A compact acousto-optic lens for 2D and 3D femtosecond based 2-photon microscopy

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Abstract

We describe a high speed 3D Acousto-Optic Lens Microscope (AOLM) for femtosecond 2-photon imaging. By optimizing the design of the 4 AO Deflectors (AODs) and by deriving new control algorithms, we have developed a compact spherical AOL with a low temporal dispersion that enables 2-photon imaging at 10-fold lower power than previously reported. We show that the AOLM can perform high speed 2D raster-scan imaging (>150 Hz) without scan rate dependent astigmatism. It can deflect and focus a laser beam in a 3D random access sequence at 30 kHz and has an extended focusing range (>137 μm; 40X 0.8NA objective). These features are likely to make the AOLM a useful tool for studying fast physiological processes distributed in 3D space.

1. Introduction

Two photon microscopy is increasingly being used to study biological processes [1-3]. Applications include imaging morphological structures, monitoring dynamic physiological processes with fluorescent reporters, triggering localized release of biologically active compounds with photolysis and controlling neuronal activity with genetically encoded light activated proteins. Many physiological processes of interest occur in small cellular structures at depths greater than 100 μm within tissue that absorbs and scatters light. The deep tissue penetration and submicrometer resolution that 2-photon microscopy provides has made this approach popular for studying such biological phenomena [4]. However, current 2-photon microscopes, that use galvanometer mirrors to steer the laser beam and build up an image, are too slow to monitor many fast spatially distributed physiological processes, which occur on the 1-100 ms time scale, since they typically take more than 100 ms to form an image [2]. Moreover, most microscopes developed to date are optimized for imaging a single X-Y plane. These constraints are particularly limiting for studying brain function, since information is encoded and transmitted as brief electrical impulses (~1 ms) in groups of neurons distributed in 3D space.

Several strategies have been employed to improve the temporal resolution of 2-photon imaging and monitoring of fluorescent reporters. These include using resonance scanners which speed up scanning and image acquisition [5], but the inevitable reduction in dwell time limits their utility except for the brightest fluorescent preparations. AODs have also been used because they provide a fast, mass-less scanning solution that is not limited by inertia. The first high speed microscopes using AODs in the linear raster scanning mode

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OCIS codes: (180.2520) Fluorescence microscopy; (180.6900) Three dimensional microscopy; (230.1040) Acousto-optical devices; (320.2250) Femtosecond phenomena
used a single AOD for fast X scanning and a galvanometer for the slower Y scanning [6, 7]. An additional advantage of AODs is that their fixed and rapid response time (10-25 μs) and high precision control and pointing stability allow them to deflect a focused laser beam from one location to another very rapidly and reproducibly (pointing mode). This enables random access multiphoton (RAMP) measurements to be made from multiple regions of interest (ROI) at high speed [8-13]. Since only ROIs are imaged, rather than the whole plane, RAMP can be orders of magnitude faster than the scanning mode and is particularly suitable for ROIs that are small and sparsely distributed. Such microscopes use 2 (or 3) AODs to make fast 2-photon fluorescent measurements from one 2D XY plane in neural tissue. Similar 2 AOD designs have been used for single photon photolysis [14] and 2-photon photolysis [15] in 2D. However, the disadvantage of using AODs is that forming an image is complicated by chromatic and temporal dispersion. An additional problem in the scanning mode is that as the speed of scanning is increased the AOD introduces a cylindrical focusing effect [16, 17]. The resulting astigmatism of the illumination point spread function (iPSF) can be corrected with a cylindrical lens, but only for a single scan speed, so most 2 AODs systems use pointing mode for full frame structural imaging. This is slow and can take several seconds per image [11].

There have been several recent advances in high speed 3D imaging. Sampling many locations (~100 neurons) within a 250 μm cube of cortex at 10 Hz has been achieved by combining fast piezoelectric control of the objective axial (Z) position and sophisticated galvanometer based XY plane scanning [18]. Faster continuous focusing has been demonstrated using a piston mirror, and an ingenious dual objective system that corrects for spherical aberrations [19]. The focusing properties of AODs have also been utilized for high speed focusing. Kaplan et al. (2001) first demonstrated that 2 AODs with counter propagating chirped acoustic waves could be used to produce a high speed (400 kHz) dynamic cylindrical lens [20] (illustrated in Fig. 1). They also proposed that 4 AODs could in principle be used to generate a spherical lens. These ideas were developed further and a proof of principle 3D 2-photon microscope was demonstrated that can focus rapidly and perform RAMP measurements [21]. However, a limitation of this system, which is based on 4 conventional AODs to form the acousto-optic lens (AOL), is that light transmission efficiency drops off rapidly if the light is focused more than ±25 μm from the natural focal plane of the NA=1 objective. Fast 3D pointing with this system was demonstrated by measuring calcium transients within neuronal processes [21] within the octahedral shaped 3D field of view [22]. The temporal dispersion of the AOL increased the 200 fs laser pulse width to the picosecond range, and resulted in reduced 2-photon excitation efficiency and thus increased the laser power required to image by an order of magnitude compared to conventional 2-photon systems. Moreover, structural imaging was slow (seconds per 2D plane) because the AOL was limited to the pointing mode rather than scanning mode [23]. This is problematic because 3D functional optical imaging using RAMP measurements requires the prior acquisition of a stack of high resolution images of the whole volume to be studied so that ROIs can be accurately selected. These and other technical difficulties (section 1.2) have prevented AODs from being widely accepted as suitable deflectors for 2-photon microscopy.

To overcome these technical difficulties we have designed and built a compact spherical AOL that can be used to scan and focus a femtosecond laser beam at high speed. At the core of our AOL are 4 AOD crystals with properties optimized for their function. By incorporating the AOL in a conventional 2-photon microscope we have developed a high speed 3D 2-photon microscope that can image rapidly in 2D raster-scanning mode and perform RAMP measurements in 3D at 30 kHz. Here we describe the crystal design and drive algorithms and quantify the spatial and temporal performance of this prototype.
AOLM. Our results suggest that this technology is likely to be useful for a range of applications that require high speed imaging and 3D RAMP measurements.

1.1 Principle of operation of cylindrical and spherical AOLS

A dynamic cylindrical lens formed of two AODs is illustrated in Fig. 1. The acoustic transducer on each AOD crystal produces an ultrasonic sound wave that propagates across the optical aperture of the crystal.

The sound wave induces changes in refractive index of the crystal which diffracts the incoming laser beam at an angle determined by the frequency of the sound wave. By changing the sound frequency with time (chirping) the optical wave front can be curved bringing it to a line focus in the XZ plane. A stationary focus requires two counter propagating AODs to cancel movement produced by the curtain of sound as it propagates across the crystal. A pair of AODs can thus form a stationary cylindrical lens in the XZ plane [20].

Increasing the positive chirp rate moves the line focus up (increasing Z), whilst adjusting the difference between the ramp centre frequencies adjusts the X position. Changing to a negative chirp rate produces a diverging optical wavefront with a virtual focus before the AODs. A second pair of counter propagating AODs orthogonal to the first is required in order to focus in the YZ plane [20]. The 4 AODs can then shape the optical wave front to give a spherically diverging or converging beam. This can be used to focus to a point above and below the natural focal plane of a subsequent fixed lens system.

1.2 Technical difficulties in using AODs for 2-photon microscopy

Although an AOL is potentially attractive for 3D pointing and high speed scanning, there are six significant technical difficulties to overcome for efficient 2-photon microscopy:

1. High efficiency AODs are made of tellurium dioxide (TeO$_2$) [16], which has a high group velocity dispersion (GVD), causing temporal chromatic dispersion of ultra-short Ti-Sapphire laser pulses. Moreover, standard AODs have a large axial depth to aperture ratio so the large aperture (9 mm) AODs required for high resolution imaging have a large axial depth (~30 mm). Four conventional AODs have a GVD of ~80,000 fs$^2$ and stretch femtosecond pulses to picosecond pulses, a level of dispersion that was considered impractical to compensate with a prism-based device [21]. This temporal dispersion contributed significantly to the increase in laser power required for 2-photon imaging from 5-10 mW for galvanometer systems to ~100 mW for such an AOD based system [21].

