4Pi microscopy deconvolution with a variable point-spread function

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To remove the axial sidelobes from 4Pi images, deconvolution forms an integral part of 4Pi microscopy. As a result of its high axial resolution, the 4Pi point spread function (PSF) is particularly susceptible to imperfect optical conditions within the sample. This is typically observed as a shift in the position of the maxima under the PSF envelope. A significantly varying phase shift renders deconvolution procedures based on a spatially invariant PSF essentially useless. We present a technique for computing the forward transformation in the case of a varying phase at a computational expense of the same order of magnitude as that of the shift invariant case, a method for the estimation of PSF phase from an acquired image, and a deconvolution procedure built on these techniques. © 2006 Optical Society of America

OCIS codes: 180.2520, 180.1790, 100.1830, 100.3190.

1. Introduction

4Pi microscopy is a form of confocal light microscopy that uses interference between the foci of two opposing objective lenses to obtain an imaging resolution significantly better than that obtainable with a conventional microscope. The method is slowly reaching maturity, and a commercial instrument is now available from Leica Microsystems Incorporated. The commercial variant is of type A (only the excitation is brought to interference) and utilizes two-photon excitation. The focus of this paper is thus on two-photon type A 4Pi microscopy. A key feature of 4Pi microscopy is the requirement for postprocessing to remove sidelobes present in the images, and to obtain the full imaging resolution. Various methods exist for sidelobe removal, ranging from a simple three-point linear filter to fully fledged maximum-likelihood (ML) deconvolution algorithms. The various methods have their corresponding pros and cons: the three or five point filters are fast, stable, and do not require a highly accurate point spread function (PSF) measurement. The filters, however, exhibit a small amount of noise amplification (or, strictly speaking, a reduction in the effective signal to noise ratio), and do not offer the advantages of ML algorithms such as noise reduction or the same degree of resolution enhancement. The ML algorithms in turn suffer from being slow, resource hungry, susceptible to poor PSF measurements, and subject to problems related to stability and convergence. It is thus possible to obtain a much higher level of noise amplification and artifact generation from the ML algorithms than from the linear filters. With an accurate PSF and a suitable choice of regularization, artifact generation in ML algorithms can be minimized, thus justifying our use of a ML technique in this paper. Pure destructive interference is also incapable of being solved by using a three- or five-point deconvolution (the appropriate matrix is singular). ML techniques get around this by providing additional information.

One area in which all current algorithms have difficulties is that in which the PSF depends on the position in the sample. The major PSF variation observed in 4Pi microscopy is a phase shift in the underlying interference pattern that is caused by a difference in the optical path length from each objective. This is manifested by a shift from constructive to destructive (or anywhere between) interference at the center of the PSF, as shown in Fig. 1. When constant over the whole specimen, this can easily be corrected with the phase adjustment piezo or by using a suitable PSF measurement with a conventional ML algorithm. When this varies throughout the object, however, it is impossible to correct for by using the existing algorithms. Such a variation is commonly seen in biological specimens in which precise control of the refractive index inside the cell is
difficult. Various hardware solutions to this problem have been presented, involving continuous compensation of the phase during measurements and monitoring both outputs of the beam-splitter prism. Neither of these options exists for the commercially available device. In this paper we present a method of monitoring both outputs of the beam-splitter prism.

2. Phase Shifted 4Pi Point-Spread Function

Most deconvolution methods require a computationally inexpensive method of computing the forward transform, \( \mathbf{d} = H \mathbf{f} \) (the mapping from the vector of object voxels, \( \mathbf{f} \), to the data voxels, \( \mathbf{d} \)), and also in several cases the conjugate transpose of this mapping. In the case of a spatially invariant PSF this is a convolution and can be inexpensively performed by using Fourier domain multiplication. When the PSF is not spatially invariant, as is the case with a 4Pi PSF in nonideal optical conditions, then this approach is not applicable. In the special case of a phase shift in the interference maxima of the 4Pi PSF, the forward transform, and its conjugate transpose, can be approximated as the linear combination of a small number of convolutions. Hereafter we will concentrate on two-photon 4Pi type A, as this the type of 4Pi microscope that is currently commercially available, and as the inverse problem is not even moderately well posed for the one-photon variants. However, the principles are applicable to all 4Pi variants.

