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Difference-of-Gaussian Annular Pupil for Extended Depth-of-Focus Three-Dimensional Imaging

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ABSTRACT

We propose a technique for 3-D microscopic imaging with extended depth-of-focus using a novel illumination scheme in a laser scanning optical microscope. The novel illumination scheme creates an effective annular pupil, called the difference-of-Gaussian annular pupil, without the critical drawback of stopping and wasting the light. Two laser beams of different Gaussian pupils with different temporal frequencies are first generated. The laser beams are then combined spatially and used to scan the specimen. The scattered light from the object is picked up by a photodetector whose output consists of a DC and an AC current (due to the optical heterodyning of the two optical beams). The DC signal is no difference from the DC output of a conventional laser scanning microscope with the processing pupil as a Gaussian function, whereas the AC signal is derived from the mixing of the two Gaussian beams and would be given by effectively a Gaussian pupil with a different size than that generated by the DC signal. The AC and the DC signals are then subtracted by electronics and hence the effective pupil function would be given by the difference of the two Gaussian pupil functions. By properly choosing the size of the two Gaussian laser beams, we could realize the difference of the two Gaussian pupils which becomes a new type of annular pupil called the difference-of-Gaussian annular pupil.

I. INTRODUCTION

Three-dimensional (3D) imaging is a formidable task in optical microscopy. It is well known that by using a higher numerical aperture (NA) of the objective lens in the microscope, one can achieve a high lateral resolution in the image. However, the improvement of the resolving power results in a depth-of-focus (DOF) reduction [1]. This trade-off problem between the resolving power and the DOF is common to optical microscopes. When the thickness of the specimen exceeds the depth of focus, only a fraction of the total information content of the specimen is imaged in focus or sharply. The result is a low resolution and contrast within the image. Hence improvement in the depth-of-focus has been of great interest in all areas of imaging, especially in high-resolution microscopy [2-5]. 3-D imaging in microscopy therefore aims to develop techniques that could provide high lateral resolution and at the same time with a large depth of field so that a thick specimen could be observed conveniently.

Current practical 3-D imaging techniques include optical sectioning microscopy (OSM) and confocal scanning microscopy (CSM). In OSM [6], 3-D information is collected by recording a series of 2-D images at various focal planes throughout the 3-D specimen. Since each 2-D image contains the in-focus as well as the out-of-focus information, reconstruction of the 3-D information, i.e., extraction of the in-focus information from these 2-D images, is required. Many reconstruction algorithms exist [6]. The difficulty of optical sectioning lies in the fact that during the recording stage it is important that exact focal spacing between adjacent 2-D images be accurately controlled. During the reconstruction, it is also critical to precisely align the 2-D images before processing. Recognizing these problems, a radically new microscope design, the scanning confocal microscope (SCM), has emerged [7]. In SCM, a doubly focused objectives lens system and a pin-hole aperture in front of a photomultiplier are used to image only a single point within the 3-D specimen. Three-dimensional information is then gathered by scanning the specimen

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in three dimensions while collecting the light transmitted through the specimen with the photomultiplier. The main difficulty of SCM is that the instrumental tolerances required to achieve high-resolution imaging are very difficult to obtain. Also, 3-D scanning is time-consuming which precludes, for example, the possibility of monitoring interactions taking place within the living cell. In addition, considerably more photobleaching using confocal microscopy will occur if the microscope operates in a fluorescent mode as it requires a time-consuming 3-D scan [6]. This may be critically important in the analysis of living cells. Therefore, confocal microscopy with a scanning laser approach is too slow to do three-dimensional for a dynamic intracellular applications.

Indeed, the major drawback of the two practical 3-D imaging techniques is the required depth- or z-scanning. In this paper, we propose a novel technique in that inspection of 3-D space is required by a single 2-D scan of the thick specimen. The proposed system employs a novel annular-illumination so that a large depth of focus can be achieved in the microscope and yet the system will not waste or stop any light as in conventional system using annular pupils. In what follows, in Section II) we first review a standard laser scanning imaging system. In Section III), we discuss three-dimensional imaging and optical transfer functions. Section IV) describes an annular pupil and its long depth-of-focus capability. Finally, in section V) we describe a novel idea to create a new type of annular pupil, called the difference-of-Gaussians annular pupil, which does not waste or stop the light when implemented in the proposed scanning imaging system. Implementation of the idea is presented at the end of the section.