2. The diffraction efficiency of standard AODs is strongly dependent on the incident angle of the laser beam. The range of angles of incidence for the second AOD of each pair is much larger than the first AOD, which receives a collimated incident beam (Fig. 1). As the designed input acceptance angle is increased, the overall AOD efficiency drops. It is important to trade off these factors to maximize the 3D field of view.

3. The spectra of the ultra short (100-200 fs) pulses of Ti-Sapphire lasers are typically 10-5 nm wide, respectively. Since the deflection angle is proportional to the wavelength for diffractive deflectors, chromatic aberration can cause significant elongation or distortion of the iPSF. This can be minimized by using a configuration that compensates the aberration at the centre of the field of view [8, 22]. However, as the AODs deflect the spot away from the centre, the residual magnification chromatic aberration increases [11, 24].
4. When AODs are used for high speed scanned imaging (above about 20 frames/second for 100×100 voxels) the iPSF becomes distorted as the scan speed increases due to focusing of the faster scanning AOD [16, 17, 25]. One solution to this is to have a set of astigmatic correction lenses optimized for each of a set of predetermined scan rates. The disadvantages of this are increased mechanical complexity and fixed scan rates.

5. The path length of the AOL is over 1 m long due to three telecentric relays that superimpose the output of one AOD grating on the input to the next [21]. This is inconveniently large unless at least three pairs of precision aligned mirrors are used to fold the arrangement. Such a system is still bulky, very challenging to align [23] and has extra losses from the numerous optical components. It is therefore not ideal for widespread use.

6. Remote focusing, in combination with a high NA objective, is likely to be limited by spherical aberration [19, 26]. However, it is uncertain how this limits the focal range for 2-photon microscopy, since commercial objective designs are proprietary. It is therefore not clear whether AOL limitations [21, 26] or spherical aberration will ultimately limit the axial range of remotely focusing AOLMs.

In the following sections, we show how a high performance AOLM was designed to minimize these technical difficulties. This excludes improvement to residual chromatic aberration, which is beyond the scope of this paper. We also derive the control equations for a new compact AOL and develop a vector based approach to simplify imaging with AOLs.

2. Design of AODs and AOL for 2-photon microscopy

As outlined above, the three most basic problems in designing an AOL for femtosecond 2-photon microscopy are the high temporal dispersion caused by the thickness of the 4 AODs, the sharp fall off of diffraction efficiency with input angle and chromatic aberrations.

2.1 Design of AODs for high resolution imaging and minimum GVD

2.1.1 Speed and resolution—The speed of the AOD is limited by the time, \( T \), it takes to fill the AOD aperture:

\[
T = \frac{W}{V} \tag{1}
\]

where \( W \) is the aperture of the AOD and \( V \) is the speed of sound perpendicular to the direction of optical propagation. The fill time is therefore linearly related to the size and type of the crystals used. The resolution of an AOD can be expressed as the number of resolvable spots. For a single photon (single wavelength) system, analysis of the two-AOD system in Fig. 1 shows that the number of resolvable spots is equal to twice the time bandwidth product of each AOD [27]. Equivalently the number of resolvable spots \( N_r \) can be expressed in terms of \( W \), \( \lambda_0 \) the optical wavelength and \( s \) the semi-scan angle that the AOD deflects between the centre drive frequency and one of the frequency limits, which typically correspond to the 80% diffraction efficiency limits. These equations are:

\[
N_r = 2TB = \frac{4Ws}{\lambda_0} \tag{2}
\]

where \( B \) is the radio frequency drive bandwidth. Beneath a high NA objective, the number of resolvable spots reduces as NA increases; however this loss of resolution is more than compensated by the square law dependency of fluorescence on light intensity, since the size of the 2-photon PSF is smaller than the iPSF. At the centre of the field of view for NA = 0.8
at 800 nm wavelength the theoretical full width half maximum (FWHM) of the 2-photon PSF is \( w = 0.375 \mu \text{m} \) [1]. Based on this, and a geometric optic derivation of the field of view at the same NA, it can be simply estimated that the number of resolvable spots for a 2-photon AOD system \( N_{\text{rt}} \) is approximately:

\[
N_{\text{rt}} = 2.12T B = \frac{4.24 W_s}{\lambda_0}
\]

(3)

In view of the restricted semiscane angle of the second AOD of each pair in the AOL (see section 2.2), for the prototype we chose a large aperture AOD (\( W = 15 \text{ mm} \)) which results in a number of resolvable 2-photon spots of \( N_{\text{rt}} \approx 80 \) per mrad semiscan angle ‘s’ at NA=0.8 and \( \lambda_0 = 800 \text{ nm} \). This gives a resolution at the centre of the field of view of one part in 240, 480 or 720 for 3, 6, 9 mrad semi-scan angle, respectively. The fill time of a 15 mm TeO\(_2\) crystal is approximately 24 μs (Eq.(1)). However with a 15 mm aperture, the axial thickness of standard 55° acoustic wave walk off angle AOD would cause a large GVD for 4 AODs (~100,000 fs\(^2\)). This was judged impractically large to compensate [21] (Fig. 2; Technical difficulty 1).

2.1.2 Origins of the acoustic wave ‘walk off’ and design of thin, low GVD, AO crystals—Sound propagation within TeO\(_2\) crystals is highly anisotropic: in the <110> crystallographic direction it is \( V_{110} = 616 \text{ m/s} \) whereas along the <001> crystal axis (optic axis) it is \( V_{001} = 2400 \text{ m/s} \). In standard AODs, the high diffraction efficiency, large scan range, uniformity and linear polarization characteristics are achieved with a crystal cut that produces an acoustic wave vector angled at 5-6° to the <110> (Fig. 2a and b).

Unfortunately, the 5° off <110> axis acoustic wave vector, coupled to the anisotropy of the acoustic velocity, causes the acoustic wave to walk off at an angle of approximately 55° - 65° as shown in Fig. 2a. This has serious side effects for our application in that it causes the crystal to have a thickness much greater than its aperture size (Fig. 2a).

To reduce the acoustic walk off, and thus the AOD thickness and GVD, we altered the crystal cut. Reducing the acoustic rotation of the crystal to 2°, produced an acoustic walk off angle to 22° because the acoustic walk off angle is 11-fold greater than the acoustic wave vector rotation from the <110> direction (Fig. 2c). However, if light propagates closer than about 3° to the <001> optical axis, it becomes increasingly circularly polarized. It is therefore difficult to couple external light efficiently to these circular propagation modes. To minimize this problem we optically rotated the crystal by 3° (see inset Fig. 2d) so that the polarization of the input and output waves are >80% linearly polarized. This optically and acoustically rotated AOD design was first described by Young et al. (1990) [28] and was fabricated for us by (Gouch & Housego, Florida, LLC).

The reduced walk off angle of the custom designed AODs reduces the axial depth of 15 mm aperture AODs to 15 mm. This reduced the temporal dispersion of the 4 AODs to 36,000 fs\(^2\) at \( \lambda_0 = 800 \text{ nm} \). This, together with the 13000 fs\(^2\) dispersion from other microscope components, was sufficiently low to compensate with a custom prism based pre-chirper, with ~50,000 fs\(^2\) GVD (APE GmbH, Berlin). This customized AO crystal design therefore overcomes Technical difficulty (1).

2.2 AOD designs for low and high input acceptance angle

The most serious problem to overcome in designing an efficient AOL concerns the steep dependence between diffraction efficiency and input acceptance angle for AODs (Technical difficulty 2). AODs typically have high diffraction efficiency over a wide drive frequency
range. This is achieved because the acoustic vector is closely tangential to the inner ordinary index wave number surface that the diffracted output wave moves along as drive frequency is varied (Fig. 2b, d). This means that for a fixed direction input optical wave vector and fixed direction acoustic wave, a wide range of output angles can be obtained with only a small error between the sum of the incident and acoustic waves and the diffracted vector, which must remain on the ordinary wave number surface (vector with green polarization ellipse, Fig. 2b, d). This efficient acousto-optic coupling is not maintained as the angle of incidence of the input wave vector is varied because the acoustic vector is much less tangential to the index wave number surface (vector with red polarization ellipse). The diffraction efficiency of the AOD thus reduces much more rapidly with variation of incidence angle than with variation of output angle. This is a major problem because, as the laser beam is tilted and curved by the first AOD, transmission through the second AOD in each pair will become inefficient. Unless optimized, this property will severely restrict the focusing range and scan volume of the AOL.

