Calculated two-photon fluorescence correction factors for reflective scan engines

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Excitation laser spatial and temporal characteristics at the objective focal point are critical to the performance of two-photon scanning microscopes. Optical aberrations in scanning systems increase the microscope objective focal spot area and introduce pulse time broadening in the deflected beam, resulting in degradation of two-photon-induced fluorescence across the scan field. The geometrical pulse broadening is investigated for what is believed to be the first time and then combined with a focused spot area to provide a normalized two-photon fluorescence intensity correction factor. This factor, calculated using OSLO optical software, is compared for four reflective scan engines and allows compensation of the detected signal with position across the scan field. This new metric highlights that a parabolic mirror afocal relay exhibits superior performance as a reflective scan engine for two-photon scanning microscopy. © 2010 Optical Society of America

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1. Introduction

Multiphoton fluorescence scanning microscopy [1] is employed in biomedical research because of its ability to produce images of biological activities and structure deep within live tissues [2–6]. When focused by a high numerical aperture objective, near-infrared ultrashort laser pulses create the very high photon flux at the focal point required for the fluorescent molecules in the sample to absorb two photons simultaneously. The average two-photon-induced fluorescence emission per fluorophore per incident photon per second is given by Diaspro and Sheppard [7] as

$$\langle I_{\text{fl}}(t) \rangle = \frac{\delta_2 P_{\text{ave}}^2}{\tau_p f_p} \left[\frac{\pi \text{NA}^2}{hc\lambda} \right]^2,$$

where δ_2 is the two-photon absorption cross section, P_{ave} is the laser average power, τ_p is the pulse duration, f_p is the laser pulse repetition rate, and NA is the objective lens numerical aperture, while h is the Planck constant and c is the speed of light of wavelength λ . When diffraction-limited performance is convolved with this expression, the two-photon-induced fluorescence intensity from the excited volume element emerges as being inversely proportional to the pulse duration of the exciting laser and the square of the focused spot area for a particular fluorophore:

$$I_{\text{fl}} \propto \frac{1}{\text{Area}^2 \tau_p} = F,$$

where F , the fluorescence intensity factor, is introduced within this research as a parameter of the optical system. The evaluation of F across the scan field is a useful metric, as for any chosen wavelength, average laser beam power, and pulse repetition rate, any increase in focal spot size and any temporal

broadening degrades the multiphoton fluorescence emission.

The minimum focal spot size, ideally the diffraction limit, can be achieved in optical systems when a planar wavefront uniformly illuminates the back aperture of an objective. However, in scanning microscopy the beam must be deflected in order to build the scanned image of the sample and, as a result, the beam wavefront is tilted with respect to the objective back entrance at some angle for all scan pattern positions except on-axis. Moreover, the beam wavefront can be distorted prior to the objective by other optical elements including the scan engine. The combination leads to the actual optical performance at the raster in the sample falling below the benchmark diffraction limit, as is evident in Fig. 1, which shows lens performance for skew incident beams.

The temporal pulse broadening in a microscope system is dominated by, and is usually ascribed completely to, the effect of group velocity dispersion (GVD) by lens materials. This effect has been well investigated [8] and arises from the different wavelengths within an ultrashort pulse propagating with different velocities through each of the different glass elements in the optical train, resulting in different chromatic elements of the pulse arriving at different times in the focal plane. The net effect is to decrease the peak intensity at the focal point. The modern multiphoton microscope can employ a pulse compressor system [9,10] or a negative chirp GVD compensator [11] to effectively precorrect for the *on-axis* temporal pulse broadening associated with the microscope objective and other refractive or coated optical elements. The GVD associated with *off-axis* beam propagation through the optical train

while scanning is impossible to compensate for dynamically and difficult to measure experimentally. Using only reflective surfaces in the scan system limits the GVD-induced pulse broadening to that associated with the atmospheric optical path distance for any system.

The contribution of the scan engine itself to pulse time broadening has, until now, been overlooked. For fast scanning by any scan engine, the mirrors used must have a low moment of rotational inertia arising from mirror mass and size [12] and effectively proportional to the cube of the aperture [13]. However, even with small mirrors, the optical path difference between the peripheral rays creates a distorted pulse front and an effective temporal broadening at the sample.

