Limits for Reduction of Effective Focal Volume in Multiple-Beam Light Microscopy

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Abstract

Recently developed microscopy techniques, such as 4Pi, break the Abbe diffraction limit and allow for imaging at unprecedented resolution. The effective focal volume is also reduced, leading to more sensitive measurements in fluorescence microphotolysis or correlation spectroscopy. In 4Pi microscopy, the improvement is due to the utilization of two interfering laser beams for illumination, rather than a single one as in conventional microscopy. We study theoretically the possibility of further reduction of the focal volume with employment of three or more interfering beams, including the limiting case of an infinite number of beams. The volume is indeed reduced, but reaches a limit quickly as beams are added. The volume obtained in the setups with three or four beams is about half of that in the 4Pi case and is close to the volume computed for the limit of an infinite number of beams. The setups suggested employ purely optical principles, and, thus, the considered limiting case arguably represents the maximal reduction in focal volume possible for a purely optical far-field setup.

Until recently, the resolution of optical far-field microscopy was limited to the size $\lambda(2n \sin \alpha)$ (or 200–300nm in practice), where $\lambda$ is the wavelength of the light used in the microscope, $n$ is the refraction index, and $\alpha$ is the half-aperture angle of the light focused by the lens. This barrier, known as the Abbe diffraction limit, has been overcome with the emergence of 4Pi [1] and 3FM [2] microscopies. For instance, the 4Pi microscope provides a resolution of 100 nm. Subsequently, methods for superresolution fluorescence microscopy have been invented [3], such as STED [4], SSIM [5], FPALM [6], PALM [7], or STORM [8], allowing for resolutions of 20–30 nm.

The optical microscope can be used not only for imaging, but also as a probe for, e.g., dynamics of cellular components, most notably in the fluorescence microphotolysis (FM) or fluorescence correlation spectroscopy (FCS) modes. These approaches are largely non-invasive and, thus, allow one to detect molecular diffusion rates, binding, and other phenomena in living cells. The smallest volumes that FM or FCS can sample are determined by the distribution of light in the focus of the microscope, described by the point spread function (PSF); generally, the higher resolution is associated with a smaller focal volume. However, although the superresolution imaging techniques, such as STED and others, provide a nanoscale resolution, they may not necessarily provide a better sensitivity for FM or FCS. The reason is that all superresolution methods employ not only optical focusing, but also additional approaches, such as photobleaching or photoswitching, to increase the resolution (also, proposed schemes for PALM, FPALM, and STORM employ wide-field techniques not compatible with FM or FCS). These additional factors are applied to volumes much larger than that defined by the nominal imaging resolution, and, thus, the effective volume sampled by, e.g., FM measurements is not reduced in comparison with conventional

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microscopy. On the other hand, the 4Pi microscope employs purely optical principles to obtain a narrower PSF, providing an ideal probe for FM or FCS measurements. Indeed, FM and FCS measurements have been recently performed using the 4Pi microscope [9], with the 4Pi effective volume being about half of that of the conventional confocal microscope.

Here, we investigate the principle limits for confining illumination using ideas similar to that behind 4Pi microscopy. The 4Pi microscope uses the interference of two focused counterpropagating coherent laser beams at the focal spot to illuminate the sample, while a conventional microscope uses only one beam. We consider setups where multiple coherent laser beams are focused at the same spot. This is achieved by using multiple objectives, i.e., one objective for one beam [10], rather than multiple beams focused by a single objective, as done to furnish an optical lattice [11]. Resulting PSFs, \( h(\vec{r}) \), are computed. The PSF resolution can be defined as the full-width-half-maximum (FWHM) of the PSF, and the effective volume is defined [12] as

\[
V_{\text{eff}} = \frac{\int h(\vec{r}) d\vec{r}^2}{\int h^2(\vec{r}) d\vec{r}^2}
\]

Microscopies with more than two interfering beams have been proposed before, such as the theta-microscopy [10], MOM [13] and MIAM [14], but, to our knowledge, the question of what is the limit of light confinement with such purely optical approaches have not been addressed. In general, different numbers of the beams have not been sampled systematically, and corresponding PSF effective volumes have not been considered.