A way around the narrow input acceptance angle problem is to increase the angular spread of the acousto-optic interaction. This can be achieved by lowering the drive centre frequency of the AOD so that acoustic wavelength is increased and by narrowing the width of the acoustic transducer in the direction of optical propagation (Fig. 3). The lower centre frequency of our custom AOD (35 MHz), compared to that of a standard commercial AOD (50-70 MHz), accounts for half the increase in acceptance angle shown in Fig. 2, but also reduces output scan angle range proportionately. For this application increasing the input acceptance angle is the limiting issue. To quantify these effects for our custom design we calculated the theoretical relationship between diffraction efficiency and transducer length normalized to 100% at 6 mm [27] (Fig. 3c). This relationship shows that as the transducer length decreases the acceptance angle increases. But, unfortunately, the diffraction efficiency drops as the transducer length decreases, because the acousto-optic interaction length reduces in proportion. To optimize both diffraction efficiency and uniformity of scanning we chose two different Lithium Niobate transducer lengths for the first and second AODs in each XZ or YZ plane pair. The first AOD of each pair, which receives light at a fixed input angle, had a relatively wide transducer (3.6 mm in our prototype) resulting in approximately 85% peak diffraction efficiency at ~3 W drive. Since we did not know the optimum transducer length for the second crystals, two transducers of 2.4 mm and 1.8 mm width were attached. These give acceptance angles of approximately 4 and 6 mrad at efficiencies of approximately 65% and 50% respectively at ~3 W drive.

In summary, the small input acceptance angle of AODs (Technical Difficulty 2) can be overcome by lowering the drive frequency and reducing transducer length for the second AOD of each pair to 2.4 - 1.8 mm, but this is at the expense of reducing diffraction efficiency as the transducer is narrowed. This trade-off between acceptance angle, which ultimately determines the field of view (FOV) and transmission efficiency, is a key design compromise that can be optimized for a particular AOL application by use of multiple transducer lengths on the second crystal of each pair.

2.3 Aberrations of AOD based deflectors

Unlike a mirror, the angle of deflection of an AOD is proportional to wavelength, introducing chromatic aberration with polychromatic femtosecond pulsed laser light (Technical difficulty 3). Chromatic aberration at the centre of the field of view can be corrected by ensuring that the deflection of the beam in each AOD of a particular pair are opposite in direction (Fig. 1a; [22, 29]). This is because, at this position, the deflections from the first AOD in each plane are exactly cancelled by the deflection of the second. The extent of the chromatic aberration increases with the radial distance away from the centre of field of view and the spectral width of the source. In the XY plane, a circular diffraction limited
iPSF at the centre of field of view becomes an increasingly elongated ellipse as its distance from the centre increases. Its long axis is aligned radially. Eq. (4) quantifies the expected dimension of the long axis, $l_{xy}$, of an imaged bead of width $b$, at radius $r$ from the centre of field of view ($X = Y = Z = 0$), for an optical wavelength $\lambda$ and bandwidth $\Delta \lambda$, assuming a linear convolution of the diffraction limited Gaussian 2-photon PSF width $w$ [1, 11, 12]:

$$l_{xy} = \sqrt{w^2 + \left( \frac{r\Delta \lambda}{\sqrt{2}\lambda} \right)^2 + b^2} \quad (1)$$

A similar aberration convolution model applied in the axial direction predicts the effect of various aberrations on the measured axial dimensions $l_z$ of an imaged bead of diameter $b$:

$$l_z = \sqrt{d^2 + \left( \frac{z\Delta \lambda}{\sqrt{2}\lambda} \right)^2 + (SA)^2 + (AstigZ)^2 + b^2} \quad (2)$$

where $d$ is the theoretical axial length of the 2-photon PSF in the Z direction [1], $z$ is the axial displacement of the 2-photon PSF from the centre of the field of view, SA is the geometric spherical aberration and AstigZ is the axial distance between the XZ and YZ plane foci. As discussed in Technical difficulty (6), spherical aberration is expected to become significant at sufficiently large AOL focus Z displacement. These equations show that chromatic aberration components in the X, Y and Z dimensions (Technical difficulty 3), can be minimized by choosing a laser with a narrow spectral width $\Delta \lambda$. However, since the spectral width and pulse length in time are inversely related, and longer pulse widths reduce 2-photon excitation efficiency, there is likely to be an optimum pulse length.

The astigmatic aberration caused by high speed scanning of a 2 AOD XY scanner (Technical difficulty 4) can be most conveniently quantified by calculating the scan speed induced astigmatism in terms of the drive parameters used to set up the raster scanning. The total scan angle in the X or Y plane is $2s/\text{zoom}$, where ‘zoom’ is the magnification factor. The additional parameters that define the angular scan rate in the fast X direction of the AOD are the number of voxels making up the image ‘Nvox’ and the dwell time per voxel ‘Dwell’. The angular scan rate is then used to estimate the degree of cylindrical focus from the AOD, which causes the astigmatism. The axial astigmatism ‘AstigZ’ is defined as the axial distance between the two orthogonal elliptical astigmatic beam waists. This can be calculated for a microscope objective in a material of refractive index $n$ using geometric optics based on the assumption that the effective NA of the objective is limited by the width of the AOD $W$ rather than any intermediate apertures. The equation is:

$$AstigZ = \frac{-W^2}{4V} \left( \frac{1}{(\text{NA})^2} - \frac{1}{n} \right) \frac{2s}{\text{Nvox} \times \text{Dwell} \times \text{zoom}} \quad (3)$$

In the XY plane, the FWHM of the long axes of these waists, AstigXY, can be estimated using a Gaussian beam model linked to the geometric model:

$$AstigXY = \frac{\text{NA} \times AstigZ}{\sqrt{n^2 - (\text{NA})^2}} \quad (4)$$

The corresponding scanning frame rate for a square image of Nvox $\times$ Nvox voxels and AOD fill time $T$ is:
These equations predict that astigmatism in the iPSF will increase as a function of the imaging speed. This prediction will be tested experimentally in a later section.

2.4 AOL with telecentric relays and the compact configuration AOL

The equations describing the acoustic drive waveforms necessary to form an AOL [20] assume zero optical distance between the AODs. A solution to this problem is to use a sequence of three 1:1 telecentric relays that project the image of one AOD onto the next [21] (Fig. 4 a). For 15 mm aperture AODs the focal length, $F$, of each lens is likely to be >100 mm in order to keep the NA sufficiently low for diffraction limited imaging with simple lenses. Since the total length of each relay is $4F$, the total length of the AOL is thus >1200 mm. Moreover, telecentric relays with a complex folding mirror arrangement consisting of 6 extra mirrors with 12 degrees of freedom in their adjustment [23] were used in the proof of principle 3D AOD system [22]. The use of six lenses and six mirrors makes this configuration complex. In order to simplify the AOL we implemented the ‘compact configuration’ (Fig. 4 b). This aligns the AODs axially with no intermediate lenses or mirrors so that the overall length of the AOL is reduced to the thickness of 4 AODs plus any air gaps and intermediate optical components (e.g. polarisers; section 3.1). However, this mechanically simple solution complicates AOL control because the drive equations that Kaplan et al., (2001) [20] derived are no longer directly applicable.

2.5 Drive equations for pointing and scanning for the ‘compact configuration’ AOL

In this section we derive the new equations required for driving the compact AOL in both the pointing and scanning mode.

