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Notch spatial filtering of image artifacts for structured illumination microscopy of cell-based assays

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ABSTRACT

Improvement of structured illumination microscopy (SIM) to remove grid pattern noise was investigated by applying notch spatial filters in the Fourier domain. We have acquired wide-field and SIM images of pollen grains and evaluated multiple reconstruction schemes on a quantitative basis. The results suggest that grid pattern noise can be reduced substantially to be smaller than that of standard SIM by more than 18 times, at the expense of little overhead in the reconstruction time. Also, if notch spatial filters are used in combination with subtractive reconstruction, the overall image quality can be enhanced by almost ten times, compared to standard SIM.

1. Introduction

Fluorescence optical detection and imaging have been of tremendous interest in cell-based assays for analyzing cellular activities: for instance, cell viability changes, specific enzymatic activities, cell invasion, and responses to treatments of drugs or cytotoxic chemicals [1–9]. For two dimensional (2D) cell-based assays, optical detection systems were generally based on wide-field fluorescence microscopy [6,10]. However, the introduction of complex three-dimensional (3D) cell-based assays that use thick extracellular matrices and complicated microfluidic channel systems requires high axial scanning resolution for the acquisition of cellular activities in 3D cell cultures [8,11]. For fluorescence optical detection of 3D microfluidic cell-based assays, fluorescent intensities were indirectly measured of a specific chemical bio-transformed by enzymatic activity of cells [8]. Also developed was optical detection for 3D cell cultures based on confocal fluorescence microscopy [9,12–15].

Recently, structured illumination microscopy (SIM) has been applied to enhance axial resolution of fluorescence imaging systems [16–19]. For example, use of subtractive image reconstruction [20–23], allowed much improved axial resolution by removing wide-field fluorescence from SIM images [24]. A critical disadvantage that affects the quality of fluorescence optical imaging based on structured light illumination is the presence of image artifacts among which the most severe is the grid pattern noise [25–27]. Causes that affect the grid pattern noise are suspected as unstable light excitation at image acquisition, non-sinusoidal illumination patterns, phase angle mismatches, and non-linear photobleaching. It was shown that the image acquisition with several phase and angular steps of structured illumination and the mean image calculation is effective for the reduction of grid pattern noise in SIM [24,28]. There are, however, shortfalls in this approach: mainly, use of multiple tilt angles increases the overall time for 3D image stack acquisition for SIM and yet cannot completely remove grid pattern noise in the image.

In this work, we apply a set of notch spatial filters for the elimination of grid pattern noise in the image post-processing of SIM. A notch filter can reject a band of spatial frequencies in the pre-defined neighborhoods around a center frequency, so it can be used for either positive or negative grid patterns, for example, 60 Hz AC noise [29–31]. The notch filtering begins with optical transfer function (OTF) of a fluorescence image that is acquired by SIM with a single tilt angle with grid pattern noise. Spectral components of the OTF are then associated with grid pattern noise. Multiple notch spatial filters are applied to eliminate grid pattern noise without any loss of processing time for 3D image stacks in SIM. Although we focus on the SIM for 3D cell-based assays, the approach described here should be applicable to super-resolved optical sectioning by SIM in general.

2. Materials and methods

2.1. SIM set-up and sample preparation

For proof of concept, a fluorescence optical imaging system was built based on SIM as shown in Fig. 1, which consists of three

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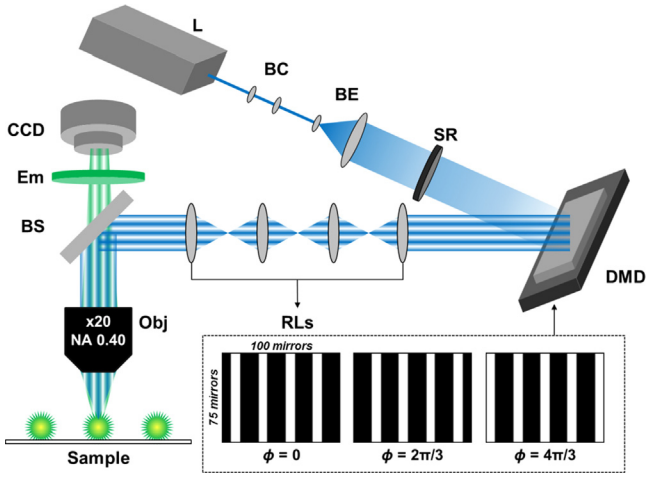