II. CONVENTIONAL LASER SCANNING SYSTEM

Fig. 1 shows an idealized version of a conventional laser scanning system. p(x,y) is the pupil function. The pupil function forms a point spread function (PSF), h(x,y), onto the object $I_0(x,y)$. The PSF is then two-dimensionally scanned the object to give an output $I_i(x,y)$ displayed on the 2-D display. Mathematically, we have

$$I_i(x,y)= \iint I_o(x',y')h(x-x',y-y')dxdy= I_0(x,y)*h(x,y), \quad (1)$$

where * denotes the 2-D convolution, and the PSF, h(x,y), is given by the absolute squared of the Fourier transform of the pupil function p(x,y), i.e.,

$$h(x,y) =|F\{p(x,y)\}|^2 =|P(\frac{x}{\lambda f},\frac{y}{\lambda f})|^2 \quad , \quad (2)$$

where F denotes the Fourier transform of and we denote that $F\{p\} = P$, i.e., the upper case function P is the Fourier transform of the lower case function p. λ is the wavelength of the scanning laser light and f is the focal length of the lens as shown in fig. 1. In this context, the optical transfer function (OTF) is defined by taking the Fourier transform of h(x,y), or

$$OTF(X,Y) = F\{h(x,y)\} \quad , \quad (3)$$

where X and Y are the spatial frequencies along the x and y direction, respectively.

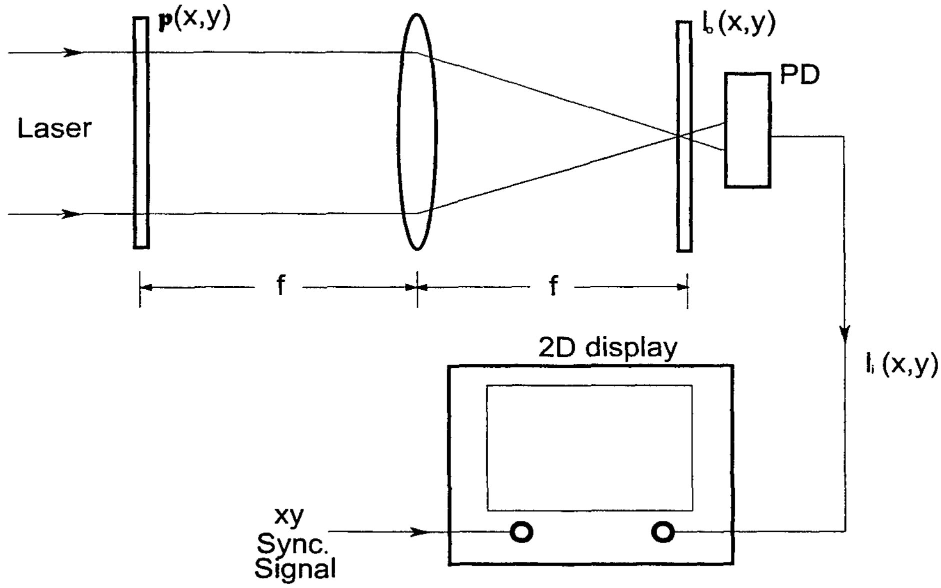


Fig. 1 Conventional laser scanning imaging system

III. THREE-DIMENSIONAL IMAGING AND OPTICAL TRANSFER

For 3-D imaging, we are interested in the defocused PSF, h(x,y,z), which can be calculated by modifying eq.(2) as follows:

$$h(x,y,z) = |F\{p(x,y)\} * \frac{1}{-j\lambda z} \exp(-j2\pi z/\lambda) \exp(-\frac{\pi}{\lambda z}(x^2+y^2)|^2$$

