Correction of cell-induced optical aberrations in a fluorescence fluctuation microscope

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We describe the effect of optical aberrations on fluorescence fluctuations microscopy (FFM), when focusing through a single living cell. FFM measurements are performed in an aqueous fluorescent solution and prove to be a highly sensitive tool to assess the optical aberrations introduced by the cell. We demonstrate an adaptive optics (AO) system to remove the aberration-related bias in the FFM measurements. Our data show that AO is not only useful when imaging deep in tissues but also when performing FFM measurements through a single cellular layer. This work paves the way for the application of FFM to complex three-dimensional multicellular samples. © 2013 Optical Society of America

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Fluorescence fluctuation microscopy (FFM) is an ensemble of techniques that in principle allows the absolute measurement of fluorescent molecule concentrations, mobility coefficients, and rates of biochemical interactions [1–3]. FFM is based on the detection of the fluctuations of the fluorescence signal and gains in accuracy for a small number of fluorescent molecules inside the observation volume, which is related to the confocal point-spread function (PSF). To achieve this condition in biological environments, samples with low concentrations of fluorochromes (nanomolar to micromolar) are observed with high numerical aperture objectives in a confocal geometry. However, the absolute character of these measurements relies on the assumption that the observation volume is well quantified [4–6]. For most biological applications of FFM, this volume, if not perfectly known, should at least remain stable for comparable measurements at different times or locations. Optical aberrations can prevent a meaningful analysis of FFM measurements by causing sample-dependent distortion of the PSF, which has an impact on both the number of molecules and the characteristic time measured by FFM. We previously demonstrated the utility of an adaptive optics (AO) system for fluorescence correlation spectroscopy (FCS) to greatly reduce this effect in aqueous solutions with various refractive indexes [7], where spherical aberrations caused by index mismatch have dramatic consequences on the measured parameters.

AO for microscopy has originally been demonstrated for imaging deep into thick tissues, where the large amount of aberrations visibly degrades image quality [8]. FFM being much more sensitive to aberrations than imaging applications, we show in this Letter that focusing through a single cell introduces aberrations that have a significant impact on the measured FFM parameters. We demonstrate an improved version of our AO system [7] that now uses FFM measurement as an optimization metric for automatic aberration correction. The ability to perform accurate FFM measurements through a cell is a first step toward applying FFM to complex three-dimensional multicellular environments, such as spheroids, which should yield relevant biophysical results in the near future.

We focus our attention on the number of fluorescent molecules N inside the observation volume (one of the outputs of FFM). Assuming that N follows Poisson statistics, it can be estimated as the inverse of the relative fluctuation of the fluorescence signal when shot noise is negligible. We obtain real-time measurements of N using the mean μ and the variance σ2 (evaluated over a few seconds) of the photon count (acquired during a few microseconds binning time): N = μ2/(σ2 − μ). The subtraction of μ in the denominator removes the contribution of shot noise in the overall variance σ2 of the signal.

The molecular brightness (defined by the count rate per fluorescent molecule μ/N) is a commonly used metric to quantify the signal-to-noise ratio of an FFM experiment [9] and scales as the Strehl ratio in the presence of optical aberrations of low amplitude [7]. Therefore, we use the measured molecular brightness as a quality metric for modal optimization in our aberration correction scheme, which is similar to the one described for AO-based image-sharpening [10]. An important advantage of our method is that it does not require acquiring an image of the sample. Instead, we perform aberration correction in a single point. In this way, the correction is not affected by possible spatial variation of the aberrations. Moreover, since FFM measurements are usually performed in regions of low contrast (where molecules are freely diffusing), image sharpness is not conveniently used as optimization metrics for aberration correction [9]. Molecular brightness, in contrast, is a sensitive metric for optical aberration correction in these dilute samples, regardless of the spatial structure.