Fig. 1. Schematic illustration of SIM (L: laser, BC: beam collimator, BE: beam expander, SR: speckle reducer, DMD: digital micromirror device, RLs: relay lenses, CCD: charge-coupled device, Em: emission filter, BS: beam splitter, and Obj: objective lens). Also shown in the inset: structured input binary pattern images with tri-phases (ϕ) used in this study.

parts: an excitation part based on a laser at $\lambda_{ex}=488$ nm, a digital micromirror device (DMD) as a spatial light modulator that produces structured light illumination, and an emission part for the detection of fluorescence with $\lambda_{em}=515$ nm. Light from a fiber-coupled Argon-ion laser (35-LAP-431-230, Melles Griot, Carlsbad, CA, USA) was collimated and expanded with a fiber collimator (F220SMA-A, Thorlabs, Newton, NJ, USA), a beam expander (BE05M, Thorlabs). Speckle was removed by a speckle reducer (LSR-3010-1, Optotune AG, Dietikon, Switzerland) in the excitation part. After spatial modulation of excitation light using a DMD (Discovery™ 3000 kit, Texas Instrument, Dallas, TX, USA), structured excitation light was delivered to fluorescent samples through a combination of relay lenses ($f_1=f_3=f_4=100$ mm, MAP10100100-A, Thorlabs; $f_2=75$ mm, PCX-25.4B-075, Lambda Research Optics, Inc., Costa Mesa, CA, USA), a beam splitter and an objective lens (LMPlanFLN ($\times 20$, NA 0.40), Olympus, Tokyo, Japan). The relay optics used four relay lenses to provide sufficient space for the delivery of expanded excitation light from illumination optics to DMD and also for the potential use in a cell culture incubator. Fluorescence images were captured by a mono-color CCD camera (10-QIC-F-M-12-A, Qimaging, Surrey, BC, Canada) with an emission filter (FF03-525/50-25, Semrock, Inc., Rochester, NY, USA). To scan the sample axially, a linear motorized stage (UTM100CC1DD, Newport, Irvine, CA, USA) was applied in the emission part. Details of the image reconstruction algorithms using the SIM optical imaging system are described elsewhere [24].

For assessment of the notch spatial filtering and the image quality, two types of samples were prepared. Fluorescent microbeads (F-8836, Invitrogen, Carlsbad, CA, USA) with 10 μm diameter were immersed on a slide-glass with a PDMS (Polydimethylsiloxane) chamber. Also, slices of fluorescent pollen grains were purchased from Carolina Biological Supply Co. (Burlington, NC, USA).

2.2. Notch filter design

Fig. 2a shows a SIM image typical of a pollen grain which was captured with three phases ($\phi=0$, $2\pi/3$ and $4\pi/3$) and a single tilt angle ($\theta=0$) and reconstructed by standard SIM. The image in Fig. 2a suffers significantly from grid pattern noise which consists mainly of three spectral components of different amplitudes and widths in the OTF. The spectral components are represented as symmetric pairs about the origin as shown in Fig. 2b (A, B, and C). The widths and the positions of the three spectral components in the Fourier domain are determined by the tilt angle (θ) and the period of input images of structured light illumination. Although the amplitudes of the OTF depend on the acquired images, both the tilt angle and the period are fixed during the acquisition of SIM images. Therefore, we have established a set of three notch filters (Fig. 2c) for the elimination of symmetrically paired spectral peaks that are responsible for grid pattern noise. Each notch filter employs a Gaussian rejection transfer function as follows:

$$H(u,v)=1-\exp\left\{-\frac{1}{2}\left[\frac{D_1(u,v)D_2(u,v)}{{D_0}^2}\right]\right\}. \quad (1)$$