Schematics of the proposed arrangements are shown in Fig. 1. The 4Pi setup features two beams of equal phase, and we extend this arrangement to 3, 4, or more beams, with collinear polarization (which decreases the illumination spot), and with all beams being in the same plane. One can add out-of-plane beams, such as in the case of 6 beams in Fig. 1. Two limiting cases are considered. The first is an infinite number of beams of the same polarization, with all wave vectors being in the same plane (“Inf. beams, 2D”). The second case is an infinite number of beams converging from all directions in space, representing the most massive focusing possible. The latter arrangement can be realized in many different ways, depending on the choice of polarization and phase for each beam. We consider the arrangement in which all possible rotations, from angle 0 to \( \pi \), of the plane with “Inf. beams, 2D” are summed up (see Fig. 1). We assume the same half-aperture angle \( \alpha \) for each beam in the same setup (larger \( \alpha \) leads to a narrower illumination spot). Theoretically, \( \alpha \in [0, 90^\circ] \), but technically it is limited to \( \approx 68.5^\circ \) [9]. For many beams, \( \alpha \) has to be limited to even smaller values due to the spatial restrictions, e.g., to 60° for 3 beams in one plane. For the limiting cases “Inf. beams, 2D” and “Inf. beams, 3D”, the value of \( \alpha \) has to be zero, which would lead to a divergence of the illumination spot. Below, we formally set \( \alpha \leq 90^\circ \) for these arrangements, as an idealized and abstract case of the most narrow illumination theoretically possible.

The PSFs are obtained as follows. Consider a light beam traveling in the \(-z\) direction and focused by a lens, resulting in the spherical wavefront converging around \( \vec{r} = 0 \) The electric field [15, 16] is

\[
\vec{E}(\vec{r}) = -i \left[ I_0(\vec{r}) + I_2(\vec{r}) \cos 2\phi, I_2(\vec{r}) \sin 2\phi, -2I_1(\vec{r}) \cos \phi \right]
\]

where

\[
l_{0,1,2}(\vec{r}) = \int_0^{\pi} \cos^{1/2} \theta \sin \theta f_{0,1,2}(\theta) J_{0,1,2} \left( \frac{2\pi n \sqrt{x^2+y^2 \sin^2 \theta}}{\lambda} \right) \exp \left( \frac{-2\pi n z \cos \theta}{\lambda} \right) d\theta,
\]

\[
\text{Eq.} \quad 2
\]
\( f_0(\theta) = 1 + \cos \theta, f_1(\theta) = \sin \theta, f_2(\theta) = 1 - \cos \theta. \) Here, \( \phi \) is the angle between the plane of oscillation of \( \mathbf{E}(\mathbf{r}) \) and the plane of observation (we assume the most common case of \( \phi = 90^\circ \)); \( J_{0,1,2} \) are Bessel functions of the first kind. For a beam with an arbitrary direction \( \mathbf{\vec{k}} \), we can define a coordinate frame \((x', y', z')\), such that the beam is traveling in the \(-z'\) direction, and the electric field is described by the above formula with \( \mathbf{r} \rightarrow \mathbf{r}' \). Then, it is simple to obtain \( \mathbf{\vec{E}}(\mathbf{r}') \) for this beam in the original coordinate frame \((x, y, z)\) through rotation of all vectors by the angle defined by the direction and polarization of the beam. For arbitrary beams \( i, i = 1, 2, \ldots, N \), we obtain the total electric field \( \mathbf{\vec{E}}_{\text{tot}}(\mathbf{r}) = \sum_{i=1}^{N} \mathbf{\vec{E}}_i(\mathbf{r}) \) and PSF \( h(\mathbf{r}) = |\mathbf{\vec{E}}_{\text{tot}}(\mathbf{r})|^2 n_{\text{exc}} \) (\( n_{\text{exc}} \)-photon excitation is used; we set \( n_{\text{exc}} = 2 \) as commonly used in 4Pi microscopy). We assume that all beams are coherent with identical phase.