We have constructed a confocal microscope, which is designed for FFM and equipped with AO. The optical layout is shown in Fig. 1(a). We use a high-speed 97-actuator deformable mirror (DM) (AlpAO, France) for aberration correction. First, the DM is calibrated in a closed-loop scheme using a 32 × 32 Shack–Hartmann wavefront sensor (SHWFS) (AlpAO) to individually generate eight...
Zernike modes (pairs of astigmatisms, comas, trefoils, and spherical aberrations) with root mean square (RMS) amplitudes of ±0.05 μm, the bias values that we use to perform the modal optimization described by Booth et al. [10]. The DM shape is sequentially optimized in terms of Zernike modes, using for each mode the parabolic interpolation of three measurements (of duration 4 s each), which we obtain with the DM set to its last optimized shape, and plus or minus a bias of the mode. Depending on the level of noise and the amount of initial aberrations, the cycle of eight mode corrections is repeated two or three times. Prior to cell measurements, the microscope inner aberrations are corrected in an aqueous fluorescent solution by performing two optimization cycles. We then measure the number of molecules ($N_0 \approx 3.4$) and the photon count rate ($\mu_0 \approx 120$ kHz) in the fluorescent solution. The corresponding Zernike modes are defined as default commands to the DM and typically correspond to a 0.030 μm overall RMS amplitude. The experiment that we describe in this Letter is illustrated in Fig. 1(b). We perform FFM measurements through mouse embryo fibroblast cells in the same fluorescent solution that we use to correct the microscopic aberrations. Doing so allows us to investigate the optical effect induced by the cell as compared to the nominal measurements in the cell-free fluorescent solution. The FFM measurements $\mu$ and $N$ are normalized with the cell-free measurements: $\tilde{\mu} = \mu / \mu_0$ and $\tilde{N} = N / N_0$. To minimize the amplitude of the initial aberrations when starting cell experiments, we use neighboring wells in the same chambered coverslip (Nunc Labtek) for system aberrations correction and cell measurements. In the following, we show typical FFM data obtained with two cells that spread differently on the glass substrate: a spherical cell1 [Fig. 2(a)] and a flat cell2 [Fig. 2(b)].

We show in Figs. 2(c) through 2(f) FFM measurements as a function of the focus position $z$, which we define relative to the apex of the cell ($z = 0$ μm). We first acquire measurements with the DM set to its default commands (dotted curves). At the cell–water interface, the number of molecules is close to the cell-free values [$\tilde{N} \approx 1.1$ at $z = 0$ μm for the two cells, Figs. 2(c) and 2(d)]. This observation suggests that the PSF is not significantly distorted by optical aberrations. However, there is a loss of photons [$\tilde{\mu} \approx 0.7$ at $z = 0$ μm], dotted curves of Figs. 2(e) and 2(f), which is presumably not related to optical aberrations for two reasons: (1) there is no significant increase of $\mu$ after aberration correction (data not shown) and (2) $\mu$ is generally less sensitive to optical aberrations than $N$. This observation holds true for any FFM measurement in a dilute sample and is related to the fact that the image of a diffuse object is only weakly affected by optical aberrations [9]. In the limit of a very large detector, $\mu$ is independent of aberrations because it scales as the incoming optical power. This loss of photons measured at $z = 0$ μm is presumably due to light scattering in the cell. To observe the effect of optical aberrations, it is necessary to focus a few micrometers above the center of cell1/cell2 before (dotted line) and after (solid line) aberration correction. (e), (f) Photon count rate above the center of cell1/cell2 before (dotted line) and after (solid line) aberration correction.

Fig. 1. (a) Optical layout. DPSSL, 561 nm diode-pumped solid state laser (Cohult); DM, 97-actuator deformable mirror (AlpAO); OBJ, 63×/1.2 water-immersed microscope objective (Zeiss); SM, 3 mm × Y galvanometric mirrors (Cambridge Technology); APD, single-photon-counting avalanche photo-diode (PerkinElmer); MF, 1× Airy multimode fiber; DF, 600 nm long-pass dichroic filter (Chroma); SHWFS, 32×32 Shack–Hartmann wavefront sensor (AlpAO); CAM, wide-field camera (ANDOR sCMOS Zyla); FM1, flip mirror for DM calibration; FM2, flip mirror for transmission microscopy. (b) Schematic of the experiment. The $z = 0$ focus position corresponds to the apex of the cell. FFM measurements are carried out in a fluorescent solution (sulforhodamine-B at a 15 nM concentration).