The 4Pi PSF can be described as an envelope corresponding to the PSF of a confocal microscope operating at the respective excitation and emission wavelengths \( I_{\text{env}}(x, y, z) = I_{\text{det}} e^{-2z} \), or in the case of two-photon excitation \( I_{\text{det}} e^{-8z} \), multiplied by the interference pattern produced by the coherent addition of light from both objectives \( I_{\text{fringes}}(x, y, z) \) giving

\[
I_{\text{PSF}} = I_{\text{env}}(x, y, z) I_{\text{fringes}}(x, y, z). \tag{1}
\]

The same general form applies to 4Pi type B when \( I_{\text{fringes}} \) is regarded as the projection of the detected light interference into the object space, and to type C when the combination of the two interference patterns is used.

These interference fringes correspond to the wavefronts of the excitation beam, which are plane in the focus of a Gaussian beam and acquire curvature at increasing distance on either side. If the envelope, \( I_{\text{env}} \), is sufficiently small, such that the detected intensity is very low before the curvature of the fringes becomes noticeable, it is possible to approximate the fringes as a plane-wave interference pattern having only a \( z \) dependence, \( I_{\text{fringes}}(z) \). This approximation forms the basis of the three- and five-point deconvolution schemes extensively used in 4Pi microscopy, and is a good approximation in the two-photon case. For plane-wave interference and one-photon excitation, the normalized fringe pattern is expected to be

\[
I_{\text{fringes}1\text{photon}}(z) = \cos^2\left(\frac{2\pi nz}{\lambda_{\text{ex}}} + \phi\right) \tag{2}
\]

where \( n \) is the refractive index, \( \lambda_{\text{ex}} \) the excitation wavelength, and \( \phi \) a phase term. In the case of two-photon excitation, the detected intensity is proportional to the square of the excitation giving

\[
I_{\text{fringes}}(z) = \cos^4\left(\frac{2\pi nz}{\lambda_{\text{ex}}} + \phi\right). \tag{4}
\]

The mapping \( \mathbf{d} = H \mathbf{f} \) responsible for the imaging process can be expressed as

\[
d(\mathbf{r}) = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} f(\mathbf{s}) I_{\text{PSF}}(\mathbf{r} - \mathbf{s}) d\mathbf{s}, \tag{3}
\]

where \( f(\mathbf{r}) \) is the fluorophore distribution in the sample and \( d(\mathbf{r}) \) the detected intensity. The dual parameterization of the PSF \( I_{\text{PSF}}(\mathbf{r} - \mathbf{s}, \mathbf{r}) \) reflects its spatial variance. Owing to the dependence of \( \phi \) on the position \( \mathbf{r} = (x, y, z) \) in the object space. Substituting \( I_{\text{fringes}} \) in Eq. (1) gives us an approximate expression for the two-photon 4Pi PSF in the presence of an arbitrary, spatially varying, phase shift

\[
I_{\text{PSF}} = I_{\text{env}}(\mathbf{r}) \cos^4\left[\frac{2\pi nz}{\lambda_{\text{ex}}} + \phi(\mathbf{r})\right]. \tag{2}
\]
Using the linearity property of integration we can now approximate the imaging through

\[
d \approx \frac{1}{4} \left( \frac{3}{2} I_{\text{env}} \ast f + 2 \cos(2\phi) \left\{ \cos \left( \frac{4\pi nz}{\lambda_{\text{ex}}} \right) I_{\text{env}} \right\} \ast f \right)
- 2 \sin(2\phi) \left\{ \sin \left( \frac{4\pi nz}{\lambda_{\text{ex}}} \right) I_{\text{env}} \right\} \ast f
+ \frac{1}{2} \cos(4\phi) \left\{ \cos \left( \frac{8\pi nz}{\lambda_{\text{ex}}} \right) I_{\text{env}} \right\} \ast f
- \frac{1}{2} \sin(4\phi) \left\{ \sin \left( \frac{8\pi nz}{\lambda_{\text{ex}}} \right) I_{\text{env}} \right\} \ast f,
\]