The PSFs (see Fig. 2) are obtained using the software \textit{fintool} [9], or, for an infinite number of beams, by numerical integration of \( \mathbf{\vec{E}}(\mathbf{r}) \) from single beams; visualization is done with VMD [17]. We describe only illumination PSF here (not combined with the detection PSF), since it is the illumination PSF that determines diffusion-bleaching dynamics on the sample in FM or FCS experiments [9]. The distances are measured in \( \lambda/n \). Typical values of \( \lambda \) and \( n \) are, e.g., \( \lambda = 910 \text{ nm} \) (the high value is due to the two-photon excitation) and \( n = 1.46 \). For the 1-beam case, we assume one-photon excitation and the wavelength is \( \lambda/2 \), where \( \lambda \) is the wavelength used for all other cases. Using two-photon excitation for the 1-beam case decreases the PSF size by the factor \( \sim \sqrt{2} \), but, due to the two-fold increase of the wavelength, the size actually increases by \( \sim \sqrt{2} \). For other cases, two-photon excitation leads to a smaller PSF volume than that for one-photon excitation, due to the constructive interference of multiple beams.

The 4Pi PSF is narrower in the \( z \)-direction than that of the 1-beam setup, and its size is further decreased by using 3, 4, or more beams (Fig. 2). However, as the number of beams grows, one has to employ smaller \( \alpha \). For example, if all beams are in the same plane, the maximal \( \alpha \) is 60° for 3 beams, 45° for 4, 30° for 6, etc., and the PSF elongates in the \( x \)-direction as more beams are used (can be noticed already for 3 vs. 4 beams). A similar effect is observed if out-of-plane beams are added (such as 6 beams in Fig. 2). We found that the PSF volume for setups with \( >4 \) beams in one plane or \( >6 \) in 3D is larger than that for 4- or 6-beam cases. Even if an idealized value \( \alpha = 68.5^\circ \) is used, the PSF size is not decreased much beyond the 3- or 4-beam case. For example, 60 beams in Fig. 2 correspond to an overlap of three sets of 20 beams uniformly distributed in the \( x, y, z \)-, and \( x, z \)-planes. The resulting PSF does not have side lobes or other outstanding features, but its size is not significantly reduced in comparison with the case of 3 beams. Likewise, the PSFs for an infinite number of beams with \( \alpha = 68.5^\circ \) are similar in size to the 3-beam PSF with \( \alpha = 60^\circ \).

Effective volumes \( V_{\text{eff}} \) are plotted in Fig. 3. The 4Pi \( V_{\text{eff}} \) is about 1/2 of that for the 1-beam microscope. Another decrease by the factor of 2 can be obtained by using 3, 4, or 6 beams. Even with \( \alpha = 60^\circ \) for 3 beams or \( \alpha = 45^\circ \) for 4 beams in one plane, or 6 beams in 3D, \( V_{\text{eff}} \) in these cases is about 1/2 of \( V_{\text{eff}} \) of the 4Pi PSF with \( \alpha = 68.5^\circ \). For 3 beams at \( \alpha = 60^\circ \), \( V_{\text{eff}} \approx 0.24 \left( \lambda/n \right)^3 \) vs. \( V_{\text{eff}} \approx 0.4 \left( \lambda/n \right)^3 \) for the 4Pi at \( \alpha = 68.5^\circ \). For an infinite number of beams in 2D or 3D, \( V_{\text{eff}} \) is not reduced much further than in the case of 3 beams, even if an idealized and unrealistic value of \( \alpha = 90^\circ \) is employed, resulting in \( V_{\text{eff}} \approx 0.14 \left( \lambda/n \right)^3 \). Thus, the realistic setups with 3, 4, or 6 beams allow for the values of \( V_{\text{eff}} \) which are about twice smaller than the best 4Pi values, and that are already very close to the minimal theoretically possible, but probably practically inaccessible, limit of light focusing.
Although $V_{\text{eff}}$ is decreased by using more than 2 beams, the imaging resolution cannot be improved beyond that of the 4Pi microscope, as suggested by the FWHM values in Table 1 (see also Supplementary Figures). The resolution along $x$ and $y$ can be somewhat improved by using 4 or an infinite number of beams, but in the $z$ direction, the 4Pi FWHM is the smallest. Interestingly, the FWHM of the 3-beam PSF is greater than that of the 4Pi PSF in each dimension, while $V_{\text{eff}}$ is smaller for the 3-beam case (the same is true for “Inf. beams, 3D” vs. “Inf. beams, 2D”). This is because the side lobes or exterior rings present in the 4Pi and “Inf. beams, 2D” PSFs contribute significantly to the value of $V_{\text{eff}}$, even though the central part of the PSF is relatively narrow.