Consider first the case of AOD1 and AOD2 in Fig. 5a. These are being driven at $f_1$ and $f_2$ respectively with chirped waveforms:

$$f_1 = f_c + a_1 t \quad (6)$$

$$f_2 = f_c + a_2 t \quad (7)$$

The distance between the AODs is $d_1$ and the distance to the focus of the converging wave of AOD1 is $d'_1$. First, referring to Fig. 5b, consider a time $t = 0$ when the diffracted wave from a position $X = 0$ on AOD1 is precisely vertical down the $Z$ axis, passing through position $X=0$ on AOD2. Next, consider a time $\Delta t$ later when the frequency of the ramp is shifted by $\Delta f = a_1 \Delta t$ so that the angle of diffraction changes by a fixed amount $\Delta \theta_1$. In this case, the focus of the converging beam is displaced in the -$X$ direction as shown. At the fixed point $X=0$ on AOD2, the angle of the ray passing through that point on its way to the focus shifts by $\Delta \theta_2$ as shown. It is clear by simple geometry that:

$$\frac{\Delta \theta_2}{\Delta \theta_1} \approx \frac{d'_1}{d'_1 - d_1} \quad (8)$$

Thus if you wish to cancel the rotation of the incoming wave from AOD1 at AOD2, in order to keep the diffracted wave from AOD2 pointing at the same focal position the rate frequency shift of AOD2, $a_2$ needs to be greater than $a_1$. For this configuration of AODs, increasing the frequency of both AODs therefore causes cancellation, not addition of the
angular deflection. The ratio of the frequency shift rates is the same as the ratio of the angles of deflection in Fig. 5b, giving:

\[
\frac{a_2}{a_1} = \frac{d_1}{d_1 - d_1} \quad (9)
\]

The two linear frequency ramp rates versus time are therefore different for the two AODs for a focal spot that is stationary in X (Fig. 5c).

2.5.1 Deriving the Equations for the pointing mode—In order to calculate the specific drive equations for a particular desired focal spot distance \(d'_2\) (Fig. 5a), we used an equation that defines the distance to the focus for a given ramp rate [20]. We also used the fact that in order to keep the focus stationary the curvature of the wavefront entering AOD2 is exactly doubled by the curvature added by AOD2. Simple rearrangement of these equations provides the relationships for setting up and controlling the AOLM:

\[
d_2 = \frac{d_1 - d_1}{2}, \quad a_1 = \frac{V^2}{\lambda (2d_2 + d_1)}, \quad a_2 = \frac{V^2}{2\lambda d_2} \quad (10)
\]

These provide the drive parameters in terms of the distance \(d'_2\) from the output face of the final AOD to the point of focus of the AOL. In these equations, \(d_1\) is always a positive value. The values of \(d'_2\), \(a_1\) and \(a_2\) are positive for converging rays for the +1 diffraction order (Fig. 5) and negative for diverging rays. It is apparent that if \(d_1 = 0\) then \(a_1 = a_2\) and the equations simplify to those presented by Kaplan et al. (2001) [20]. For the −1 diffraction order (diffraction towards the transducer) the sign of deflection and curvature is reversed. Note that in the equations and analysis above, the distances are apparent optical thicknesses.

These principles can be extended to a system that uses four AODs to focus in the X, Y and Z dimensions. Fig. 6 shows two orthogonal views of a four AOD system. The third and fourth AOD3 and AOD4 are interleaved with AODs 1 and 2 as shown, the distance between AOD3 and AOD4 being \(d_3\) and the distance from AOD4 to the focal point being \(d'_4\). The ramp rate equations for \(a_3\) and \(a_4\) are identical to those for \(a_1\) and \(a_2\) with \(d_3\) and \(d'_3\) replacing \(d_1\) and \(d'_1\). This derivation shows that a compact configuration AOL can be used to achieve a stationary focal point at any chosen value of Z within the field of view.

2.5.2 A graphical, vector-based method for finding start and stop frequencies of AODs—Since AODs have a limited acoustic frequency range, and the frequency ramp rates for Z focusing are high, the drive frequencies often reach the acoustic limit and have to be reset. It is therefore necessary to understand how to offset the drive frequencies without causing any movement of the focused spot for pointing mode (or restart an interrupted scan for raster scanning mode). Since the control of 4 AODs is a high dimensional problem, we developed a graphical vector approach to aid calculation of these parameters (Fig. 7).

For the case of XZ deflection and focusing with AOD1 and AOD2, the average drive frequencies \(f_{av_2}\) and \(f_{av_1}\) can be plotted on a graph (Fig. 7a). These ‘average’ symbols represent the frequency at the centre of the AOD at the particular time being considered. As the ramp rates \(a_1\) and \(a_2\) are linear in both time and space, the frequencies at the centre are the spatial averages of the frequencies currently in the AOD aperture and in turn determine the average deflection angle of the AOD. Any pair of average frequencies can be represented by a point in the \(f_{av_2}, f_{av_1}\) plane. A gradual or step change in the pair of drive centre frequencies can be represented by a vector lying in the plane. Its direction determines
what happens to the focal spot position. If \( f_{av_2} \) and \( f_{av_1} \) are changed in such a way that there is no change in the position of the spot, this corresponds to a vector that we refer to as a common mode drive gradient \( R_{comm} \) (Fig. 7a, blue arrow). A common mode drive frequency is defined as:

\[
R_{comm} = \frac{\Delta f_{av_1}}{\Delta f_{av_2}} = \frac{2d_2}{2d_2 + d_1} \quad (11)
\]

The ratio of the frequency changes is equal to the ratio of ramp rates that give a stationary focal spot at a distance \( d_2' \) from the last AOD. It has a fixed value for any particular chosen \( Z \) focal plane. For a stationary focal spot, if you plotted the two frequencies at the centre of the two AODs as they varied with time, they would move along a line parallel to the vector shown. It also implies that it does not matter what pair of frequencies you start the ramp at; as long as they are on this common mode deflection line, the focal spot will always be at the same position.

The converse applies to the differential mode line on this plot defined by:

\[
R_{diff} = \frac{\Delta f_{av_1}}{\Delta f_{av_2}} = \frac{-2d_2}{2d_2 + d_1} \quad (12)
\]

Changes in this direction produce changes in the X position of the focal spot with no changes in common mode frequency difference. Its gradient is by definition \(-1\) times the common mode gradient. Referring to Fig. 7a and b, in \( f_{av_2}, f_{av_1} \) frequency space the vectors \( R_{comm} = [1, R_{comm}] \) and \( R_{diff} = [1, R_{diff}] \) (where vectors are shown in bold and scalars in normal type face) can be used as the unit vectors of a 2D basis. Any vector in this plane can be analyzed in terms of its common mode and differential mode basis vector components.

In Fig. 7b, the red point ‘P’ represents a particular \( f_{av_2}, f_{av_1} \) coordinate. This can equally well be represented by the vector equation:

\[
[f_{av_1}, f_{av_2}] = [f_c, f_c] + A \times R_{comm} + B \times R_{diff} \quad (13)
\]

where \( f_c \) is the centre frequency of the AOD drive range and \( A \) and \( B \) are the scalar multipliers for the unit vectors pointing in the \( R_{comm} \) and \( R_{diff} \) directions as illustrated by the red and blue arrows. The blue dotted line through the point ‘P’ with a gradient \( R_{comm} \) is the line of all the other points that equally well point at the same position. Using this vector analysis, it is clear that for pointing to the same position in 3D space (e.g. point P), the ramps must start at the pair of frequencies defined by the lower left end of the dotted blue line and stop at the top right end to keep within the acoustic bandwidth limits. Jumping back to the start of the line allows repeated pointing to exactly the same position.

### 2.5.3 Algorithms for raster scanning mode

These ideas can be extended to raster scanning by simply considering a consecutive series of points in space. The graphical representation of this is shown in Fig. 7b, where the green line represents a time sequence of \( [f_{av_2}, f_{av_1}] \) coordinates that produce a focused beam slowly moving in the X direction. This can also be broken down into the vector components, \( C \times R_{comm} + D \times R_{diff} \) as shown by the red and blue arrows forming a triangle with the green arrow. The small differential component reflects the slow speed in X of the focused spot, while the common mode component determines the \( Z \) focal plane.

The most commonly used raster scan is to move the focal point in the X direction, keeping the Y and Z values constant. Then the Y position is incremented by some small amount, to...
perform another scan in the X direction, and so on until a two-dimensional image is built up. The Z direction is then incremented and another two-dimensional grid is scanned until a three dimensional volume has been built up.

Consider now the drive frequency sequence necessary to scan a focal spot along the maximum possible distance in the X direction without exceeding the drive frequency limits of the AODs. Fig. 8 shows the two X AOD centre frequencies plotted against time whilst scanning in the X direction. In order to produce the required focusing in Z, the ramp rates on both AODs are high. Since there is a limited frequency range, it is not possible to do a single scan right across the maximum possible X scan range (except at Z=0). The scanning process therefore has to be broken up into a sequence of ‘miniscans’. Two of these miniscan frequency traces are plotted in the figure showing the transition between them.