$$= |P(\frac{x}{\lambda f}, \frac{y}{\lambda f}) * \frac{1}{-j\lambda z} \exp(-j2\pi z/\lambda) \exp(-\frac{\pi}{\lambda z}(x^2+y^2)|^2, \tag{4}$$

where the quantity inside the absolute value represents the diffraction pattern of the function $P(\frac{x}{\lambda f}, \frac{y}{\lambda f})$ propagating at a distance z away from the focal plane of the lens. The 3-D OTF can be calculated, similar to eq.(3), by taking the 3-D Fourier transform of h(x,y,z) and that gives

$$OTF(X,Y,Z) = F\{h(x,y,z)\}, \tag{5}$$

and Z is the spatial frequency along the z direction.

IV. ANNULAR PUPIL AND ITS LONG DEPT-OF-FOCUS

It has been known that annular pupil can increase the depth-of-focus and lateral resolution simultaneously as compared with a circular pupil [8,9]. An annular pupil is defined as a clear circular pupil with a central obstruction as shown in fig. 2.

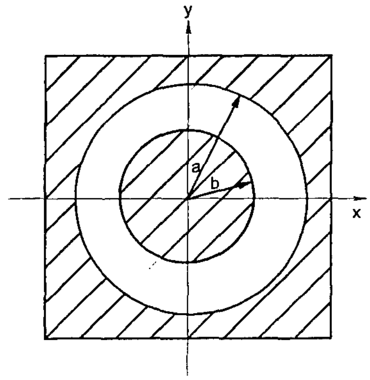


Fig. 2 Annular pupil

If the pupil is an annulus with outer radius a and inner radius b, we can define a central obscuration ratio of $\varepsilon = b/a$ [9]. The depth-of-focus δz_{annu} of an annular pupil has recently been derived by using the Heisenberg's uncertainty principle [9]:

$$\delta z_{ann} \approx \frac{1}{1-\varepsilon^2}\delta z_{cir}, \tag{6}$$

where $\delta z_{cir} = \lambda / NA^2$ is the depth-of-focus when using a circular pupil and NA is the numerical aperture of the objective lens. For the lateral resolution, it is $\delta x_{annu} = c(\varepsilon) \times \delta x_{cir}$, where $\delta x_{cir} = \lambda / 2NA$ is the resolution of the optical system when a circular pupil with its radius equal to the outer radius, a, of the annulus is used. Note that $c(\varepsilon)$ depends on ε and is smaller than 1. Hence we see that annular pupil provides a better resolution and at the same time a longer depth-of-focus as compared with the circular pupil.

Let us now pay some attention to the depth-of-focus of the annulus. By inspecting Table 1, it is clear that for small ε, the effect of long-depth-of-focus is minimal. However, for ε=0.95, the depth-of-focus of the annulus is more than a factor of 10 than that of a circular pupil with the radius of the circular aperture equal to the outer radius of the annulus. At ε = 0.99, a factor of more than 50 is achieved. As an example, for NA = 0.3 and at illumination wavelength $\lambda = 0.6\,\mu m, \delta x_{cir} = 1\mu m$ and $\delta z_{cir} = 6.67\mu m$, whereas $\delta x_{ann} \approx 1\mu m$ and $\delta z_{ann} = 50.25 \times \delta z_{cir} = 50.25 \times 6.67 \approx 335\mu m$ for $\varepsilon = 0.99$. Hence, any thick specimens of thickness less than 300 μm would be able to be imaged sharply by this annular pupil without any z-scanning and at the same time without any out-of-focus contamination. Indeed, the 3-D OTF has been calculated for a laser-scan fluorescence microscope [11]. An annular illumination microscope has also been constructed and investigated recently [12]. However, due to the low value of ε being used, the gain in depth-of-focus was not significant enough and depth scanning was required for 3-D inspection [12].