where \( \ast \) represents convolution. The five integrals obtained when substituting Eq. (4) into Eq. (3) all involve a spatially invariant PSF term, and can thus be expressed as convolutions. These convolutions are easily calculated, for instance by using the Fourier space multiplication technique. Similar reasoning can be applied to the conjugate transpose of the transformation.

The concept is easily extended to the 4Pi C variants through the introduction of an additional sinusoidal term in \( I_{\text{fringes}} \) and a second phase map, which is related to the original through a scaling and offset.

3. Phase Estimation

To be able to perform a reconstruction based on our variable phase PSF model, as outlined in Section 2, we require knowledge of the PSF phase at every point in the image. It is possible to obtain information about the phase during image aquisition by using methods such as those presented by Hell et al.\(^8\) and Blanca et al.\(^9\) Unfortunately, the hardware implementation of the commercial 4Pi microscope does not allow these techniques to be used and one must attempt to extract this information from the resulting images. Given a method for calculating the forward transformation that maps the fluorophore density and PSF phase in the object space to an image it is theoretically possible to invert the problem and simultaneously estimate the object and phase through some form of maximum-likelihood optimization. As one must estimate two parameters for each data point and as the vast majority of voxels contain absolutely no information about the phase, being either dark or part of a larger structure, this problem is both underdetermined and poorly posed, and it is difficult to envisage a regularization technique capable of improving the conditioning enough to allow a ML algorithm to produce a sensible result. One alternative is to independently estimate the phase from the structures in the image in which the phase information is present, such as reference beads or thin membranes aligned perpendicular to the optical axis, and interpolate this over the whole image before deconvolving by using this phase estimate.

The technique for phase extraction presented here is one of several possible methods, but the requirements for most methods will be similar: structures that are axially small, separated from other structures in the axial direction and having a good signal-to-noise ratio. Our method simply attempts to measure the distance between the central (or highest) maximum, and the center of the envelope. In the case of destructive interference, where both maxima are equally high, the choice of which distance to measure becomes arbitrary. To be robust in the presence of noise, the images are filtered before processing. When only the position of an object is important, optimal filtering is obtained by making the cross correlation of the image with the PSF. To extract the center of each object, we thus perform a cross correlation of the image, \( d \) with the envelope of the PSF, \( I_{\text{env}} \) giving us an image \( d_{\text{env}} \). The position of the fringes is then extracted by using the cross correlation with \( \cos(4\pi nz/\lambda_{\text{ex}})I_{\text{env}} \) giving us \( d_{\text{fringes}} \) the \( \cos(4\pi nz/\lambda_{\text{ex}}) \) term from the expansion above being a sufficiently good approximation for this purpose. The distance from the peaks of \( d_{\text{env}} \) to the correspondingly nearest peak in \( d_{\text{fringes}} \) then gives us the phase at that point in the image. Because \( \cos(4\pi nz/\lambda_{\text{ex}})I_{\text{env}} \) averages to zero on a distance scale greater than a few periods of the cosine term, the envelope of \( d_{\text{fringes}} \) tends to zero over areas of the image with no high frequency components (corresponding to small structures) in the axial direction. A threshold on the envelope of \( d_{\text{fringes}} \) can thus tell us which parts of the image are interesting for phase extraction. However, this is not entirely sufficient, as fringes will also be visible at the edges of extended structures, and where several small structures are in close vicinity or periodically spaced. While it should theoretically be possible to estimate the phase from such structures, additional information about the structure would be required. To remove structures where the assumptions of negligible axial thickness and isolation from neighboring objects are not fulfilled, we can examine the height of the maxima on either side of the central peak. In the case of an extended or periodic object, these will be elevated. Figure 2 illustrates the application of phase estimation using these principles to a line scan through the simulated 2D test image shown in Fig. 5.