It takes one AOD fill time for the sound wave to fill the AOD aperture so that data recording can begin, and the time at which a particular frequency reaches the midpoint of the AOD is half the AOD fill time after it was transmitted by the transducer. Since the frequencies are defined at the centre of the AOD, recording of fluorescence data from a full AOD is only possible for frequencies sent by the transducer more than half an AOD fill time from either the beginning or end of the frequency ramp. This further reduces the available frequency range as shown by the bold dotted lines (Fig. 8). The total time from the end of data gathering of one miniscan to the start of data gathering of the next is thus the AOD fill time plus any reset time for electronically resetting the drive frequencies.

The key point in developing an algorithm for calculating the precise start and stop frequencies of each miniscan is that the position of the focal spot at the start of the first voxel of a new miniscan must be in exactly the same position as it was at the trailing edge of the last voxel of the previous miniscan. This is illustrated in the lower part of Fig. 8 (red line). The frequency offsets $\Delta f_1$ and $\Delta f_2$ must therefore be in the exact ratio $R_{\text{comm}}$.

Fig. 9 illustrates how the fastest possible X scan is built up by a sequence of miniscans in the $\theta_1 \theta_2$ plane. The green arrows show the path in this frequency space of the two drive frequencies at the centre of the AODs. The blue common mode dashed arrows show how the frequencies are reset from the end of one data gathering sequence to the beginning of the next. The start and stop frequencies of the miniscans are then defined by extending these ramps out to the drive frequency limits as shown. Note that as in the pointing mode, the precise direction of the blue common mode unit vector and the red differential mode unit vector are dependent on the chosen focal Z plane according to Eq.(11).

It is now a fairly simple extension of the equations already derived to calculate the full equations for each important parameter. The X scan rate $\frac{\delta \theta}{\delta t}$ is set at a rate so that it takes one dwell time to scan across one voxel:

$$\frac{\delta \theta}{\delta t} = \frac{2s}{N\text{vox} \times \text{Dwell}}$$

(14)

where $s$ is the semiscan angle of the AODs, Nvox is the number of voxels in the scan, typically 100 to 500, and Dwell is the dwell time of the focal spot as it scans across each voxel. It is also assumed that the Z focal distance of the AOL, $d_2'$ is also predetermined, so that the common mode offset frequency ratio $R_{\text{comm}}$ is also fixed. These assumptions give a set of simultaneous equations that can be solved to give the following equations for the precise ramp rates $(a_1, a_2)$ of AOD1 and AOD2 in the desired form:
These equations apply where there are two AODs for focusing in the XZ plane or, as shown in Fig. 6, when there are four AODs. In this case, the angular scan rate \( \frac{\delta \theta}{\delta t} \) is that measured about the AOD 2 (which is third in Fig. 6). Since the focal spot is at a finite distance from the AOL, the apparent angular rate as measured about the centre of the last AOD 4, which we use as the reference point for calculating drive parameters, can be obtained by multiplying this scan rate by \( \frac{d_2'}{d_4'} \).

Referring to Fig. 6, the appropriate equations for the YZ plane are found in a similar manner:

\[
a_3 = \frac{V / \lambda}{\left(2 + \delta \phi / \delta \theta \right)} \quad \text{and} \quad a_4 = \frac{V^2}{2 \lambda d_4'} + \frac{V \delta \phi}{2 \lambda d_4'} \tag{16}
\]

Here, \( \phi \) is the angle as measured from the centre of AOD 4. These derivations form the basis for the initial set of algorithms we developed to pre-compute the sequence of ramps for all 4 AODs to either raster scan or point to an arbitrary sequence of points of interest within the 3D FOV. Note that these equations independently define the position of the focus in the XZ and YZ planes so that irrespective of whether the 3D focal spot is stationary, or scanning at arbitrary scan speed, the astigmatism can be set to zero simply by correctly specifying \( d_2' \) and \( d_4' \). In this way the 4 AOD 3D scanner can overcome the longstanding complications of scan speed induced astigmatism in conventional 2D XY scanners (Technical difficulty 4).

3. Design implementation and experimental Results

In this section we describe the implementation of our compact spherical AOL and the initial experimental results we have obtained. These include a demonstration of focusing at 30 kHz, the use of pointing mode for imaging over a large range of axial focus and a comparison of 2 AOD and 4 AOD imaging at different frame rates.

3.1 Design and assembly of the spherical AOL

Each AOD was mounted on a custom made goniometer that allowed fine control of the crystal orientation (Fig. 10). This was important for setting up the AOL because the X and Y deflectors must be orthogonal to a small fraction of a degree in order to avoid measurable astigmatism when focusing or defocusing strongly. Accurate alignment of the input angle is also critical in optimizing the transmission efficiency, because the correct diffraction mode must be selected from multiple diffraction modes on either side of the transmitted zero order mode. For the present application AODs were designed for \(-1\) order mode with a nearly vertical polarization incident laser beam at the Bragg angle and nearly horizontal polarization for the diffracted beam (with reference to a horizontal deflector). To achieve the best alignment we used a polarizer, set near to the polarization of the diffracted beam, and optimized the crystal angle to get the maximum efficiency in the wanted diffraction mode. The same protocol was followed for the subsequent crystals. A half wave plate after the polarizer was used to adjust the polarization of the diffracted beam before it enters the next crystal in the series, enabling maximum efficiency in the desired mode. This combination of polarizer and half wave plate blocked the unwanted zero order mode after every crystal. The first AOD in each XY pair has an efficiency of 85% and the wide acceptance angle of the
second AOD (using 2.4 mm transducers) in each XY pair has an efficiency of 65%. The overall efficiency of the AOL with polarizers and half wave plates is approximately 15% (all peak figures).

In order to drive the AOL with the required sequence of high speed radio frequency (RF) ramps a computer controlled digital synthesizer was used (iDDS, Isomet (UK) Ltd) to generate up to 20,000 trigger controlled pre-stored RF ramps. The parameter for each ramp was pre-computed using the algorithms described in section 2.5 in MatLab embedded in 3D imaging software developed in the LabView environment (National Instruments Inc.).

3.2 Speed of Pointing in 3D space

To determine the speed at which the AOL could focus to arbitrary positions in 3D space, we combined it with a single fixed lens (F = 200 mm) and used 3 silicon detectors placed in different focal planes with pinholes in front of each, as illustrated in Fig. 11a.

The location of each of the pinholes was determined by imaging in pointing mode, using the silicon detector signals. Random access pointing mode was then used to focus on each pinhole in turn repeating the sequence four times in different orders. The light intensity through each pinhole increased as focus was attained (Fig. 11b). The plot shows that the AOL focused the laser onto the three detectors in any order within 100 μs, thereby demonstrating random access pointing in 3D at 30 kHz.

3.3 Design and assembly of AOLM

To add the AOL to a standard 2-photon microscope, we relayed the image of the output face of the last AOD of the AOL to form a conjugate image on the galvanometer mirrors of a standard two photon microscope (Ultima, Prairie Technologies Inc., USA). A 4:1 demagnification was required to just fill the galvanometer mirrors. The subsequent field lens and tube lens form a conjugate image of the galvanometer mirrors on the back aperture of the microscope objective. We also used a Pockels cell to control the laser power, a custom designed, prism based pre-chirper with −50,000 fs² GVD at 800nm (APE GmbH, Berlin) before the AOL. A diagram of the complete AOLM is shown in Fig. 12.

3.4 Temporal dispersion compensation

Once all the components of the prototype AOLM were in place, we used a Carpe autocorrelator (APE GmbH, Berlin), introduced in the optical path immediately after the laser (λ = 800 nm, MaiTai, Newport Spectra Physics), and measured the pulse width after the 40X objective with a 2-photon detector. The pulse length on leaving the laser was 100 fs. Without the pre-chirper the large dispersion of the AOL and microscope optics spread the pulse width to 1.52 ps (Fig. 13). We then adjusted the pre-chirper to minimize the measured pulse length after the 40X objective at the centre of the field of view. The pulse length could be brought back to 115 fs after the objective by introducing ~50,000 fs² GVD with the pre-chirper. Thus the combination of thin, optically rotated AODs and a pre-chirper enables femtosecond 2-photon imaging.