ε	$\frac{1}{1-\varepsilon^2}\delta z_{cir}$
0.99	$50.25\delta z_{cir}$
0.95	$10.25\delta z_{cir}$
0.9	$5.26\delta z_{cir}$
0.8	$2.78\delta z_{cir}$

Table 1: Depth-of-focus of annular pupils
in terms of the depth-of-focus of circular pupils when ε varies

However, the use of an annular aperture for $\varepsilon \approx 1$ has a major drawback in that it stops and wastes a large amount of light and, therefore, in particularly, it has not been used effectively for fluorescence microscopes for the purpose of achieving larger depth of focus for 3-D microscopic imaging.

V. PROPOSED NOVEL ILLUMINATION

We develop a technique for 3-D microscopic imaging with extended depth-of-focus using a novel illumination scheme in a laser scanning optical microscope. The novel illumination scheme creates an effective annular pupil, called the difference-of-Gaussian annular pupil, without the critical drawback of stopping and wasting the light. Two laser beams of different Gaussian pupils with different temporal frequencies are first generated. The laser beams are then combined spatially and used to scan the specimen. The scattered light from the object is picked up by a photodetector whose output consists of a DC and an AC current (due to the optical heterodyning or mixing of the two optical beams). The DC signal is no difference from the DC output of a conventional laser scanning microscope with the processing pupil as a Gaussian function, whereas the AC signal is derived from the mixing of the two Gaussian beams and would be given by effectively a Gaussian pupil with a different size than that generated by the DC signal. The AC and the DC signals are then subtracted by electronics and hence the effective pupil function would be given by the difference of the two Gassuian pupil functions. By properly choosing the size of the two Gaussian laser beams, we could realize the difference of the two Gaussian pupils which becomes a new type of annular pupil called the difference-of-Gaussian annular pupil. Since no stopping of the light is used to create the novel annular aperture, the proposed system could prove to be more robust and practical than currently available methods in 3-D imaging when extended depth- of- focus is required. In addition, the proposed system does not require depth- or z-scanning for 3-D imaging which is important for many practical applications.

i) Design Idea

The novel pupil we want to synthesize is of the form of difference-of-Gaussians. The idea is as follows. Let the pupil function, p(x), of the form of the difference of two Gaussian function:

$$p(x) = g_1(x) - g_2(x) \quad , \tag{7}$$

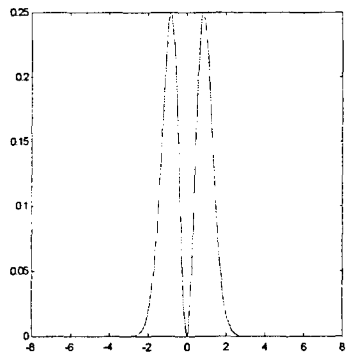
where we denote that $g_1(x)$ and $g_2(x)$ are two Gaussian functions of different size. The corresponding PSF is then given by, according to (2),

$$h(x) = |F\{p(x)\}|^2 = |F\{g_1(x) - g_2(x)\}|^2 = |G_1(\frac{x}{\lambda f}) - G_2(\frac{x}{\lambda f})|^2 , \tag{8}$$

where G_1 and G_2 are Fourier transforms of g_1 and g_2, respectively, and they are also Gaussian functions as the Fourier transform of a Guassian function is also a Gaussian function. In fact, Gaussian functions are the so-called self-Fourier transform functions [13]. Expanding eq.(8), the PSF now becomes

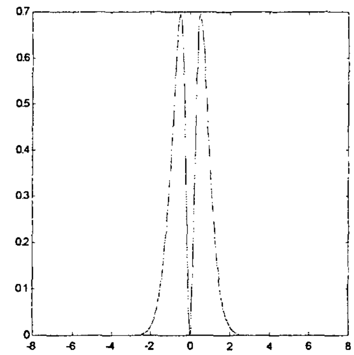
$$h(x) = G_1^2 + G_2^2 - 2G_1G_2 . \tag{9}$$

