Each microsphere was excited individually. Each wavefront sensor measurement was collected over a period of 500 milliseconds, much longer than the typical AO loop bandwidth. This insures that there is little noise on the data. The standard deviation for each individual wavefront was measured to be better than 1% of the wavelength at 647 nm. Table 2 shows three different measurements taken with a 40X (0.75 NA) objective lens. The first measurement shows that the wavefront error for the bead located at the center of the field of view RMS(1) is 1.60 radians, the wavefront error for the bead located 14 μm from the center RMS(2) is 1.90 radians, and the wavefront error between the two measurements RMS(1-2) is 0.73 radians. Taking the average of three measurements shows the isoplanatic half width is 19 ± 5.6 μm. This results show that a reference microsphere together with an AO system can help to improve the quality of the images taken, not just at the location of the microsphere but also within a circle 10 microns in radius.

Table 2. Isoplanatic angle measurements for the 40X magnification, 0.75 NA objective lens

#	*d* [μm]	Angle [arcmin]	RMS(1) [rads]	RMS(2) [rads]	RMS(1-2) [rads]
1	14	10.7	1.60	1.90	0.73
2	18	13.8	0.98	1.24	0.77
3	25	19.1	1.69	1.10	1.30
Mean	**19 ± 5.57**	**14.5 ± 4.25**	**1.42 ± 0.39**	**1.41 ± 0.43**	**0.93 ± 0.32**

4. Discussion & conclusions

One of the challenges in designing a Shack-Hartmann wavefront sensor is imposed by the amount of light the reference source can provide. Polystyrene microspheres are loaded with fluorescent dye and the light emitted is proportional to the radius cubed, thus smaller beads provide less light. The size of the beads should be smaller than the diffraction limit of one subaperture of the Hartmann wavefront sensor. Note that this is larger than the diffraction limit of the microscope aperture by the ratio D(size of the aperture)/d_{LA}. Since the diffraction limit of the microscope is inversely proportional to the numerical aperture (NA), smaller beads are needed for higher numerical aperture systems. Fortunately the light gathered by the objective also increases with increasing NA (light gathering power ~NA^2). Thus increasing the wavefront sampling by a factor of 4 increases the size of the microsphere radius by a factor of 2, and the amount of light emitted by a factor of 8. Current results show that for a 40X objective with a NA of 0.75, a 1 micron fluorescent microsphere provides enough light to run the AO system loop at 10 ms bandwidth. If the size of the bead were reduced by a factor of 10 to 100 nm in radius we could still obtain a good correction by using the AO system but with the disadvantage of lower signal to noise ratio. This could be compensated by increasing the AO loop bandwidth to 100 ms or by reducing the number pixels used for each subaperture. Both of these approaches come from the fact that the signal to noise ratio of the Shack-Hartmann wavefront sensor can be improved by increasing the integration time of the CCD camera or by using less pixels to detect the movement of the centroids.

The microsphere solution concentration injected into the embryo in this study insures that there is at least 1 microsphere within 10 microns of the injection site. This can be used to accurately target different embryo locations for imaging. Higher microsphere concentrations have also been tested and the results showed that beads can spread much more densely without impacting embryo development. Having multiple microspheres relatively close to each other does not present a problem since the confocal illumination set-up used in this experiment can accurately target one bead at a time. Experimental results showed that even if two or more microspheres are in the same focal plane and are “relatively” close to each other (i.e. within 5 PSF’s FWHM) the wavefront sensor would see an extended object and the resulting measurement would be the average wavefront seen from each microsphere [20]. Microspheres that are in different focal planes present even less of a problem since the finite focus of the confocal illumination prevents them from being fully illuminated (due to the high

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NA) and hence they are not the brightest object in the field of view. Microspheres that are in different focal planes do show up as a background light in the wavefront sensor camera. It has been shown by Thomas et al. [21] that a robust centroiding algorithm, like the cross-correlation technique used here, does not sense the background and only detects the brightest object in the field of view, be it an extended or point source. This still holds true even for a very small signal to noise ratio (i.e. the background would add to the peak of the Hartmann spot centroid but it does not move it). The same can be said for light that is being scattered inside the embryo. It adds to the background but it does not change the wavefront sensor measurement.

An emerging field in adaptive optics is tomography AO, where multiple light sources together with multiple SHWS are used. The information from each wavefront sensor is then processed using a reconstructor to acquire a tomographic image of the changes in the index of refraction in the optical path [7]. One of the advantages of using tomography AO is that it can provide information on the depth dependence of variations in the index of refraction in the tissue thus allowing for the AO system to correct for the wavefront aberrations only in the optical path. This technique can also extend the isoplanatic angle by correcting wavefront aberrations that are common to a larger field of view. By depositing multiple fluorescent beads into the biological sample and using multiple wavefront sensors we can also apply the tomographic techniques that have been developed for astronomical AO.

An adaptive optics microscope was designed using a Shack-Hartmann wavefront sensor and a Micro-Electro-Mechanical Systems (MEMS) deformable mirror to directly measure and correct the wavefront error induced by a *Drosophila* embryo. The wavefront measurements were taken by using a new method of seeding an embryo with fluorescent microspheres that are used as "artificial guide-stars." The maximum wavefront error for a 40X magnification, 0.75 NA objective lens was 1.37 μm and 0.19 μm for the peak-to-valley and root-mean-square, respectively. The residual wavefront error after correction was 7 nm RMS. The measurements also show that the isoplanatic half-width is approximately 19 μm resulting in a field of view of 38 μm in total. Analysis of the data demonstrated that this approach can improve the Strehl ratio by 2 times on average and as high as 10 times when imaging through 100 μm of tissue.

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