3.5 3D Structural imaging in the pointing mode

To test the 3D AOL drive algorithms and determine the image quality that can be attained with the AOLM, we used pointing mode to build up 2D images at different focal planes. To demonstrate 3D imaging we mechanically set the objective lens (water immersion 40X 0.8 NA lens, Olympus) to different focal planes above and below a pollen grain and compensated the focus with the AOL so that the image was of an equatorial section of the same pollen grain.
Fig. 14 shows single frame and averaged 125×125 voxel images of the same pollen grain at different focal planes over a 137 μm axial focusing range. Visual inspection shows intensity across the pollen grain is reasonably uniform and spines that taper to sub-micron diameter are clearly visible at each focal plane. Indeed, there is relatively little difference in the image quality or the intensity at the different focal planes even though the images were taken at the same laser power and PMT settings. Furthermore, the 8 mW laser power (measured at objective back aperture) and 4 μs dwell time per voxel are comparable to those for a well set up conventional galvanometer based 2-photon microscope. These results show that the AOLM produces high quality 2-photon images at low laser powers over a wide range of focal depths.

3.6 Resolution and aberrations of the point spread function

To examine the optical resolution of the AOLM, we measured the 2-photon PSF with 200 nm fluorescent beads using a water immersion 40X 0.8 NA lens. To compare the predicted distortion of the PSF by chromatic aberration (Eq. (4), Fig. 15a) and the properties of the PSF of our prototype AOLM, we compared images of beads for two different spectral bandwidths. Fig. 15b shows a montage of beads measured at the centre, edges and corners of the field of a 100 μm square at Z=0 using a Ti-Sapphire laser with a 100 fs pulse length and 10.6 nm FWHM spectral width (MaiTai, Spectra Physics). The red line on each figure indicates the FWHM of an elliptical Gaussian function that best fit the data. Note that the chromatic aberration is approximately radial and centered on X = Y = 0, as predicted (Fig. 15a, b). The FWHM for the bead image at the centre was 0.47×0.50 μm compared to 0.42 μm in theory (at 800 nm). Beads at the diagonal corners had an average long axis FWHM of 0.82 μm compared to 0.76 μm in theory from Eq. (1).

Fig. 15c shows beads imaged with a laser with a longer pulse length (140 fs) and shorter spectral width (7 nm FWHM, Chameleon Ultra II, Coherent). The radial elongation of the bead images is noticeably smaller than for the wider bandwidth laser. Indeed the FWHM for the bead at the centre of the field was 0.55×0.58 μm, while the diagonal corner beads had an average long axis FWHM of 0.63 μm compared to 0.608 μm from Eq.(4). These results show that chromatic aberration can be minimized by reducing the laser bandwidth, but this comes at the cost of longer pulse lengths, which require a higher average power to achieve similar 2-photon excitation.

We then examined how the 2-photon PSF dimensions varied along the Z axis at X=Y=0 by imaging fluorescent beads (Fig. 16). The XY dimensions of 200 nm beads were close to the diffraction limit at Z=0, and the long axis of the elliptical bead image remained below 0.75 μm (FWHM) over the full 160 μm range of the AOL axial focus. The Z dimension of bead images was also close to the diffraction limit at Z=0 (1.9 μm). The Z dimension of the 2-photon PSF increased with distance from the natural focal plane. The formation of cone shaped structures in addition to the core PSF indicates that spherical aberration is the dominant aberration at large displacements. However, there was also some asymmetry in the relationship between the Z dimension of the 2-photon PSF and axial focal depth, which is presumably due to unidentified residual aberrations. Nevertheless, it remained below 5 μm over the full 160 μm range of the AOL axial focus. These values are suitable for high resolution imaging within the 3D field of view.

3.7 High speed 2D imaging: comparison of a 2 AOD scanner and a 4 AOD AOLM

To compare the properties of our prototype 3D AOLM and conventional 2D 2-AOD scanning, we imaged pollen grains and 1 μm beads at the natural focal plane (Z=0) at different speeds. The top row in Fig. 17a shows raster scan images of a pollen grain acquired with 2 AODs at 3 different frame rates, with the objective focused at the equatorial plane in
each case. The total light collection time for each image has been kept at 8 μs by integrating multiple frames at higher scan speed. Focusing by the fast scanning AOD becomes more pronounced as the scan rate increases introducing astigmatism and blurring the image. In contrast, raster scan imaging with the 4 AOD AOLM produced identical images at the different scan speeds, with no sign of any change in focus. We quantified the magnitude of the astigmatism by imaging 1 μm fluorescent beads. The measured astigmatism for 2 AOD scanning matched that predicted from theory (Eq. (3)) and AstigZ reached 12 μm at 155 Hz (0.4 μs/pixel). There was no measurable astigmatism for the 1 μm bead images for 4 AOD scanning at any frame rate tested (i.e. up to 155 Hz). These results confirm that the scan speed dependent focusing and astigmatic distortion that occurs in 2 AOD scanning systems is not an issue in our prototype 4 AOD AOLM, therefore enabling flexible high speed line and raster scanning.

4. Discussion

Here we describe the design of a high performance spherical Acousto-Optic Lens (AOL) that can be used for high speed 3D femtosecond based 2-photon imaging and random access point measurements. The improved performance over a previous AOL microscope design [21] was achieved by making a number of technical advances. These include using custom designed, thin, optically rotated AODs with optimized transducer lengths and a low acoustic centre frequency to form the AOL. This overcame two technical difficulties. Firstly, it substantially lowered the GVD introduced by the 4 AODs allowing femtosecond imaging with a commercial pre-chirper. Secondly, it increased the input acceptance angle by ~3-fold over standard AODs. We have also reduced the complexity and large size of the previously proposed AOL configuration by designing a telecentric relay-free and mirror-free compact design with dimensions of less than 25×25×20 cm. Lastly, we have derived new control algorithms for the compact AOL and have developed a novel vector based approach that simplifies the calculation of the complex RF waveforms required to drive the 4 AODs during pointing and raster scanning. With our prototype system, we demonstrate random access pointing of the laser beam in 3D at 30 kHz and 2-photon imaging over 137 μm axial focal depth with a 40X 0.8 NA objective. This compares with the 50 μm range for a 1 NA lens previously reported [21]. We show that temporal dispersion of the AOL microscope can be compensated with a prism based pre-chirper, allowing imaging with laser pulses in the 100 femtosecond range. We confirm earlier proposals that the AOL is self compensating against chromatic aberration at the centre of the field and that the observed residual chromatic aberration fits approximately to theory for two lasers with different spectral widths. Moreover, we show that the scan rate dependent astigmatism present in 2 AOD based systems is eliminated in our 4 AOD AOL, and demonstrate for the first time high speed raster scanning (>150 Hz) in 2D that does not exhibit scan rate dependent astigmatism. These results show that our AOLM design has a substantially improved performance over that previously reported [21] and allows 3D random access and raster scan 2-photon imaging with an order of magnitude lower laser powers.

4.1 Comparison to previous 2D AOD imaging

A new feature of our AOL and drive algorithms is the ability to perform high speed raster scanning without the scan rate dependent astigmatism that has impaired high speed AOD based scanning to date [16, 17] (Fig. 17). Astigmatism occurs because the cylindrical focusing property of AODs becomes more pronounced as the scan rate increases. Interestingly, this effect has been used to produce a 3D two-AOD microscope. However, since this microscope’s focusing is cylindrical not spherical (unless the AODs are scanning at the same rate), and since its scan rate is inextricably linked to the degree of focus, its 3D capabilities are rather restricted [25]. The scan rate dependent astigmatic effects introduced
by AODs can be compensated with fixed astigmatic lenses, but these only compensate properly at one particular scan rate which severely restricts the flexibility of imaging. Although this limitation is rarely discussed, it is sufficiently problematic that most papers using 2 AODs to image [8, 10-14, 21-23, 30] and the first 4 AOD 2-photon system [21, 30], use sequential pointing mode, rather than raster scanning to form ‘structural’ images. This takes of the order of 1s for a 200×200 pixel 2D image (20 μs/point), which is significantly slower than imaging with conventional galvanometers. Here we develop the appropriate drive algorithms for the AOL to perform high speed raster scanning. We demonstrate, for the first time, variable rate raster scanning with an AOD-based scanning microscope that is free of scan rate dependent astigmatism. Moreover, we show that the astigmatism present with two-AOD scanning at rates up to 155 Hz is absent when the 4 AOD spherical AOL is used. High speed raster scan or line scan imaging is therefore an important design feature of the compact AOL.

