation profile, and the recorded figure is a convolution of the PSF and the object function. For larger-sized objects, however, the proportionality between excitation and emission is lost, and the convolution procedure is no longer warranted.

In optical microscopy of biological materials, the sample is in general composed of many objects that vary in size, ranging from pointlike objects to large bulky structures. Between these extremes, we may encounter entities whose magnitudes compare with the focal excitation volume. In this intermediate regime the signal is affected by the coherent buildup process as well as by the spatial profile of the object. Unless the illuminated object falls within the point-source limit, the image monitored is not a simple convolution of the object with a PSF. At a given point, a simulation of the signal in this regime needs full evaluation of the wave equation over the spatial extent of the object. This implies that a straightforward deconvolution procedure as is commonly applied in confocal fluorescence microscopy is inappropriate in reconstructing the original contours of the object when it is measured with CARS microscopy.

The object-dependent coherent buildup of the signal complicates the experimental determination of spatial resolution in a FWM microscope. However, the axial-edge response of a thick layer is a convenient measure for the axial resolution in the coherent as well as in the incoherent nonlinear optical microscope. Figure 6 depicts the axial-edge response for both the coherent CARS signal and the corresponding nonlinear incoherent signal when the axial-edge response of a thick layer is a convenient measure for the axial resolution in the coherent as well as in the incoherent nonlinear optical microscope. Figure 6 depicts the axial-edge response for both the coherent CARS signal and the corresponding nonlinear incoherent signal...
when a thick slab of optically active material is scanned axially through focus. Both profiles show a sheer increase near the focal point and a gradually decreasing growth of the signal after passage of the focal plane. The apparent differences in the responses reflect the origin of the way in which the signals are generated. Whereas the incoherent signal saturates when the excitation intensity diminishes, the coherent signal still shows moderate growth. This phenomenon can be explained by the fact that the growing interaction length counterbalances the decrease of the excitation intensity. The profile that is measured by scanning of a thick slab of optically active material along the optical axis proves to be insensitive to NA. The shape of the contour hardly changes within the NA range of 0.25–0.9. Differences are subtle and will be obscured by experimental uncertainties in a practical realization of the z scan. This finding establishes the method of scanning a thick layer as a measure for axial resolution in a coherent nonlinear microscope.

In a FWM microscope the signal is detected in the forward direction (Fig. 1). In general, a second objective is used to capture the divergent signal beam to optimize for maximal signal-detection efficiency. The amount of signal detected depends on the characteristics of the collimating objective as well. In Fig. 7 the relative amount of CARS signal collimated by the second objective is plotted as a function of the NA when identical NA’s are used for excitation and collection. At low NA, approximately 84% of the signal is collected by the detection lens. The losses can be ascribed to signal waves that are diffracted under larger angles that are no longer intercepted by the second lens. For a NA that exceeds 0.5 the fraction of collimated signal is reduced. This result is in concert with the observation that diffraction of signal waves becomes more pronounced for higher NA. Given the NA of the excitation objective, more than 99% of the signal may be collected if the NA of the second objective is 1.4 times larger.

So far we have considered the spatial distribution of the coherent signal that was evaluated by use of the exact solution of the wave equation. Next we shall compare these results with calculations based on the SVEA. The SVEA states that the change in the growth of the coherent signal is insignificant within the size of an optical wavelength. For low-NA lenses the SVEA is expected to predict reliably the contours of the signal field. However, when the focal interaction length compares with an optical wavelength the SVEA is no longer valid. Figure 8 compares the profiles of a z scan calculated either by an exact analysis or by imposing the SVEA [Eq. (12)] for a NA objective of 0.9. As one can judge, the SVEA slightly underestimates the growth of the signal intensity; the overall agreement between the curves is, however, sensible. For lower NA values, the profile predicted by the SVEA gradually approximates the correct result. At a NA of 0.5 the difference between the curves is nearly indiscernible. Although omission of the second-order derivative term introduces apparent dissimilarities in the z scan, invoking the SVEA does not lead to dramatic changes in the coherent emission characteristics. Spatial confinement of the optical field, as is achieved with high-NA lenses, does not cause an exceptional discrepancy between the result based on the SVEA and the exact
4. CONCLUSIONS

Nonlinear coherent four-wave mixing microscopy reveals some profound differences from the commonly used incoherent (nonlinear) fluorescence mode of the microscope. In examining a model that includes the detailed process of coherent signal accumulation in diffraction-limited focal volumes, we found that the signal volume is shaped both by coherent signal buildup along the optical axis and diffraction-induced flow of energy away from the optical axis. The final contours of the signal field are dictated by the details of the excitation field as well as by the spatial extent of the object. Moreover, the ultimate resolution of the FWM microscope is affected by the magnitude of the extent of the object. Moreover, the ultimate resolution of the FWM microscope is the axial scan of an optically active layer through focus, which proves to be practically independent of numerical aperture. Finally, we stress that although the slowly varying envelope approximation underestimates the rate of signal accumulation along the optical axis, the use of the SVEA does not lead to erroneous predictions of coherent signal intensities in optical microscopy.

APPENDIX A

To derive an expression for the signal field in the SVEA we assume that the optical axis is the major forward-propagation axis and that the amplitude can be written as

\[ E(\vec{r}, t) = E_0(\vec{r}) \exp[i(k_z z - \omega t)]. \]  

(A1)

Substitution of Eq. (A1) into the wave equation [Eq. (6)] leads to the following result, expressed in cylindrical coordinates:

\[ \frac{\partial^2 E_0(\vec{r})}{\partial z^2} + 2ik_z \frac{\partial E_0(\vec{r})}{\partial z} + \left( \frac{1}{r} \frac{\partial}{\partial r} r \frac{\partial E_0(\vec{r})}{\partial r} + \frac{1}{r^2} \frac{\partial^2 E_0(\vec{r})}{\partial \phi^2} \right) E_0(\vec{r}) \]

\[ = \frac{4\pi\omega^2}{c^2} P(\vec{r}) \exp(-ik_z z). \]  

(A2)

The left-hand side of Eq. (A2) can be simplified by a requirement that the first term be much smaller than the second:

\[ \frac{\partial^2 E_0(\vec{r})}{\partial z^2} \ll k_z \frac{\partial E_0(\vec{r})}{\partial z}. \]  

(A3)

This approximation is the SVEA. When this approximation is made, the equation can be rearranged in the spatial-frequency domain as

\[ \frac{\partial E_0(\rho, z)}{\partial z} = \frac{2i\pi n^2 p^2}{k_z} E_0(\rho, z) + 2i\pi n^2 p^2 \rho \frac{\partial E_0(\rho, z)}{\partial \rho} \exp(-ik_z z). \]  

(A4)

Solving Eq. (A4) leads to Eq. (12) of Section 2.

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REFERENCES