This research investigates the optimum scan engine design to achieve the best possible two-photon fluorescent signal by comparing and contrasting four scan engines. These are evaluated by spot size within and across the scan field as well as, for the first time to our knowledge, the wavefront distortion associated with the scan engine, allowing the calculation of the effective temporal broadening induced by the scan engine itself. We introduce the new fluorescence intensity factor as a system metric and calculate these values across the scan field for three scan engines normalized to an ideal reference system of a single mirror. The analysis further highlights the advantage of a new parabolic reflective scanning engine design [14] that employs two parabolic reflectors as an afocal relay between two scan mirrors.

2. Approach

This paper uses OSLO optical design software (Lambda Research Inc) to calculate the spot diagrams, point spread functions, and wavefront distortion of input Gaussian beams based on the propagation of a grid of rays launched from an on-axis object point and evenly spaced through the system entrance pupil with an evaluation of the rays at the intermediate image plane. The beam spot areas at the different scan field points evaluated were calculated using the astigmatic Gaussian beam analysis option within OSLO. The input beam profiles are representative of the TEM₀₀ titanium-doped sapphire near-infrared ultrashort pulsed laser used in two-photon microscopy. Pulse time broadening arising from the difference between peripheral rays was calculated based on geometrical analysis of OSLO results for spot footprint and angle of incidence. The fluorescence intensity factors were calculated from the spot area and the pulse time broadening.

The performance advantages of particular scan engines are shown through comparison with other known and commonly used systems. There are many scanner designs in optical scanning microscopy from single mirror deflectors to very complex multiple element arrangements designed to reduce beam aberration and field distortion [12,15,16]. Not all of them can be applied in fast imaging microscopy due to

Fig. 1. (Color online) (a) Incident angle dependence of focusing lens optical performance. (b) Four spot diagrams and (c) four intensity PSFs represent incident beam angles at the lens of (1) 0°, (2) 10° in the horizontal direction, (3) 10° in the vertical direction, and (4) 10° simultaneously in both directions relative to the optical axis. The scale on the spot diagrams is different, and relative size may be gauged by the black Airy disk circle on the spot diagrams.

the mass of moving elements and, particularly in the two-photon fluorescence microscopy case, the fact that engines with only achromatic metallic-coated reflective elements are optimal as they do not introduce additional GVD to the propagated beam.

Three established reflective designs and the recent parabolic scan system have been simulated. All systems deflect the incoming beam in two perpendicular directions, as shown in Figs. 2(a)–2(d). They represent (a) a single flat scan mirror; (b) two flat scan mirrors as a close coupled engine (CCE); (c) the spherical mirror based SSE system with two flat scan mirrors, each with its rotational axis in an orthogonal direction and two spherical mirrors between them as reflective relay optics; (d) the PSE system, including two flat scan mirrors with rotational axes in two orthogonal directions and two parabolic reflectors between them as reflective relay optics. The 25 points of the scan pattern used to analyze performance of the scan engines at the intermediate image plane are shown in Fig. 2(e).

A one-mirror scan engine [12] is ideal in microscopy, as gimbaling a single mirror introduces the lowest possible aberration to the propagating beam with the scan pattern symmetric about the X and Y axes and its pivot point is easily located in the objective conjugated telecentric plane. This engine is modeled and used as a reference system (RS) in this paper. In reality, this design is not practical for fast scanning as the mirror must pivot about the optical center of the reflective face and the combined mass of mirror and mount leads to a very high moment of inertia. The next design, two close coupled mirrors (CCE) [12,15], is also a simple system but is limited in speed by the size and moment of inertia of the second mirror. Moreover, the effective pivot point of close coupled mirrors is between the two mirrors so it cannot provide microscope telecentricity. The third design, based on spherical mirrors (SSE), and the new parabolic mirror design (PSE) are afocal relays and are simulated according to the Amos patent and associated paper [17,18] and our paper [14], respectively. In both these systems, the optics relay the image of the first scan mirror to the surface of the

second scan mirror, ensuring that the scan line created by the first deflecting mirror is imaged to one spot on the surface of the second scan mirror. For the case of the PSE, this is illustrated graphically in Fig. 3, where the relay of a circular arc emerging from a scan mirror located at the parabola focal point is relayed through a second arc and down to the focal point of the second parabolic mirror. The PSE and SSE designs provide one effective pivot point to align within the conjugated telecentric objective plane and allow a small fast resonant scanning mirror to be used so as to increase the imaging speed. However, the SSE system suffers from spherical aberration and astigmatism because of the tilted spherical surfaces employed.