The form of the PSF given in (9) is the effective PSF expression that we are looking for to be implemented. Before we discuss how to implement the PSF shown in eq.(9), let us first discuss briefly the difference-of-Gaussians annular pupil of the form given by (7). Fig. 3) shows two examples of the difference-of-Gaussians function. For the Gaussian function given by the expression e^{-wx^2}, we define w as the width of the Gaussian. Fig. 3a) shows the difference of two Gaussians with width=1.0 and 2.0, and fig. 3b) with the widths =1.0 and 10.0. Fig. 3 shows only a 1-D plot of the pupil function. We can recover a full 2-D pupil function by changing x^2 to $x^2 + y^2$ in the Gaussian function. Note that the spatial extent of the graphs in fig. 3 is from -3 to +3 and the separation of the two peaks of the graph in fig. 3b) is closer than that of fig 3a). Hence by choosing different widths of the two Gaussians, one could synthesize the difference-of-Gaussian annular pupil with different obscuration ratio.



exp(-1.0*x.^2)-exp(-2.0*x.^2)

Fig. 3a) Difference-of-Gaussians annular pupil (w =1 and 2)



exp(-1.0*x.^2)-exp(-10.*x.^2)

Fig. 3b) Difference-of-Gaussians annular pupil (w=1 and 10)

ii) Implementation method:

In the novel illumination scheme, two laser beams with different temporal frequencies are first generated. The optical beams are then combined spatially and used to scan the specimen. The scattered light or the transmitted light from the object is then picked up by a photodetector. The photodetector has two outputs, one is a DC current and the other is an AC current. The two currents represent the two scanned and processed version of the original object and can be sent to a monitor for real-time display or to a digital storage device for possible further processing. Let us now discuss an optical implementation of the idea. Referring to Fig. 4, a spatial fileter (SF) cleans up the laser beam to give a clean Gaussian beam. The Gaussian beam is then collimated by lens L. The two beamsplitters (BS) and the two mirrors (M) then form an interferometer. u and v represents two pupil functions along the two paths of the interferometer. However, the temporal frequency of the laser beam along the path where the pupil v is located has been shifted to the amount equal to $\omega_0 + \Omega$, where ω_0 is the frequency of the laser, and Ω is the frequency shift provided by an acousto-optic frequency shifter (AOFM) [14]. The AOFM is a device, which accept the incident laser beam at frequency ω_0 and give out a laser beam at frequency $\omega_0 + \Omega$ [14]. Now, the two pupils u and v are combined by beamsplitter BS2 to give an effective overall PSF of the form, at the focal plane of lens L1,

$$h(x,y) = |F\{u\}e^{j\omega_0 t} + F\{v\}e^{j(\omega_0+\Omega)t}|^2 = |Ue^{j\omega_0 t} + Ve^{j(\omega_0+\Omega)t}|^2 \qquad (10)$$

Lenses L2 and L3 just form an optical relay system such that the PSF formed at the focal plane of lens L1 is projected and focused onto the specimen.