The method detailed above is limited in accuracy to one voxel. An alternative method of phase estimation, offering subvoxel phase resolution, extends the above by fitting a suitable model function to the data at each reference point rather than simply looking for the maxima. One could also interpolate \( d_{\text{env}} \) and \( d_{\text{fringes}} \) in the \( z \) direction to achieve the same result. In any case, to accurately estimate the phase over the entire image the values from the resulting measurement points must be interpolated. As an alternative to the automatic selection of reference points, manual selection could be desirable in poor signal-to-noise conditions, or when speed and/or memory usage is an issue. In this case the judgment as to whether a structure is isolated or not is based on the experience of the user.

In the examples presented here, a smoothly varying phase is assumed. It could be advantageous to further develop the phase estimation procedure to allow discontinuities in the first derivative, such as...
would occur at a refractive index step. Such a model would however, be likely to require a very large number of control points, or additional information such as could be obtained from differential interference contrast (DIC) images. It is in any case unlikely that the small errors introduced in the assumption of smoothness will be noticeable in the resulting images, although they could be significant for subresolution distance measurements.

4. Deconvolution Algorithm

Conventional deconvolution algorithms for light microscopy are optimized for either Gaussian or Poisson noise, with algorithms such as Richardson–Lucy\textsuperscript{10} that are based on Poisson noise being mathematically superior for almost all cases of confocal imaging. Whether this is also the case for 4Pi images remains to be established. The avalanche photodiodes used in the commercial 4Pi have a very limited (approximately 3 bits) dynamic range. This is due to the fact that they act in photon-counting mode, combined with a limited bandwidth and relatively short (~1 μs) per voxel integration time. To overcome the limited dynamic range, it is thus common practice in 4Pi microscopy to significantly oversample (~20 nm voxel size) and to smooth the resulting images with a Gaussian filter.

This averaging will alter the noise characteristics, with the central limit theorem implying that the noise should become more Gaussian.

The algorithm we have used solves a Tikhonov regularized, weighted least-squares problem

\[
\arg \hat{f} \min \left[ \|W(d - H\hat{f})\|^2 + \lambda^2 \|L(\hat{f} - f_{data})\|^2 \right] \quad (6)
\]

using a modified conjugate gradient (CG) solver.\textsuperscript{11–13} This attempts to find an estimate of the object, \(\hat{f}\), which minimizes both a misfit term, \(\|W(d - H\hat{f})\|^2\), describing how well the object matches the data, and a likelihood term \(\|L(\hat{f} - f_{data})\|^2\) that represents some prior knowledge (most often an assumption of smoothness) about the form of the object. The parameter \(\lambda\) determines the respective weighting of the data misfit and likelihood terms. In contrast to ordinary least squares, which is the solution in the presence of uniform Gaussian noise, choosing \(W_{ij} \propto 1/\sqrt{\varnothing_i}\), \(W_{ij} = 0\) gives a scaled Gaussian noise model in which the noise amplitude scales with the square root of pixel intensity. This allows us to approximate the Poisson noise, and might indeed be more appropriate than a pure Poisson model in cases in which the signal is averaged. These weights are in practice not particularly useful as they give infinite weight to zero valued voxels in the image. A combination of \(\tilde{n}\) and uniform Gaussian noise, \(W_{ii} \propto 1/|\beta + \tilde{n}|\), achieved by putting a constant \(\beta\) in the denominator, yields better results. For a likelihood function, \(L\), we use a \(3 \times 3 \times 3\) approximation to the second derivative, as this yields the qualitatively best results on both simulated and real images. The forward mapping \(H\) and its transpose are evaluated as indicated in Section 2. A positivity constraint is also introduced by clipping negative values to zero at each iteration.