4.2 Comparison with previous 3D 2-photon imaging technologies

Experimental results show that our prototype 2-photon AOLM is capable of high resolution 3D imaging and of high speed random access pointing in 3D space at 30 kHz. Indeed, focusing with our compact AOL is three orders of magnitude faster than recently reported for 3D 2-photon imaging using a piezoelectric device to move the microscope objective [18]. However, the focusing range achieved is smaller (250 μm and 137 μm, respectively). Since the AOL can jump from point to point much faster than galvanometer mirrors, a larger fraction of the time can be used to collect photons from sparse regions of interest. These AOLM features should therefore enable considerably faster functional optical imaging application in neuroscience. For example, it should allow recordings from 30 neurons distributed in 3D space at 1 kHz sampling per location, which is considerably faster than current galvanometer based 3D scanning approaches [18]. AOL focusing is also considerably faster and has a larger range than can be achieved with deformable mirrors [31]. A recent approach, involving piston mirrors and the use of two objectives to compensate for spherical aberrations, can also be used to focus rapidly (<1 kHz) over a larger range than can presently be achieved with AOLS [19]. However, like galvanometers, piston mirrors have the disadvantage of being continuous, which limits the duty cycle for sparse ROIs. The random access pointing with an AOLM therefore has some significant advantages over other proposed methods for the problem of high speed sampling of sparse distributed processes in 3D.

One of the key advances reported here is the use of optically rotated AODs with less than half the axial length of standard crystals, which reduce the temporal dispersion to a level that can readily be compensated with a prism based pre-chirper, allowing femtosecond imaging. These design features enable 2-photon imaging with 4-10 mW at the back aperture, rather than the 40-100 mW reported for the first 3D 2-photon imaging system [21]. To date such temporal dispersion compensation has only been possible for 1 AOD [6, 7] and 2 AOD [8, 10-13, 15] scanners, but not for a 3D 4 AOD microscope, because the standard AODs employed have been considered too thick, introducing ~80,000 fs² GVD [21]. Thin AODs are also key in enabling a new compact AOL design, that discards the complex telecentric relays and folding mirrors used previously [15, 21], placing the AODs adjacent to one another. Although there is a small increase in the required acceptance angle range for adjacent AODs, compared to telecentric coupled AODs (<8% over the focus range tested here), the imaging results we report demonstrate that the new AOD drive equations that compensate for the physical spacing of the AODs produce high quality images over a wide range of focal planes.

It is perhaps surprising that the remote AOL focusing into a 0.8 NA water immersion objective gives clear high resolution images of the pollen grain spines with little drop-off in
imaging sensitivity over the whole 137 μm range. We believe that there are three main factors why our AOLM can focus over a wider range than for the previous design [21]: 1) Assuming the NA of both systems are limited by AOD aperture, reducing the NA at the focal point from 1.0 to the 0.8 used here would be expected to increase the axial range by 50%, thereby increasing the axial focusing range of the previous system [21] from 50 μm to 75 μm. 2) Under strong focusing conditions, the larger AOD aperture in our system (15 mm compared to 9 mm [21]) enables lower magnification coupling optics to be used. This reduces the acceptance angle required at the second AOD of each pair for a given axial displacement. This, when combined with the improved acceptance angle of the second AOD in each pair, gives a more uniform aperture illumination. Our analysis agrees with the previous proposal that the acceptance angle is the main factor limiting the axial scan range for the AOL based on standard AODs [21]. 3) Simple geometric optical modeling suggests that under strongly focusing conditions the large acoustic walk off angle (55°) of the conventional AODs previously used introduces a significant coma aberration. Indeed, even if the acceptance angle was increased by narrowing the acoustic transducer [27], the axial displacement may still be limited to a similar range by the coma aberration caused by the high walk off angle. This effect is substantially reduced with the 22° walk off angle of our AODs. Our imaging results at high axial displacement confirm optically rotated AOD design does not cause significant coma, even at an axial displacement of ±68.5 μm at NA=0.8. Indeed, the appearance of cone shaped structures above and below the core PSF at such large displacements indicates that spherical aberration is the dominant aberration. Nevertheless, neither spherical aberration nor aberrations introduced by the compact AOL prevent our system from producing high quality images over a 137 μm axial range at NA=0.8.

4.3 Current limitations of the compact AOLM and potential solutions

The features and performance of our 2-photon AOLM are sufficiently advanced to make the current prototype useful for studying fast processes in 2D and 3D. However, a number of limitations still remain. While raster scanning can be performed away from Z=0, the complex variations in diffraction efficiency that occur during the scanning process make it difficult to produce artifact-free images. Since our solutions to this problem are beyond the scope of this paper, the images showing the 3D scan volume in Fig. 14 were taken in the pointing mode. Increasing the volume over which effective 2-photon excitation can be achieved would also improve the utility of this technology. This is limited by a number of factors, including scan angle, AOL light transmission efficiency at large scan angles and distortion of the iPSF by aberrations. While some of these factors require further developments in AOD design, our predictions and measurement of significant chromatic aberration at the edges of a 100 μm square field of view suggest that the effective 2-photon imaging volume could be significantly increased by reducing this aberration. Although our AOLM compensates for spatial chromatic aberration at the centre of the field of view as previously proposed [21], we have not corrected for residual magnification chromatic aberration. This could be corrected for further either by reducing the spectral bandwidth of the laser [21], or by compensating this aberration with a dispersive lens system we have recently proposed [24].

5. Conclusion

We present a new design for a 3D 2-photon microscope based on a compact AOL, that has a significantly improved performance over the first proof of principle 2-photon AOLM [21, 30]. Its features make it suitable for both high speed 2D imaging applications and 3D pointing applications such as 2-photon functional imaging and photolysis. The small size and mechanical simplicity of our compact configuration AOL, make it possible to simply
add it, together with a pre-chirper, to an existing galvanometer based 2-photon microscope thereby enabling high speed 2D and 3D 2-photon imaging.

Acknowledgments

The work described in this paper was supported by MRC (GO400598), EPSRC (EP/D501199/1), UCL-Capital Infrastructure Fund and the Wellcome Trust. PAK was supported by Complex and the Beloe fellowship from the Worshipful Company of Scientific Instrument Makers. RAS is in receipt of a Wellcome Trust Senior Research Fellowship. We thank Stephan Dieudonné for helpful early discussions on AODs, Warren Seal for advice on AOD design, Mike Hillier for advice on iDDS design, Duncan Farquharson and Alan Hogben for building the mechanical mounts for the AOL, Bruno Pichler for early programming help and advice, Thomas Mrsic-Flogel for support and Prairie Technologies for the loan of equipment. We thank Stéphane Dieudonné, David DiGregorio, Michael Szulczewski and Emmanuelle Chaigneau for helpful comments on the manuscript.