Let us now estimate the limit for light focusing using multiple beams, assuming the “Inf. beams, 3D” setup (Fig. 1). The total electric field $\vec{E}(\vec{r})$ is obtained by integrating fields from individual beams (see Eq. 2) over all angles for two rotations: in the $y$, $z$-plane (“Inf. beams, 2D”), and rotation of that plane around the $z$-axis (rotation of the auxiliary $x'$ axis by $\pi$, from $x' = x$ to $x' = -x$). The resulting PSF is generally not symmetrical (Fig. 2), but we will enforce spherical symmetry for the estimation; in this case (see Supplement), only the $y$ component of $\vec{E}(\vec{r})$ is not zero, and it is given by the integration of the 4Pi $\vec{E}(\vec{r})$ over the rotations mentioned, so that $E_y(\vec{r}) = F_0(\vec{r}) - F_2(\vec{r})$, where

$$F_{0,2}(\vec{r}) = \int_0^\alpha d\theta \int_0^\pi d\psi \sin \theta \sin \phi \int_{f_{1,2}} (\theta) J_{0,2} \left( \frac{2\pi n r \sin \psi \cos \chi}{\lambda} \right) \cos \left( \frac{2\pi n r \cos \psi \sin \chi}{\lambda} \right).$$

(3)

Here, we define $r = (x^2 + y^2 + z^2)^{1/2}$ and the angle $\phi$ describes integration in the plane (“Inf. beams, 2D”); see Supplement for details of the derivation. Using numerical integration, we found $F_0 \gg F_2$ for all values of $\alpha$ in the relevant interval. We further notice that the narrowest $F_0(r)$ is obtained if one sets $0 = \alpha$ and $\psi = 0$ for the functions in the integral. Thus, we neglect $F_2$ and approximate $\vec{E}(\vec{r})$ as

$$\vec{E}(\vec{r}) \approx \left[ 0, J_0 \left( \frac{2\pi n r \sin \psi}{\lambda} \right), 0 \right].$$

(4)

We found that in fact $F_0(r)$ is almost the same (within a few percent deviation) for all values of $\alpha$, and that an accurate representation of $F_0(r)$ (with any $\alpha$) is given by Eq. 4 if $\alpha$ is set to $\approx 60^\circ$ in this equation (a reasonable agreement is found if $\alpha$ is between $\approx 60^\circ$ and $90^\circ$).

Then, the PSF ($n_{\text{exc}} = 2$) is $h(\vec{r}) \approx J_0 \left( 0.87 \frac{2\pi n r}{\lambda} \right)$, its resolution (FWHM) is $\approx 0.3\lambda/n$ (cf. Table 1). This approximation is valid only for the central peak of the PSF, because the function in Eq. 4 features side lobes that are missing in most of the space around the central peak of the real “Inf. beams, 3D” PSF (Fig. 2). If the side lobes are ignored, the estimate for $V_{\text{eff}}$ based on Eq. 4 is $V_1 \approx 0.069 (\lambda/n)^3$. However, the real PSF is about 1.5 times wider in the $y$-than in the $z$- or $x$-directions, and the estimate for $V_{\text{eff}}$ with $r \rightarrow 1.5 r$ is $V_2 \approx 0.231 (\lambda/n)^3$. Using these values, we estimate $V_{\text{eff}} = (V_1^2 V_2)^{1/3}$, which gives $V_{\text{eff}} \approx 0.1 (\lambda/n)^3$, close to the computed value (Fig. 3).