This deconvolution approach was chosen due to its robustness, fast convergence, the ability to improve the conditioning of the problem through regularization, and the availability of MATLAB code\textsuperscript{12} for the CG solver. While the Richardson–Lucy algorithm is to some extent regularizable by limiting the number of iterations, or filtering the images between steps,\textsuperscript{14} it is easier to guarantee convergence with the Tikhonov–least-squares approach.

To test the proposed methods for image generation, phase estimation, and image reconstruction, we have applied them both to a test pattern and to real images.

A. Deconvolution of Simulated Data

The test pattern used was the 2D arrangement of geometrical shapes shown in Fig. 3 and its corresponding phase map (Fig. 4). Note that the phase shift applied in this case is fairly extreme—shifting from constructive to destructive interference and back to constructive again as one moves down the z axis (vertically). A 2D image was used here as the computation is quicker, and the results are easier to

![Fig. 2](image)
display and interpret than in the 3D case. The algorithm is however, the same and is easily generalized to 3D, as will be shown with the biological results. An image, shown in Fig. 5, was obtained by applying the procedure in Section 2 to the test image by using the phase map and an approximation to the theoretical PSF envelope, and passing the result through a Poisson noise process. The PSF envelope was approximated with sinc²(9z/5w) sinc⁴(z/w) axial component, the width of which was chosen so as to give a sidelobe height of approximately 50 percent, multiplied with a Gaussian lateral component. Note that the sinc² and sinc⁴ in the axial component correspond to detection and two-photon excitation, respectively. The use of sinc functions ensures that the axial PSF remains band limited and avoids any artificial resolution improvement. The width of the component was chosen so as to give a sidelobe height of approximately 50%, multiplied with a Gaussian lateral component. The simulation was performed for an excitation wavelength of 800 nm, a voxel size of 10 nm in the axial direction and 100 nm laterally, and the noise was applied to give a desired photon count of 30 in the brightest voxel of the image. Two counts of background noise were also added to the entire image.

Applying the phase extraction procedure described in Section 3 gives the reconstructed phase shown in Fig. 6. The automatically selected control points are shown as crosses on the image, and it can be clearly seen that, while not completely perfect, the reconstruction provides a good estimate of the original phase map. The discrepancies that are seen come from a variety of factors including interference from nearby structures, noise, and the interpolation procedure used (the nearest neighbour interpolation followed by Gaussian smoothing, the width of which, σ = 700 nm, was chosen to ensure a smooth phase change between the reference points). It can be seen from the image reconstruction in Fig. 7 that it is still possible to obtain an accurate result despite these discrepancies. Figure 7 shows the effective phase compensation, sidelobe removal, and noise reduction offered by the restoration procedure. A detailed comparison is afforded by the line profile taken along the z axis close to the center (x ~ 0.5 μm) of the image through the test object, simulated data, and the deconvolution result that is shown in Fig. 8. The deconvolution was performed both unweighted (i.e., uniform Gaussian noise) and with intensity dependent weights. As there was no appreciable difference in the quality of the resulting images, and as the exact noise characteristics of the 4Pi images are unclear, the results presented here use an unweighted solution, i.e., a uniform Gaussian noise model. To quantify the improvement obtained through phase
compensation, we have compared our test object with the simulated data and deconvolution results both with and without phase compensation by performing a cross correlation between each of the these images and our original test pattern. The results of these comparisons are shown in Table 1. The correlation value shows how similar each image is to the original object, with a value of 1 indicating 100% agreement. It is clear that phase-compensating deconvolution offers a significantly better match than either the data or the deconvolution performed without phase compensation.