A Gaussian notch spatial filter suffers from less severe intensity degradation than a butterworth filter and less noise associated with the discontinuity at the boundary of the impulse response compared to an ideal notch spatial filter. In Eq. (1), $D_i(u,v)$, $i=1, 2$, is the distance from the center frequency of the notch filter to the origin and given by

$$D_1(u,v)=\left[\left(u-\frac{M}{2}-u_0\right)^2+\left(v-\frac{N}{2}-v_0\right)^2\right]^{\frac{1}{2}} \quad (2)$$

$$D_2(u,v)=\left[\left(u-\frac{M}{2}+u_0\right)^2+\left(v-\frac{N}{2}+v_0\right)^2\right]^{\frac{1}{2}} \quad (3)$$

where u and v represent the axis in the Fourier domain. M and N are the number of spatial frequencies in the Fourier domain in the u and v axis, respectively. u_0 and v_0 denote the center frequency of each notch filter. D_0 is the distance between the band center and the origin. In the normalized Fourier space ($-\pi\le k_x<\pi, -\pi\le k_y<\pi$), the center frequencies of the three notch spatial filters are (0.0718π,-0.0788π), (0.121π,-0.114π), and (0.160π,-0.148π). Each notch spatial filter has a width of (0.0144π,0.0192π) in the Fourier space. A wider bandwidth increases image intensity degradation, while grid pattern noise still remains for a narrower bandwidth. Multiplication of fluorescence images with the designed notch filter in the Fourier domain eliminates the spectral components derived from grid pattern noise (Fig. 2d and e) and mostly reduces grid pattern noise in the reconstructed fluorescence image.

3. Results and discussion

To evaluate the performance of notch spatial filtering in SIM, we have reconstructed images under four distinct scenarios: (I) standard SIM image acquired at a single tilt angle ($\theta=0$), (II) reconstruction by averaging partial images acquired at four tilt angles ($\theta=0$, $\pi/4$, $\pi/2$, and $3\pi/4$), (III) image that was acquired at a single tilt angle and notch-filtered, and (IV) image that was acquired at a single tilt angle, notch-filtered, and reconstructed with a subtractive approach ($I_{IV}=I_{III}-\gamma I_{wide\text{-}field}$, $\gamma=0.07$). γ represents the amount of wide-field fluorescence in a SIM image and was chosen as 0.07 for subtractive image reconstruction with a minimum loss of in-focus components and effective enhancement of axial resolution.

Fig. 3 presents the images reconstructed under each scheme as an axial projection from 3D image stacks, where each axial projection is based on the maximum intensity selection of pixels in fluorescence image slices. Compared to the standard SIM image presented in Fig. 3a, averaging of partial images is moderately successful to reduce grid pattern noise. The best result is achieved with the use of notch spatial filters. For quantitative comparison of the enhancement, we have defined noise standing-wave ratio (NSWR) as $NSWR=(I_{peak}+I_{trough})/(I_{peak}-I_{trough})$, where I_{peak} and I_{trough} denote the highest and the lowest local fluorescence intensity in a single sinusoidal fluctuation that arises from grid pattern noise, respectively, [32–34]. Reduced grid pattern noise would increase NSWR. Fig. 3 also presents NSWR for the four