In summary, the light confinement in the focus of a microscope can be improved in comparison with the 1-beam and 4Pi cases, if one uses $\geq 3$ beams. For 3 or 4 beams, $V_{\text{eff}}$ can be decreased two-fold in comparison with the 4Pi $V_{\text{eff}}$, which itself is $\approx 1/2$ of $V_{\text{eff}}$ for the conventional 1-beam microscope. With more than 3 or 4 beams, further decrease in $V_{\text{eff}}$ is
not significant. In the limiting case of an infinite number of beams, \( V_{\text{eff}} \) is decreased by another 30 to 50\% but this is achieved only if one employs an abstract and idealized assumption of arbitrary \( \alpha \). The smallest possible \( V_{\text{eff}} \) achieved under this assumption is \( \approx 1/2 \) of that in the 3-beam case. Thus, the 3-beam setup provides the smallest value of \( V_{\text{eff}} \) for realistic \( \alpha \), and this value is close to the theoretical limit.

The resolution of the 4Pi microscope in one (z) dimension is still the highest one. The techniques such as STED [4], SSIM [5], STORM [8], FPALM [6], and PALM [7] provide even better resolution by using photochemical effects. Thus, even the maximal light confinement using purely optical principles, as considered here, cannot compete with these techniques for imaging resolution. However, purely optical confinement may be necessary for many applications in FM and FCS, and in such cases the smaller \( V_{\text{eff}} \) furnished by, e.g., 3-beam setup, might be useful.

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**Supplementary Material**

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**References**

[1]. Hell S, Stelzer EHK. Fundamental improvement of resolution with a 4Pi-confocal fluorescence microscope using two-photon excitation. Optics Comm. 1992; 93:277–282.

[2]. Gustafsson MGL, Agard DA, Sedat JW. Sevenfold improvement of axial resolution in 3D wide-field microscopy using two objective lenses. Proc. SPIE. 1995; 2412:147–156.

[3]. Hell SW. Far-field optical nanoscopy. Science. 2007; 316:1153–1158. [PubMed: 17525330]

[4]. Hell SW, Wichmann J. Breaking the diffraction resolution limit by stimulated emission. Optics Lett. 1994; 19:780–782.

[5]. Gustafsson MGL. Nonlinear structured-illumination microscopy: Wide-field fluorescence imaging with theoretically unlimited resolution. Proc. Natl. Acad. Sci. USA. 2005; 102:13081–13086. [PubMed: 16141335]

[6]. Hess ST, Girirajan TPK, Mason MD. Ultra-high resolution imaging by fluorescence photoactivation localization microscopy. Biophys. J. 2006; 91:4258–4272. [PubMed: 16980368]

[7]. Betzig E, Patterson GH, Sougrat R, Lindwasser OW, Olenych S, Bonifacino JS, Davidson MW, Lippincott-Schwartz J, Hess HF. Imaging intracellular fluorescent proteins at nanometer resolution. Science. 2006; 313:1642–1645. [PubMed: 16902090]

[8]. Rust MJ, Bates M, Zhuang X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). Nat. Methods. 2006; 3:793–796. [PubMed: 16896339]

[9]. Arkhipov A, Hüve J, Kahms M, Peters R, Schulten K. Continuous fluorescence microphotolysis and correlation spectroscopy using 4Pi microscopy. Biophys. J. 2007; 93:4006–4017. [PubMed: 17704168]

[10]. Lindek S, Pick R, Stelzer EHK. Confocal theta microscope with three objective lenses. Rev. Sci. Instrum. 1994; 65:3367–3372.

[11]. Betzig E. Excitation strategies for optical lattice microscopy. Opt. Express. 2005; 13:3021–3036. [PubMed: 19495199]

[12]. Schwille, P.; Haustein, E. Fluorescence correlation spectroscopy: A tutorial for the biophysics textbook online (BTOL). 2002. http://www.biophysics.org/education/techniques.htm

[13]. Haeberle O, Xu C, Dieterlen A, Jacquey S. Multiple-objective microscopy with three-dimensional resolution near 100 nm and a long working distance. Optics Lett. 2001; 26:1684–1686.
[14]. Swoger J, Huiskan J, Stelzer EHK. Multiple imaging axis microscopy improves resolution for thick-sample applications. Optics Lett. 2003; 28:1654–1656.