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Fig. 1.
The principle of operation for a cylindrical AOL. a) pair of AODs in which the sound waves are counter propagating. A collimated laser beam is incident on AOD1 and is diffracted into the +1 mode (away from the transducer) as illustrated. b) drive frequency versus time. A positive chirp rate is applied to both AODs producing a chirped wave propagating across the crystals, c) drive frequency versus distance across the crystals for the ramps shown in b). The dashed lines in b) and c) show the limits of AOD drive frequency where diffraction efficiency drops to 80% of its peak value.
Fig. 2.
Acousto-Optic Deflector Designs. a) a standard high efficiency AOD with 55° walk off angle and ±20 mrad deflection angle. Note this has only approximately ±1 mrad input acceptance angle. b) Wave vector and refractive index sphere for standard AOD. The axes represent refractive index for the gray surfaces and wave number for the wave vector arrows. (The number of waves/cm in the material is proportional to refractive index). The acoustic wave vector (red arrow) of the standard AOD is rotated 5° off the <110> direction. The polished crystal is thus described as having 5° of acoustic rotation (blue arrow). The inset shows that the incident light wave vector is an extraordinary wave with its polarization shown as the red ellipse above. The output wave vector is an ordinary wave (green ellipse). The design of our custom AODs is shown in c). There is only 2° of acoustic rotation so that the walk off angle is reduced to 22°. This makes the crystal much thinner. However the 3D wave vector diagrams d) show that in order to keep the incident and diffracted wave vectors more than 3° off axis (inner pair of blue latitude circles) the crystal must be optically rotated by 3° as well as acoustically rotated by 2°. The red and green ellipses show the polarization of the incident (extraordinary) wave and diffracted (ordinary) output wave.
Fig. 3.
a) Schematic of custom AOD with wide transducer, b) schematic of custom AOD with narrow transducer, c) plot of diffraction efficiency for an AOD model with approximately 3W of drive power showing how efficiency drops as the transducer is narrowed and the input acceptance angle increases [27].
Fig. 4.
a) Top view of an AOL with telecentric relays. The drive on all four AODs is at the centre frequency. b) Top or side view of the compact configuration AOL.
Fig. 5.
Geometric arrangement of optical wavefronts and the drive parameters required for use with one axis of the compact configuration of AOL. a) definition of distances and focal lengths of converging waves from AODs, b) illustrates how as the sound wave in the first AOD progresses to the left it causes the deflection angle $\Delta \theta_1$ at X=0 to increase. However the angle of deflection $\Delta \theta_2$ of the dashed and dotted ray at X=0 on AOD2 which passes through the same focus is greater according to the geometrically derived equations in the text. c) shows how the ramp rates of AOD1 must be less than the ramp rate of AOD2 if the resulting focused spot is to be stationary.
Fig. 6.
Sequence and orientation of four AODs forming an AOL. Distances $d$ are defined for the equations of the compact configuration. Note the interleaved numbering of AODs, enabling the same equations to be used for both the XZ and YZ pairs of axes.
Fig. 7.
a) A graphical way to analyse drive frequencies applied to the XZ pair of AODs. The common mode ratio shown in blue is the gradient in frequency space that produces no movement of the focal spot. The orthogonal differential mode gradient shown in red produces only X deflection of the spot with no common mode deflection. b) Shows how any pair of coordinates (e.g. point P, red star) or difference in pair of frequencies (green arrow) can be analysed into common mode (blue) and differential mode components (red). $f_{\text{min}}$ and $f_{\text{max}}$ are the drive frequency limits of the AODs, and A and B are appropriate scalar multipliers of their respective unit vectors.
Fig. 8.
Diagram to illustrate AOD X1 and AOD X2 drive frequencies vs. time for part of a sequence of miniscans deflecting in X and focusing a spot at a constant Z plane across one line of a raster at a fixed Y value. The pair of green lines corresponds to a single vector in the frequency space diagram (Fig. 9). The red line shows that there is no X deflection in the flyback period between the end of one data gathering sequence and the beginning of the next.
Fig. 9.
Diagram illustrating the full sequence of miniscans making up one full X scan of one line of a raster scan for an AOD pair. The green arrows correspond to a green pair of drive frequencies as shown vs. time in Fig 8. As the green vector has a small differential mode component it is scanning in X as well as focusing at a particular Z plane. This is shown by the red differential mode lines. The frequency limits for data collection have to be inset with respect to the frequency limits because data can only be gathered from full AODs. The common mode (blue dashed) reset lines ensure that when data gathering starts again in the next miniscan it does so from the same position.
Fig. 10.
Compact configuration of AOL showing mounts used for precise positioning of each AOD. The AOL has a total length of only 150 mm from the centre of the first AOD to the centre of the last. Inset is a close up of the first AOD showing the gold transducer pad along the left hand (front) edge of the AOD. It is followed by a polarizer and half wave plate. The AOD is 15 mm thick and approximately 20 mm square external dimensions.
Fig. 11.  
Focusing of a laser beam to arbitrarily chosen focal points at 30 kHz. a) Simplified diagram of 4 AOD AOL, 200 mm focal length lens, 2 partial reflectors and 3 silicon detectors with 20 μm pinholes. Detectors were placed at arbitrary XY positions and axial distances from the lens of 175 mm (red), 200 mm (green) and 235 mm (blue). b) The signal from each detector, plotted with the appropriate colour code for its detector, during random access pointing where the laser beam is focused sequentially into each of the pinholes.
Fig. 12.
Schematic diagram of complete acousto-optic lens (AOL) 2-photon microscope. Inset shows details of AOL. The four AODs are placed as close to one another as possible (40-60 mm centre to centre). Before each AOD is a half wave plate (H). After each AOD is a polarizer (P) to absorb the residual undiffracted zero order light. By fixing the galvanometers, AOL deflection and focusing can be used to focus anywhere within the octahedral shaped scan volume below the objective. Fluorescence was either collected through the objective onto a photomultiplier tube (PMT), or additionally through the condenser (not shown).
Fig. 13.
Experimental measurements of the laser pulse width at various positions through the system. The pulse width at the Ti-Sapphire laser (red, 100 fs at 800 nm, MaiTai, Newport Spectra Physics) for the full acousto-optic lens microscope, measured after the 40X objective lens, without pulse compensation (blue, 1.52 ps) and with −50,000 fs$^2$ GVD introduced by the prism based pre-chirper (green, 115 fs).
Fig. 14.
Pointing mode images of a pollen grain optically sectioned through its equator. Each image is 125×125 voxels with a dwell time per voxel of 4 μs. Images were acquired at the same settings, with no subsequent image processing so that the images represent the true range of brightness over the Z focusing range. The height of the AOL focal plane with respect to the natural focal plane of the objective was measured by mechanically refocusing by moving the objective axially. The rows of images correspond to a single frame and 16 frames average, respectively. The scale bars are 10 μm.

*Opt Express. Author manuscript; available in PMC 2010 October 01.*
Fig. 15.
a) Raster scanned images showing resolution and effect of chromatic aberrations. Expected
distortion of 2-photon PSF across the Z=0 focal plane predicted from Eq. (1). b) a montage
of bead images measured at the centre, edges and corners of a 100 μm square field of view
at Z=0 using a Ti-Sapphire laser with a 100 fs pulse length and 10.6nm FWHM spectral
width. c) Same as b, but imaged with a laser with a 140 fs pulse length and a 7 nm FWHM
spectral width. Fluorescent bead diameter b= 200 nm, λ=800 nm, theoretical 2-photon PSF
w = 370 nm [1].
Fig. 16.
Measurement of the 2-photon PSF along Z axis at X=Y=0 acquired with pointing mode structural imaging of 200 nm beads with a semiscan angle $s = 4.3$ mrad using the algorithms described in section 2.5 a) XY FWHM dimensions of beads imaged with a 40X NA=0.8 objective lens at 800 nm. Red points show long axis of 2D Gaussian fit and blue points show short axis plotted against focus depth (mean, n=3). b) Relationship between Z dimension of bead image and axial focus depth (mean, n=3). The FWHM was calculated at each Z plane from the intensity from 80×80 nm regions in 31 planes spaced 0.5 μm apart in Z (centered on the bead at the mid plane). A single weak astigmatic lens (±0.05 m−1) was introduced before the AOL for these measurements, to correct for a small fixed astigmatic component introduced in the optical train. This brought the PSF closer to the diffraction limit (dashed lines for 200 nm beads) than the data in Fig. 15, which was acquired without this lens.
Fig. 17.
a) Raster scan images of pollen grains sectioned through their equator with the AOL set for Z=0 so that the focal plane is the natural focal plane of the objective. The top row is for conventional 2 AOD XY scanning and the bottom row using the 4 AOD AOL as a scanner. The columns are in order of increasing frame rate 12, 80 and 155 Hz. Scale bars are 10 μm. The image contrast of all the images is equally enhanced with ImageJ to show spines more clearly. b) Comparison of astigmatism between theoretical (solid red and blue lines based on Eqs. (3) and (4)) and experimental (red and blue circles) for the two-AOD scanning at frame rates 10-155 Hz. The experimental astigmatic data points are measured by eye and by ImageJ Gaussian curve fitting from a Z stack of images of 1 μm beads. Their accuracy is approximately ±1 μm. The inset diagram illustrates four rays (black arrows) and the 2-photon astigmatic beam waists (green ellipses) at the focus and shows the astigmatism parameters AstigZ and AstigXY in the Eq. (6) and (7) (red and blue arrows).