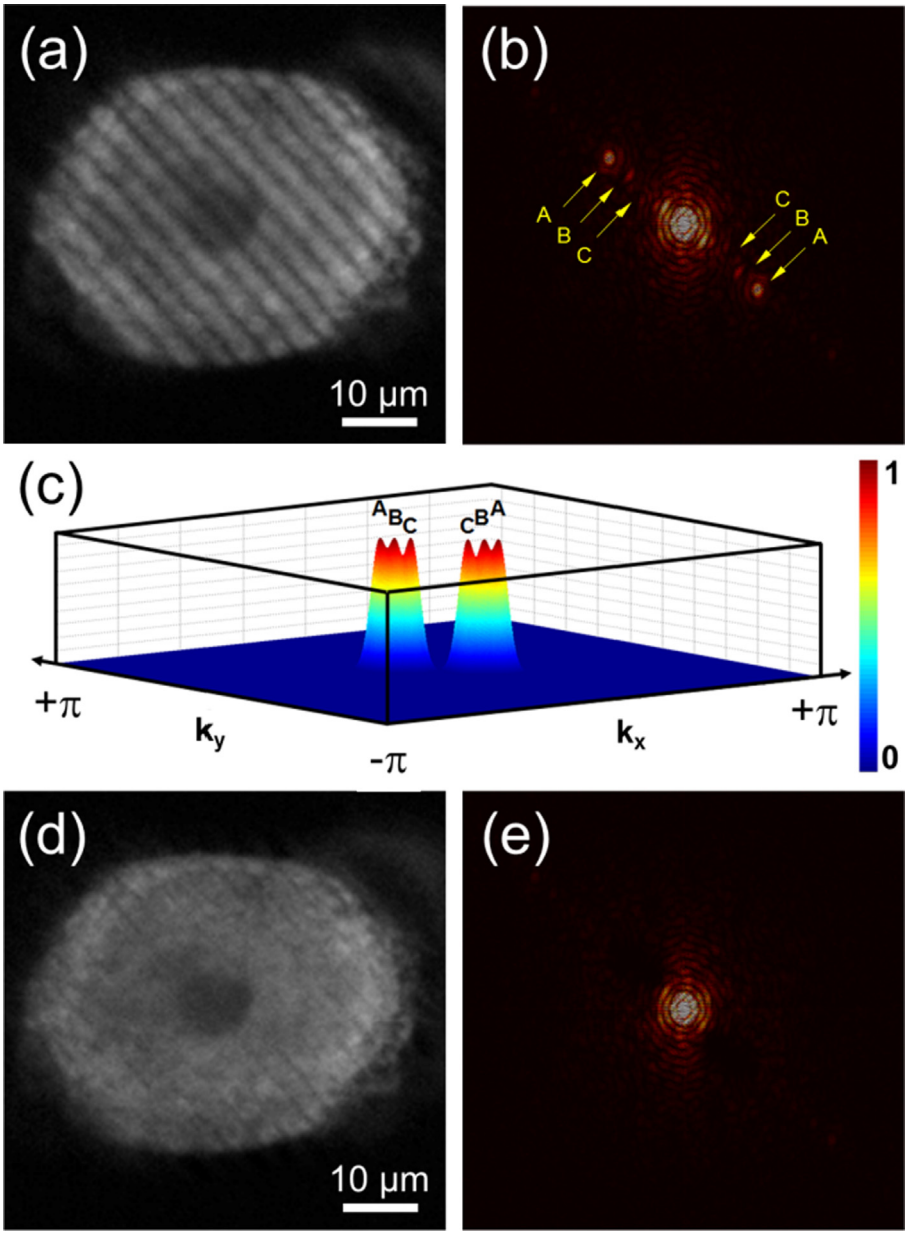

Fig. 2. (a) SIM image, (b) OTF of a fluorescent pollen grain. Arrows indicate spectral components associated with grid pattern noise, (c) Rejection characteristics of the designed notch spatial filter, (d) SIM image and (e) OTF of the fluorescent pollen grain after notch filtering.

reconstruction scenarios. The results confirm the visual observation: averaging produces moderate improvement by twice, while notch spatial filtering reduces pattern noise significantly by more than 18 times. Surprisingly, subtractive image reconstruction, while effective in removing background fluorescence and enhancing axial resolution over standard SIM, does not have a noticeable positive effect in terms of reducing grid pattern noise. In fact, there seems to be slight penalty by 14% for using subtractive reconstruction.

In other words, the result suggests that while notch spatial filtering significantly reduces grid pattern noise, axial resolution may be a setback. The effect of notch filtering on image resolution has been investigated by comparing 3D image stacks of fluorescent pollen grains acquired by wide-field microscopy (Fig. 4a), SIM with a single tilt angle (Fig. 4b), SIM with four tilt angles (Fig. 4c), subtractive SIM at $\gamma=0.07$ with four tilt angles (Fig. 4d), SIM with a single tilt angle and notch spatial filters (Fig. 4e), and subtractive SIM with a single tilt angle and notch spatial filters (Fig. 4f). Fig. 4 shows that the subtractive reconstruction algorithm for SIM, if combined with notch spatial filters, tends to enhance the axial resolution in the measured 3D fluorescence image stacks.

Another aspect of noise spatial filtering for the reduction of grid pattern noise is the time overhead to the overall reconstruction of axial sections of SIM images. Compared to the scenario *I* as a reference, partial image acquisition and averaging need more reconstruction and post-processing time because the amount of matrix calculation increases as the number of tilt angles. Notch spatial filtering may impose an additional reconstruction load for applying image filters in the Fourier domain.