B. Reconstruction of Biological Images
To demonstrate the procedure on biological data, we have applied the procedure to a 3D image stack acquired with the 4Pi microscope from a specimen in which one class of potassium channels in the cell membrane was labeled through the expression of Kir2.1-eYFP (see Appendix A for the details of the preparation and acquisition parameters). The deconvolution was performed on the smoothed data (Gaussian blur, \( \sigma = 1 \) voxel), using a theoretical PSF envelope, and a phase map interpolated from 20 manually selected control points on the cell membrane. The PSF simulation was based on vectorial theory and assumed unpolarized light. The parameter selection was based on the experimental wavelength, voxel size, and pinhole settings, and a refractive index of 1.46. To obtain the correct sidelobe height, however, a reduced NA of 1.1 was required. Such a large reduction in NA seems unlikely; one possibility would be a misalignment of the two objectives. Interpolation of the phase map was performed by fitting a linear function in \( z \) to the control points. Figures 9, 10, and 11 show an \( xz \) section through the stacks corresponding to the raw data, the result of normal ML deconvolution (using our algorithm with the phase set to zero over the whole image), and the result of ML deconvolution when applying phase correction. In both cases the number of iterations was 20. Figure 12 shows a line profile taken vertically through the center of the images. The asymmetry in the PSF, and the refractive index mismatch induced phase change through the image are clearly visible. The improvement to the deconvolution ob-

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<th>Correlation With Object</th>
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<td>Simulated data</td>
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<td>ML - No phase compensation</td>
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<td>ML - With phase compensation</td>
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*The simulated data are from Fig. 5 and the reconstructions from Fig. 7. Reconstructions without phase compensation are not shown. The test pattern is from Fig. 3. The values presented are at zero shift, and the correlation coefficients are normalized so that autocorrelation gives a coefficient of 1.*
tained through phase correction is easy to see. The residual sidelobes in the deconvolved image can probably be attributed either to overregularization or to an inaccuracy in the estimation of the PSF Envelope. Nevertheless, they are certainly low enough to facilitate an unambiguous analysis of the images.

C. Performance of the Algorithm

In its current state, our algorithm is both relatively slow and relatively resource hungry. On the test machine (AMD AthlonXP 2500+, 1 GB RAM, MATLAB R14, Windows 2000) the maximum data size that can be computed in one go is approximately $2 \times 10^6$ voxels. On a stack of this size, 30 iterations take approximately 45 min. The large memory usage can be explained by the fact that floating point representation is used for all the data and that it is necessary to keep track of several working variables, including 10 (five forward and five transpose) part OTFs being the Fourier transforms of the terms in the LHS of each of the convolutions in Eq. 5, each of which is the full size of the image. The memory usage is thus approximately five times that of a similar deconvolution algorithm with a spatially invariant PSF. The slow speed is due to the relatively large number of calculations that must be performed at each step. This could be improved somewhat, at the expense of higher memory usage, by precalculating the position dependent weights. The relatively large amount of computation involved in the forward (and transpose) transforms makes the fast convergence of a CG solver particularly attractive (normally the computation of the forward transform is fast compared with the relatively large per step time required to calculate the new search direction in CG methods, and fast convergence is partly offset by the fact that another algorithm, e.g. steepest descent, can take many more steps in the same time).

To overcome the memory related limitations on image size, it is possible to segment the image and deconvolve each segment independently. To avoid the edge effect artifacts that would otherwise be present, the segments can be made to overlap and the edge regions of each segment discarded. In theory, due to the $O(n \ln n)$ complexity of the fast Fourier transforms used to compute the forward–transpose mappings, such segmentation should also offer a performance gain although this is likely to be offset by the extra computation introduced by the segment overlaps. It should also be noted that a segmentation-based technique lends itself well to parallelization, falling into the class of embarrassingly parallel problems (those that can be split into many independent tasks with no need for data exchange except at the beginning and
end of computation). We are currently developing an algorithm based on these principles, and preliminary tests show an execution time of \(~2\ h\) on the machine above for 10 iterations on a \(512 \times 370 \times 256 \approx 5 \times 10^7\) voxel image, and a linear decrease with an increasing number of nodes (\(~40\ minutes\) on a three-node cluster).

