Apart from wavelength, the immersion medium is also important. For a water-based sample such as biological tissue, a water immersion lens should be used, whereas the use of glycerin embedded samples require the use of a glycerin immersion objective. To be able to make best use of high-NA oil immersion lenses, care must be taken to match perfectly the refractive index of the embedding medium of the fixed sample to the refractive index of the immersion oil. While several publications exist about correcting aberrations arising from violating this basic rule, only little work has been carried out to correct true sample-induced aberrations in 2PLSM caused by the small refractive index mismatch between water and tissue [3–4, 6]. In some of these latter cases, aberrations due to the shape of the sample surface were dominant. In 2PLSM of the brain, a coverslip is generally used to protect the tissue. If the bone around the craniotomy is appropriately thinned, the coverslip can be brought in direct contact with the brain tissue, ensuring a flat sample surface and removing surface-shape-associated aberrations.

Our results now show that even in this best-possible configuration with a water immersion objective, flat brain surface and assuming a homogeneous brain refractive index, correction of sample induced spherical aberration could drastically improve 2PLSM in the animal brain.

Appendix

The marginal ray calculation performed by Tearney et al. [11] assumes that the OCT signal is dominated by the light traveling at the highest angle. However, due to the coherent summation, it is rather the contribution from the area or areas of stationary phase that will dominate the integral in Eq. (2). Furthermore, their calculation assumes z_A to be fixed in relation to z_N based on the refraction of marginal rays; this need not necessarily be the case. In their experiments, the low NA (0.175) used implied a deviation from paraxial theory of only 0.5%, well below their experimental uncertainty. However, had their formula been applied at the NA of 0.8 used in this work, it would have caused a significant systematic error. Generalizing their formula (2) to the case with water immersion

$$n'_{marginal} \sin\left\{ \arctan\left[\frac{n'_{marginal}\, z_N \tan\left(\arcsin \frac{NA}{n} \right)}{n z_N + \delta/2} \right] \right\} = NA \tag{7}$$

we can calculate a “marginal ray refractive index”. For the mean defocus slope δ/zN=0.064886, this would lead to n'=1.3486, which is an underestimation by 0.004 compared to the value found with our model, as mentioned in the main text.

Acknowledgements

This work was supported by ANR RIB grants MICADO n° ANR-07-RIB-010-02 and ANR-07-RIB-010-04. J.B. was funded by a PhD fellowship from the Fondation Pierre-Gilles de Gennes. We are deeply grateful to Boris Barbour and Sarah Mikula for their careful reading of the manuscript.

#139111 - $15.00 USD Received 7 Dec 2010; revised 24 Jan 2011; accepted 2 Feb 2011; published 28 Feb 2011