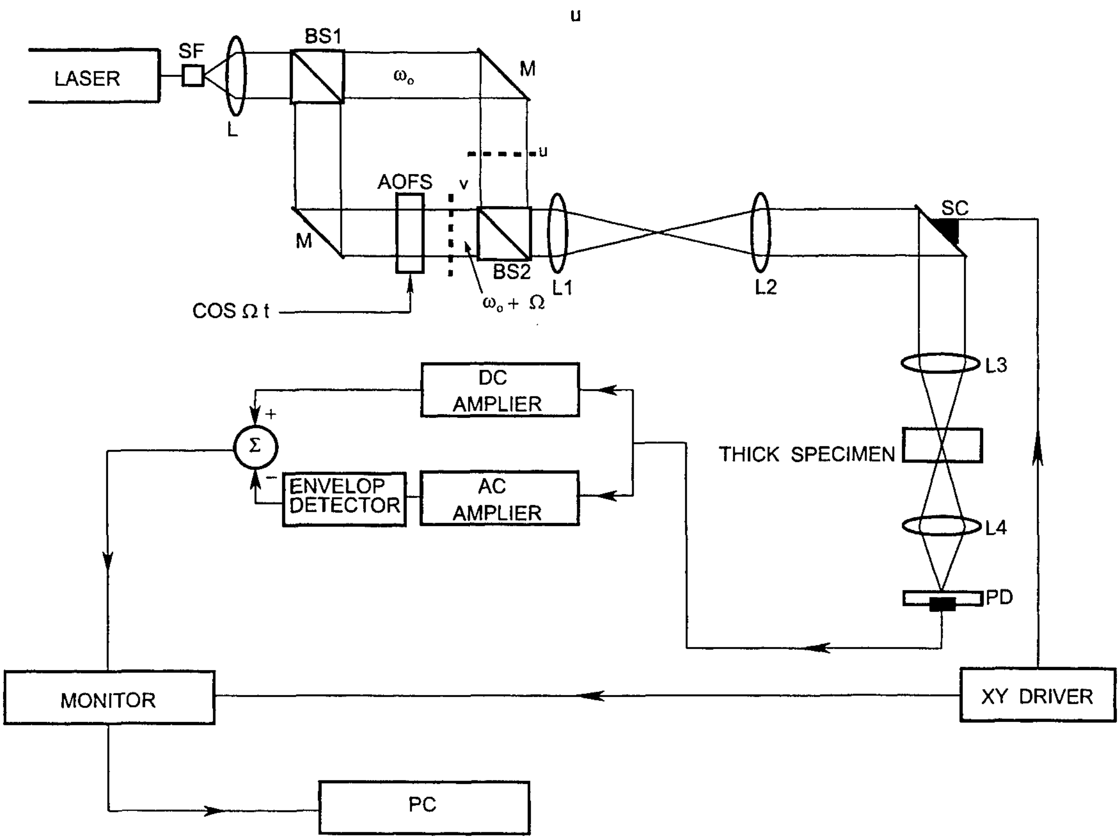


Fig. 4 : Proposed system for implementating difference-of-Gaussians annular aperture The AC amplifier has a gain of β. Σ is an electronic summer. u and v are the two Gaussian pupils

Note that by comparing to the conventional PSF of the laser scanning system [see equation (2)], the PSF in the proposed system has two terms contributing to the effective PSF. In addition, the two terms are carried by two different frequencies. As we can see that, by expanding (10), we have

$$h(x,y) = |U|^2 + |V|^2 + U^* V e^{j\Omega t} + U V^* e^{-j\Omega t}$$

$$= |U|^2 + |V|^2 + 2|UV| \cos[\Omega t + \arg(V) + \arg(U)]$$

$$= h_{dc}(x,y) + h_{ac}(x,y) |\cos[\Omega t + \arg(V) + \arg(U)], \quad (11)$$

where U and V are Fourier transforms of u and v and arg(.) stands for the argument of. We now clearly see that the effective PSF consists of a DC term and an AC term at frequency Ω due to the mixing (or heterodyning) of U and V as indicated in (10). Now, by substituting (11) into (1), we have

$$I_i(x,y) = I_0(x,y) * h(x,y) \quad (12)$$

$$= I_0(x,y) * [|U|^2 + |V|^2 + 2|UV| \cos[\Omega t + \arg(V) + \arg(U)]]$$

$$= I_0(x,y) * (|U|^2 + |V|^2) + I_0(x,y) * 2|UV| \cos[\Omega t + \arg(V) + \arg(U)].$$

We see that the photodetector's output consists of a DC and an AC signal. Both of the signals contain the scanned and processed version of the object, $I_o(x,y)$, and can be separated by a lowpass filter and a bandpass filter tuned at frequency Ω, giving outputs as

$$(I_i(x,y))_{dc} = I_o(x,y) * (|U|^2 + |V|^2) \quad ; \qquad (13a)$$

$$(I_i(x,y))_{\Omega} = I_o(x,y) * 2|UV|\cos[\Omega t + \arg(V) + \arg(U)], \qquad (13b)$$

respectively. Note that $(I_i(x,y))_{\Omega}$ is an amplitude modulated signal with temporal frequency at Ω. To demodulate the signal, one can use an envelope detector. The demodulated signal then carries the processed version of $I_o(x,y)$ at the AC channel and is now given by