[15]. Wolf E. Electromagnetic diffraction in optical systems I. An integral representation of the image field. Proc. R. Soc. Lond. A. (Math. Phys. Sci.). 1959; 253:349–357.

[16]. Richards B, Wolf E. Electromagnetic diffraction in optical systems II. Structure of the image field in an aplanatic system. Proc. R. Soc. Lond. A. (Math. Phys. Sci.). 1959; 253:358–379.

[17]. Humphrey W, Dalke A, Schulten K. VMD – Visual Molecular Dynamics. J. Mol. Graphics. 1996; 14:33–38.
Figure 1.
Schemes of multi-beam interference setups. Each beam is characterized by its wave vector $\vec{k}$, polarization $\vec{E}$, and half-aperture angle $\alpha$. For 2, 3, and 4 beams, and “Inf. beams, 2D”, all $\vec{k}$-vectors are in the same plane and all $\vec{E}$-vectors are collinear. For 6 beams, the beams 1 to 4 are the same as in the case of 4 beams, $\vec{k}_5$ and $\vec{k}_6$ are perpendicular to the plane formed by $\vec{k}_1$ to $\vec{k}_4$, and $\vec{E}_5$ and $\vec{E}_6$ are collinear with $\vec{k}_5$. “Inf. beams, 2D” means infinite number of beams converging to a point uniformly from all directions in one plane. “Inf. beams, 3D” is achieved by replicating the plane with the beams from “Inf. beams, 2D”, rotating the replicas around one axis, and summing up all contributions for rotation angles from 0 to $\pi$; this results in the infinite number of beams converging to one point from all directions in 3D.
Figure 2.
(Color) PSF isosurfaces for the geometries with various numbers of beams. The PSFs are shown as viewed in the y, z- and x, y-planes (arrangement of the beams is the same as in Fig. 1). The isosurfaces are drawn at 0.5 (red), 0.1 (blue), and 0.01 (gray) of the PSF maximum (i.e., red isosurface corresponds to the FWHM). It is assumed that $\alpha = 68.5^\circ$ is the maximal value of $\alpha$ technically possible for a single beam. The maximal realistic values of $\alpha$ are used for 1, 2, 3, 4, and 6 beams, and $\alpha = 68.5^\circ$ for the idealized cases of 60 and infinite number of beams. The scale bar shows $\lambda/n$, $\lambda$ being the wavelength used. For all cases except 1-beam, two-photon excitation is assumed, while for the 1-beam case, we assume one-photon excitation and the wavelength is $\lambda/2$. 
Figure 3. Effective focal volumes $V_{\text{eff}}$. The values of $V_{\text{eff}}$ vs. $\alpha$ are plotted for 1 and 2 beams, and for the limiting cases of infinite number of beams distributed in 2D or in 3D. For 3, 4, and 6 beams, $V_{\text{eff}}$ at the maximal realistic values of $\alpha$ are shown. For “60 beams” and “20 beams, 2D” (the latter denoting 20 beams uniformly distributed in a plane), $\alpha = 68.5^\circ$ for an idealized comparison with the infinite number of beams.
Table 1
FWHM $\Delta l$ of PSFs in $x$, $y$, and $z$ dimension (see also Supplementary Figures). The setups considered are the same as in Fig. 2; $\Delta l$ is measured in $\lambda/n$.

| Setup              | $\Delta l_x$ | $\Delta l_y$ | $\Delta l_z$ |
|--------------------|--------------|--------------|--------------|
| 1 beam             | 0.36         | 0.36         | 1.09         |
| 4Pi (2 beams)      | 0.37         | 0.37         | 0.25         |
| 3 beams            | 0.41         | 0.37         | 0.37         |
| 4 beams            | 0.51         | 0.28         | 0.28         |
| 6 beams            | 0.39         | 0.30         | 0.30         |
| 60 beams           | 0.33         | 0.33         | 0.33         |
| Inf. beams, 2D     | 0.38         | 0.30         | 0.29         |
| Inf. beams, 3D     | 0.35         | 0.43         | 0.30         |