In all scan engines modeled, the scan mirrors were moved $\pm 10^\circ$ in two orthogonal directions. The radii of concave surfaces for the SSE and PSE engines were chosen to be the same to compare their performance with the off-axis parabola specifications from Newport Corp. model 50338AL.

3. Scan Engine Position within Optical Path

The illumination path in a typical two-photon scanning microscope is schematically illustrated in Fig. 4, where the laser beam traverses the scan system, an eyepiece or f -theta scan lens [19–21], and the tube lens employed in the case of infinity-corrected objectives [15,22] before reflection or passage to the objective lens and ultimately the sample. The incident laser beam is deflected by the scan engine and is usually parallel to the objective optical axis in the undeflected position. The position of the focused spot in the objective focal plane depends on the incident beam angle at the telecentric plane (the objective back aperture), therefore, the scan engines' center of all deflecting movement (pivot point) must be positioned in the conjugated telecentric plane of the objective lens [15,23]. The scan pattern created is then focused to the microscope intermediate image plane and projected to the objective focal plane where the sample is positioned.

Following the scan engine, the beam is focused by a scan lens or f -theta lens to the intermediate image

Fig. 2. (Color online) Four simulated scanning designs: (a) single mirror engine used as the reference design RS, (b) close coupled engine CCE, (c) spherical reflector engine SSE, and (d) parabolic reflector engine PSE. (e) Scan pattern points analyzed.

Fig. 3. (Color online) (a) PSE optical relay. (b) and (d) Circular arc traced on surfaces 4 and 6, respectively. (c) Scan angle dependent parallel ray paths between the first and second scan mirrors.

Fig. 4. (Color online) Scan engine position in a typical two-photon scanning microscope arrangement. The scan lens used in this work was an optimized scan lens design from the OSLO database as shown in the expanded detail.

plane in the microscope. An optimized scan lens design from the OSLO database, as detailed within Fig. 4, was used in this work. This choice is optimal for optical aberrations, the thrust of this paper, but it should be noted would be a poorer choice for GVD due to the number and thickness of glass elements. In practice a two-photon system may well use an eyepiece with reduced wide field performance or even a singlet lens for better GVD performance. The distance from the last surface of the scan lens to the intermediate image plane was determined on the basis of minimal RMS spot size for the on-axis beam in position 13 from Fig. 2(e).

As microscope objectives, and any tube lens for an infinity-corrected objective, are well-corrected optics located inside the microscope body, the scanner performance analysis presented here is calculated at the intermediate image plane, in essence a magnified replica of the scanned beam in the objective focal plane.

4. Results and Discussion

Results are shown through comparison of spot diagrams, point spread functions (PSF), maximum PSF values, wavefront errors, calculated spot areas, pulse front aberrations, related pulse time dispersion, and the relative fluorescence intensity factors. The relative scan geometries for the various engines are shown in Fig. 5.

A. Spot Diagrams and PSF

The spot diagrams for four examined scan engines at 25 points are shown in Fig. 6(a). It can be seen that diffraction-limited performance is achieved only for the RS, CCE, and PSE designs at the central nine configurations of the examined scan area and performance degrades at the extreme angles. These results are reflected on the appropriate normalized intensity PSF map graphs for the same points, Fig. 6(b). Note the bell-shaped spots distributed around the peak for the central nine configurations with the maximum peak close to 1 and distorted spots with reduced maximum value at the sides. The intensity PSFs for the CCE along scan lines are distorted faster in compar-

Fig. 5. (Color online) Center of mass points for 25 configurations of the four scan engines. The RS system scan pattern is symmetrical and shows symmetrical pincushion distortion. The PSE engine creates straight scan lines with pincushion distortion in both sides, similar to the RS system. The CCE system produced elongated scan lines with pincushion distortions on the sides. The SSE created scan lines have different curvatures, which increase down the scan area. The created scan lines are straight only for the PSE system.

ison with the RS and PSE designs as the CCE produces elongated scan lines for the same deflecting angle compared to both the RS and PSE designs as may be seen in Fig. 5. The SSE design produces the worst performance with aberrated spot footprints due to astigmatism arising from the tilted dual spherical reflectors. The maximum PSF value is only 0.12 in the off-center configurations 12 and 14 arising from extreme astigmatism in these scan positions.