$$(I_i(x,y))_{ac} = I_o(x,y) * 2|UV| \qquad (14)$$

Now, by performing subtraction of the two outputs $(I_i(x,y))_{dc}$ and $(I_i(x,y))_{ac}$ as shown in fig. 4 by an electronic summer, we can construct or synthesize a final processed output $I_i(x,y)$ of the proposed system as, using (13),

$$\begin{aligned} I_i(x,y) &= (I_i(x,y))_{dc} - \beta(I_i(x,y))_{ac} \\ &= I_o(x,y) * (|U|^2 + |V|^2) - \beta\, I_o(x,y) * 2|UV| \\ &= I_o(x,y) * PSF_{novel} \quad , \qquad (15) \end{aligned}$$

where $PSF_{novel} = (|U|^2 + |V|^2) - 2\beta\,|UV|$ is the novel point spread function of the proposed system, and β is the gain of the AC amplifier. Note that this synthesized novel PSF has the same functional form of the desired PSF given by (9) when U and V are Gaussians. Indeed, when we employ a laser beam. The laser beam shape has a Gaussian profile and that means the two pupils u and v are Gaussians and hence U and V are Gaussians, as again $F\{u\} = U$ and $F\{v\} = V$.

iii) Computer Post-Processing

The PSF proposed in (15) should allow us to synthesize an obscuration ratio associated with the novel annular aperture such that a long depth-of-focus can be achieved and yet at the same time without the drawback of stopping or wasting light. However, the use of annular-type pupils has the effect of lowering the contrast in the obtained image. Fig. 5 shows the OTF associated with the conventional annular aperture of outer radius equal to a. In the figure, f is the focal length of the objective lens. The solid line represents the OTF corresponding to the annular aperture, whereas the dotted line represents the OTF corresponding to a circular lens with a as the radius. Note that, for the annular pupil, high frequency content of the information of the object has been attenuated as compared to the use of a circular lens. Hence the proposed annular aperture will also lead to low contrast images. Fortunately, the loss in contrast can be restored by using a computer effectively. Indeed an inverse filter operation can be performed on the computer. To investigate 3-D filtering of the imaging system, we need to find the 3-D OTF of the proposed system, and this can be done in general by using (4) and (5) where p(x,y) is given by (7) when (7) is written in x and y coordinates. Since the pupil function given by (7) involves the difference of two Gaussians, the OTF therefore can be calculated analytically quite easily. To restore contrast, we use 1/OTF, an exact inverse filter.

However, since the use of this novel annular aperture leads to a longer depth-of-focus, I speculate that the use of in-focus PSF would serve the purpose of restoring contrast as the OTF would not change appreciably during de-focus. This needs to be investigated for this type of novel pupil. If this turns out to be true, OTF can be calculated simply by taking the Fourier transform of the in-focus PSF_{novel} given in (15) and that would save a lot of computation time when restoring contrast.

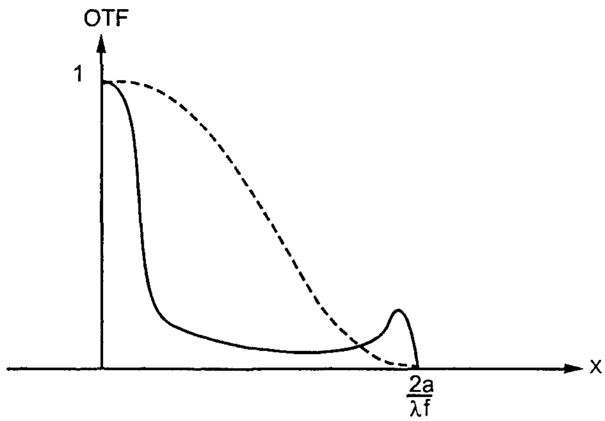


Fig. 5 : OTFs of conventional annular lens and the circular lens.
Solid line: annulus, Dotted line: circular lens
X is the spatial frequency along the x-direction.

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