5. Conclusion

Techniques capable of compensating for phase shift should be of considerable advantage in the routine biological use of 4Pi Microscopy. Despite considerable effort it is not always possible to achieve a perfect refractive index match. This is especially so for in vivo measurements where the options for controlling the intracellular refractive index are particularly limited. An unfortunate characteristic of the current generation of 4Pi microscopy techniques is a rather high rate of photobleaching. In normal measurements one would align the PSF for constructive interference on a reference bead or membrane near the object to be measured before commencing the acquisition. For particularly photosensitive samples a "shoot first, ask questions later" approach, such as is facilitated by an adaptive reconstruction algorithm, could present a decisive advantage.

We have presented a method of image reconstruction for 4Pi microscopy capable of compensating for the commonly occurring problem of a position dependent phase shift in the 4Pi PSF. This technique is demonstrated, both on synthetic data and on real 4Pi microscopic images. The ability to separate the forward mapping, which owing to its lack of shift invariance poses a difficult problem for both image generation and reconstruction, into the spatially weighted sum of a small number of shift invariant mappings represents a considerable simplification. Although only the two-photon 4Pi A case is addressed here, the concept can easily be extended to the other 4Pi variants. The CG solver used for the results presented in this paper is most likely less than ideal in terms of performance, regularization method, and the underlying noise model. It is, however, more than adequate to demonstrate our concept for phase compensation, and delivers results that are comparable to those of the leading commercial offerings in the absence of a phase shift.

Appendix A: Materials and Methods

1. Cell Biology

N-terminal fusion constructs of Kir 2.1 channel subunits with enhanced yellow fluorescent protein (eYFP) were designed by inserting the respective cDNA into the pEYFP-C1 eukaryotic expression vector (Clontech) by using the EcoRI and BamHI restriction sites as described in Stockklausner et al.15 Human embryonic kidney 293 cells were grown in minimum essential medium (Invitrogen), supplemented with 10% fetal calf serum and 1% penicillin/streptomycin at 37 °C and 5% CO₂. The cells were grown on coverslides provided by Leica Microsystems. At 80% confluence, the cells were transfected with the respective cDNA by using Fugene 6 (Roche) following the supplier's instructions.

2. Sample Preparation

The two coverslips specifically designed for use with the Leica 4Pi Microscope were provided by Leica Microsystems (Mannheim, Germany). Transfected cells were fixed in 4% paraformaldehyde in phosphate-buffered saline for 20 min at 4 °C on one of the coverslips. The fixed cells were embedded in 87% (volume) glycerol buffered with 50 mM Tris-HCL to obtain a pH = 7.4. On the other coverslip we immobilized green fluorescence beads (diameter 100 nm, G100, Duke Scientific) to implement reference objects for evaluation of the surface structure. For sealing the coverslips we used nail polish and dental glue (Twin-sil A + B).

3. 4Pi Microscopy

The measurements presented here were performed on a commercial prototype 4Pi Microscope at Leica Microsystems Incorporated, Mannheim, Germany. This is a two-photon 4Pi microscope of type A (the excitation foci are brought to interference, but the interference of the detected light is not used). Two-photon excitation was provided by using a Ti:sapphire laser producing picosecond pulses and tunable between 740 and 990 nm. A matched pair of glycerol immersion objectives (100×, NA=1.35) were used with quartz coverslips and 87% glycerol as a mounting and immersion medium. A voxel size of 26.2 nm in x and z and 30.5 nm in y was chosen and an xz scan performed resulting in a final image size of \(512 \times 25 \times 512\) voxels. The pinhole and beam expander were set to 1 Airy and 3, respectively, a line average of 16 and an accumulation of 2 were used. The excitation wavelength was approximately 970 nm.

The authors thank S. M. Tan (Department of Physics, University of Auckland) for the skeleton code of a conjugate-gradient solver, C. Karle and E. Zitron (Department of Cardiology, Medical University Hospital, Heidelberg, Germany) for providing the biological specimen, Leica Microsystems Mannheim, where the measurements were carried out, and U. Birk for stimulating discussion and help with the proofreading. This work was supported by grants from the Deutsche Forschungsgemeinschaft and the state of Baden-Württemberg.

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