Figures 6(c) and 6(d) show the PSF maximum across the whole scan field at a resolution of 0.1° . Diffraction-limited performance, as determined from PSF max values between 0.75 and 1, is produced by RS and PSE designs across the central area while for the CCE this ideal is reached only in the middle of scan lines. The SSE design does not approach diffraction-limited performance across the whole area with PSF maximum values less than 0.16. The 2D panels in Fig. 6(d) are instructive in highlighting the symmetry of the PSF variation. The panel for the PSE shows a very slight performance decrease in the upper half due to the optical magnification experienced by the expanding spherical wavefront interacting with identical parabolic surfaces spaced symmetrically, while the RS is ideal and the CCE reflects scan elongation.

B. Wavefront Contours and Wavefront Error

The resultant geometrical wavefront contours for an on-axis object point for the 25 scan field points are shown in Fig. 7(a). The wavefront error, defined as the peak-to-valley optical path difference in wavelength (P-V OPD), across the whole scanning field is shown in Fig. 7(b). These results confirm the diffraction-limited performance of RS, CCE, and PSE for the nine central points of the scanning pattern where the wavefront error for RS and PSE systems is less than one quarter-wave. For CCE the wavefront distortion is approaching a half-wave with increasing distortion towards extremities, and for the SSE, distortion is more than three wavelengths for all configurations.

The wavefront aberration in the close-coupled mirror design arises from the distance separating the two flat mirrors and the deflection angle of both mirrors. The CCE result shows similarity with the

Fig. 6. (Color online) (a) Spot diagrams and (b) intensity PSFs for four scan engines in 25 scan configurations. Scan angle range in both directions is from -10° to $+10^\circ$. (c) and (d) PSF maximum values as contour and 2D maps across the whole scanning field at a resolution of 0.1° . The color scale bar is common for (d) and (c) of each column.

reference system for the central vertical axis where the first scan mirror is at 0° deflection and the beam position is defined only by the deflection of the second

scan mirror. The P-V OPD distortion increases rapidly with deviation from center along horizontal zones and is only comparable to the RS result when

Fig. 7. (Color online) Wavefront aberrations for four scan engines. (a) Wavefront contours, (b) $\ln(P-V\text{-OPD})$, (c) scaled 2-D maps of central ($\pm 5^\circ$) scan field.

Fig. 8. (Color online) Spot areas at the intermediate image plane for (a) RS, (b) PSE, (c) CCE, and (d) SSE systems.

the second scan mirror is within $\pm 5^\circ$ of the central position.

The worst wavefront distortion results are observed in the SSE system, where even in the central position the P-V OPD error reaches the value of 3.37λ . The P-V OPD is higher at the top two corners in comparison to the lower two, which arises from the SSE system not being completely symmetrical compared to the other systems as the two scanning mirrors are angled differently to obtain an unobstructed 2D scanner. When those scan mirror tilt angles are close in value to the static mirror tilt as at configurations 21° to 25° , the wavefront error decreases.

The panels in Fig. 7(c) are of the central field within $\pm 5^\circ$ of the central position and show the wavefront error to be lowest for the RS and PSE designs.

C. Fluorescence Intensity Factor

The fluorescence intensity factor F , as described in Section 1, has been calculated and the performance of the scan engines normalized with respect to the RS system evaluated. The necessary data are the spot area and the pulse time broadening.

A spot area has been calculated using astigmatic beam analysis data for a propagated Gaussian beam, which presents the beam radii at the image surface in two perpendicular directions, X and Y , where the beam spot is an ellipse; the spot size is calculated as $\text{Area} = \pi ab$, where a and b are the two beam radii. The spot area plots for four systems are shown in Fig. 8. The spot areas for three systems, RS, PSE, and CCE are nearly the same in the $\pm 5^\circ$ scanning range. With the CCE design, the spot area increases up to 25 times toward scan extremities. The SSE result is from 5 to 15 times larger at the central configurations along all scan lines and sharply increases at the extremities.