For quantitative assessment of the overall image quality and the timing load imposed by the image acquisition and the reconstruction, a figure-of-merit (FOM) is defined as FOM=NSWR/[AR (μm)·t (s)], where t is the time consumed for each reconstruction scenario (I, II, III, and IV). Axial resolution (AR) was measured as a full-width-at-half-maximum of an axial point-spread function when a fluorescent bead was imaged and reconstructed. A larger FOM is preferred while FOM is related to the exposure time of a single

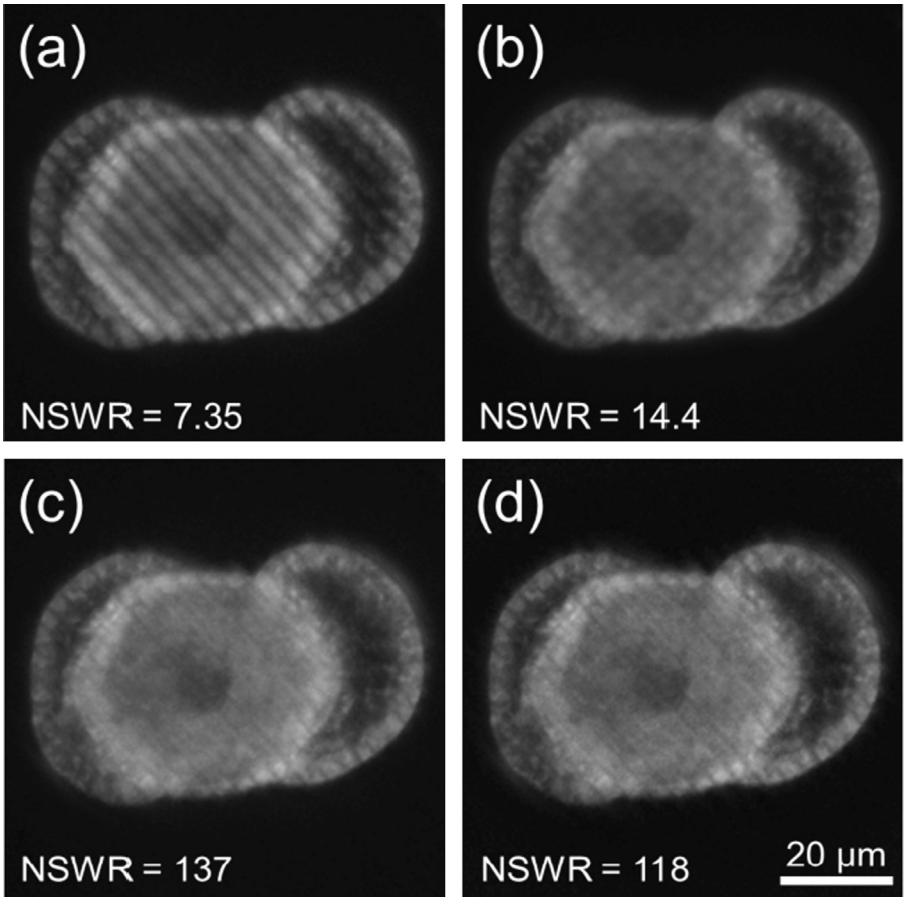

Fig. 3. SIM images of pollen grains: (a) acquired at a single tilt angle, (b) reconstructed by averaging four partial images (θ=0, π/4, π/2, and 3π/4), (c) acquired at a single tilt angle and notch-filtered, and (d) acquired at a single tilt angle, notch-filtered, and reconstructed with a subtractive approach.