Fig. 2. (a), (b) Wide-field images of cell1/cell2 over a 50×50 μm field of view, observed with transmission microscopy. FFM measurements are carried out at the center of the field of view. (c), (d) Number of molecules as a function of the focusing distance above the center of cell1/cell2 before (dotted line) and after (solid line) aberration correction. (e), (f) Photon count rate above the center of cell1/cell2 before (dotted line) and after (solid line) aberration correction.
Aberration correction at $z = 8 \mu m$ (cell1) and $z = 13 \mu m$ (cell2) reduces the number of molecules to $\tilde{N} \approx 1.5$ [factors 5 and 2 for cell1 and cell2, respectively, see the solid curves of Figs. 2(c) and 2(d)]. Larger aberrations are corrected for cell1 (overall aberration is $0.11 \mu m$ RMS, including $0.09 \mu m$ of spherical aberration $Z_{4,0}$) than for cell2 ($0.07 \mu m$ RMS, including $Z_{4,0} = 0.05 \mu m$). It is clear from the curves of Figs. 2(c) through 2(d) that the optimal wavefront strongly depends on the focus position. The aberration correction performed at $8$ and $13 \mu m$ above cell1 and cell2 strongly distorts the PSF at other focus positions. In particular, it increases $\tilde{N}$ at $z = 0 \mu m$: $\tilde{N} \approx 7.5$ and $\tilde{N} \approx 4.0$ for cell1 and cell2, respectively, [solid curve of Figs. 2(c) and 2(d)].

It is also possible to see the effects of optical aberrations on the number of molecules using the raster image correlation spectroscopy (RICS) technique [11,12]. This technique consists in analyzing the spatiotemporal correlation of confocal images acquired at a relatively slow scan speed ($5 nm/\mu s$) and with a pixel size much smaller than the PSF, so that the probability of detecting the same diffusing molecule at two consecutive pixels is nonzero. Each subregion of the original confocal images, typically $32 \times 32$ to $128 \times 128$ pixels, provides a pixel in the $N(x, y)$ 2D map. A RICS analysis in a fluorescent solution provides homogeneous maps of $N(x, y)$ in the absence of instrumental artifacts, such as optical aberrations.

As in nonscanning FFM techniques (such as FCS), $N(x, y)$ is proportional to the observation volume. Figure 2 shows the effect of optical aberrations on $N(x, y)$ maps recorded above cell1 [Fig. 3(a)] and cell2 [Fig. 3(b)], at $z = z_{\text{max}}$ (with the DM set to the default commands). The “shadow” of each cell is visible in these maps because of optical aberrations but barely appears on the raw confocal images (data not shown). At the periphery of the maps, $\tilde{N}$ is minimal ($\tilde{N} \approx 1.2$ for both cells) because a smaller fraction of the beam propagates through the cell. $\tilde{N}$ is larger at the center of the cells [cell1: $\tilde{N} \approx 7.0$, cell2: $\tilde{N} \approx 3.0$, as in Figs. 2(c) and 2(d) without AO]. After aberration correction, the RICS analysis shows the reduction of $\tilde{N}$ in the central region of the two maps [Figs. 3(c) and 3(d)]. For cell1, the region for which aberration correction is beneficial is very small and corresponds to the center of the cell. This region is larger for cell2 and is elongated along the direction of the cell. This latter observation illustrates the idea that the full benefit of AO in a microscope is only achieved when the aberration correction can be updated across the field of view, as illustrated in [13]. The measurement of molecular brightness is local and could therefore be used as an optimization metric for aberration correction at different points of the field of view. However, two major problems occur to perform a variable aberration correction across the field of view: (1) The measurement time scales as the number of points (a major technological breakthrough to solve this problem would be the development of a direct method of aberration measurement with the property of optical sectioning). (2) It is necessary to develop an accurate synchronization between the imaging scan and the update of the DM shape, which can be very challenging and might require slowing down the scan.

To conclude, we demonstrated that the optical aberrations introduced by a single living cell can have a significant impact on FFM, and that AO is a promising technique for performing robust FFM measurements in complex biological samples, such as multicellular layers. FFM provides a metric that is directly related to optical aberrations, even in dilute regions of the sample. This feature makes FFM potentially useful for aberration correction in other microscopy techniques.

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