The concept of pulse time broadening calculation at any scan field point is presented in Appendix A. The resultant time delay induced by scan engines is shown in Fig. 9. The PSE system has shown a very similar result to the RS system with both below 14 fs maximum at scan extremities and below 0.3 fs within $\pm 5^\circ$ of the central position. These engines show a difference of less than 0.07 fs across the central area.

The CCE induces a 1.1 fs broadening, which is 5.5 times larger in the central area compared to RS and

Fig. 9. (Color online) Time delay introduced by scan engines for (a) RS, (b) PSE, (c) CCE, and (d) SSE.

Fig. 10. (Color online) Relative fluorescence intensity factor for (a) PSE, (b) CCE, and (c) SSE across the investigated area.

increases towards the scan extremities. The SSE system produces up to 1.84 fs pulse broadening for the central region and sharply increases at the scan extremities.

Calculated relative fluorescence intensity factors are plotted in Fig. 10, and value ranges are shown in Table 1. Results confirm that the performance of the new PSE scan engine design is similar to RS and if employed in two-photon scanning microscopy will produce higher two-photon fluorescence

intensity signals. The evaluation of F across the whole scan area reveals more uniform and even values for the PSE design compared to the CCE and SSE designs. Within the $\pm 5^\circ$ scan range, the calculated two-photon fluorescence signal is up to 1.74 times higher for the PSE design compared to a single mirror scanning system, which is 10% higher than for a CCE system and 32 times higher than in an SSE system, which shows a maximum of 0.06 in all configurations, indicating that it suffers a 94% reduction in signal compared to the RS.

Table 1. Minimal and Maximum Relative Fluorescence Intensity Factors for PSE, CCE, and SSE Compared to the RS Range of Values for the Central Section of the Investigation Area

	PSE/RS	CCE/RS	SSE/RS
min	1.2	1.14	0.005
max	1.74	1.64	0.055

5. Conclusions

This paper introduces a new metric for scan engine performance evaluation, the fluorescence intensity factor, which was used to evaluate the performance of four scan engines. The factor is determined by spot area, as determined by spot diagrams and PSFs, wavefront distortion, and the associated temporal broadening. This temporal broadening attributable to the scan engine was calculated for what is believed to be the first time and found to be small (< 2 fs) in the central regions of the scan field but much higher in the extremities. The central field wavefront distortion as a contributing proportion of overall pulse temporal characteristics will grow to be more significant as shorter incident laser pulses are employed in two-photon microscopy.

It was found from the study above that the scan engine design employing two off-axis parabolic reflectors, appropriately positioned relative to the rotational axis of the scan mirrors, is the best choice of scan system to apply in two- and multiphoton fast imaging microscopy. The wavefront aberrations introduced to the pulsed laser beam over the range of the scan angles are minimal compared to existing systems and hence minimize pulse distortion. The geometrical spot diagrams and PSFs of the on-axis object point are equal to the reference system,

Fig. 11. (Color online) Calculating optical path difference between peripheral rays for any scan point.

confirming that optically the PSE design is very similar to an ideal single mirror scanner. The PSE is a practical realizable design with the unique ability to employ small-sized scan mirrors and hence scan rapidly and represents an ideal choice for multi-photon scanning microscopy.

Appendix A: Calculating a Beam Time Delay at Any Point of a Scan Field

A beam interacting with some surface will have a wavefront distortion (or time delay) as a result of the optical path length for different peripheral rays. At any scan position the spot intersection with the surface is elliptical with a major axis, as shown in Fig. 11. The highest OPD is created by the peripheral beam rays OC and OA, which generate the longest major radius CA. Hence, OPD is equal to BC. For known beam incidence angle α to a surface the OPD can be geometrically calculated. OSLO provides the elliptical Gaussian spots major axis values and the value of the incident angle. Hence, the OPD can be calculated from

$$\text{OPD} = BC = AC * \sin \alpha.$$

The time delay induced is $\Delta t = \text{OPD}/c$, where c is the speed of the light.

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