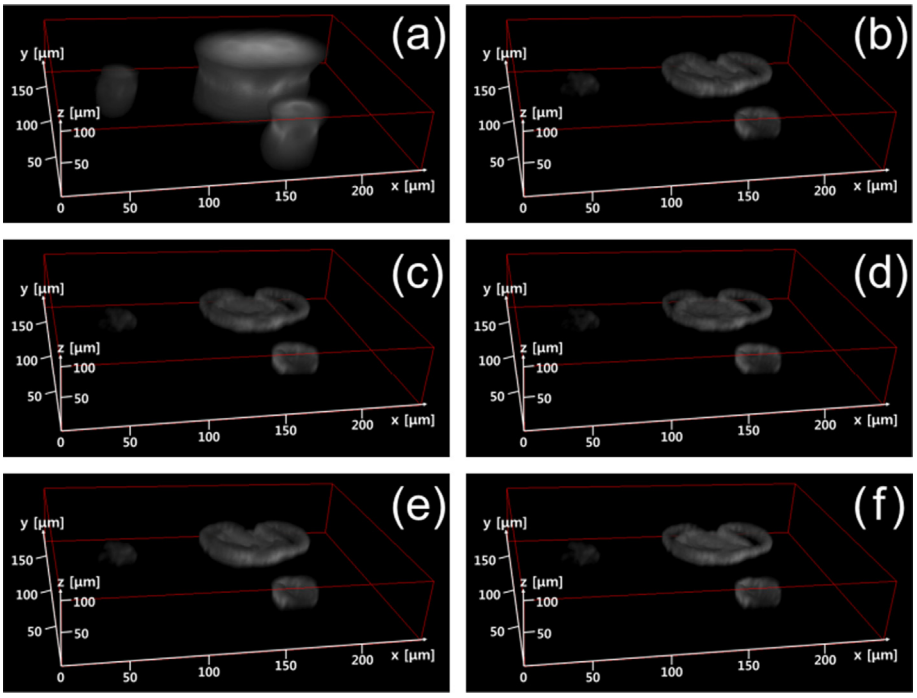

Fig. 4. SIM images of pollen grains: (a) acquired at a single tilt angle, (b) reconstructed by averaging four partial images (θ=0, π/4, π/2, and 3π/4), (c) acquired at a single tilt angle and notch-filtered, and (d) acquired at a single tilt angle, notch-filtered, and reconstructed with a subtractive approach.

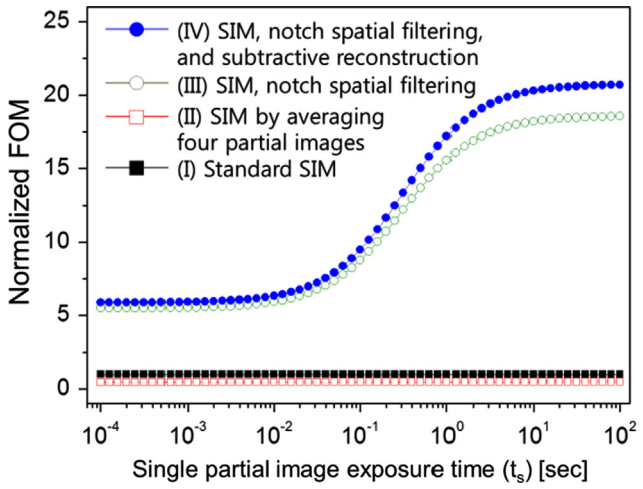

Fig. 5. FOM normalized by standard SIM with respect to the exposure time of a single partial image (t_s).

partial image (t_s) through t. Fig. 5 shows FOM for the four reconstruction schemes with respect to t_s. First of all, averaging of partial images with multiple tilt angles shows a much lower FOM compared to the reconstruction using a single tilt angle because of additional time consumption needed for both the partial image acquisition and the reconstruction. On the other hand, notch spatial filtering produces a remarkably higher FOM by more than 8.77 times compared to that of standard SIM. FOM in the case of using notch filters combined with a subtractive approach for axial resolution enhancements is also higher than that of standard SIM by more than 9.46 times, even higher by 8% than that of using only notch spatial filters, because of the enhancement in the axial resolution. This result indicates notch spatial filtering, if used in combination with subtractive reconstruction algorithm, may enhance overall image quality of SIM by almost 10 times with very little processing overhead.

4. Concluding remarks

In summary, we report reduction of grid pattern noise in SIM by application of notch spatial filters in the Fourier domain. The results based on wide-field and SIM images of pollen grains indicate that notch spatial filtering reduces pattern noise significantly by more than 18 times compared to that of standard SIM. Overall image quality can be enhanced by almost ten times in terms of a FOM when notch spatial filters are combined with subtractive image reconstruction. The approach is expected to provide a simple method of removing SIM image artifacts for various cell-based assays and can benefit other optical imaging methods with structural patterns such as confocal microscopy.

Acknowledgments

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