Adaptive illumination reduces photobleaching in structured illumination microscopy

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Abstract: Photobleaching is a major factor limiting the observation time in fluorescence microscopy. We achieve photobleaching reduction in structured illumination microscopy (SIM) by locally adjusting the illumination intensities according to the sample. Adaptive SIM is enabled by a digital micro-mirror device (DMD), which provides a projection of the grayscale illumination patterns. We demonstrate a reduction in photobleaching by a factor of three in adaptive SIM compared to the non-adaptive SIM based on a spot grid scanning approach. Our proof-of-principle experiments show great potential for DMD-based microscopes to become a more useful tool in live-cell SIM imaging.

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References and links
1. Introduction

The field of fluorescence microscopy experienced rapid development in the past two decades. A number of techniques that outperform standard widefield fluorescence microscope in terms of resolution were developed. These super-resolution methods can be roughly divided in three groups: localization microscopy, which relies on photo-switchable fluorophores and localization of single molecules [1–3], structured illumination microscopy, which employs a non-uniform illumination [4–9], and stimulated emission depletion microscopy (STED), which uses partial quenching of the fluorophores to narrow down the point spread function [10]. An ideal super-resolution fluorescence microscopy technique should allow biologists to observe live cells with improved resolution over extended periods of time. In reality, however, the resolution improvement is achieved at the cost of longer acquisition times, higher illumination intensities, or higher cumulative illumination doses. These conditions lead to enhanced photobleaching of the fluorophores and increased phototoxicity for the cells under study, which poses limitations on live-cell imaging. Therefore, deceleration of the photobleaching process is an important step for improvement of super-resolution microscopy.

In this work we address the issue of photobleaching reduction in Structured Illumination Microscopy (SIM). SIM is one of the most promising candidates for live-cell imaging amongst the super-resolution techniques [11–13]. It offers a rather modest resolution improvement of up to 2 × compared to standard widefield imaging (for linear SIM), however, it is compatible with most of the standard fluorescence dyes and requires less illumination light than localization.
Unlike widefield microscopy, where the sample illumination is uniform, in SIM the sample is exposed to a series of non-uniform illumination patterns. The spatial frequency spectrum of the illumination patterns is convolved with that of the sample, which makes the high spatial frequency components of the sample observable. The final high resolution SIM image is reconstructed from the entire series of acquired images corresponding to the different illumination patterns used. Besides the classic line grid patterns, speckle [15] and multi-spot [16] patterns have been applied in various realizations of the resolution-doubling SIM. We adopt here the nomenclature that is currently in vogue according to which "SIM" refers to the entire family of techniques that uses a plurality of images acquired using non-uniform illumination for achieving resolution improvement and/or optical sectioning. This family of techniques is broader than the classical line pattern based SIM.

In one of the SIM implementations a spatial light modulator (SLM) – it may be either a digital micro-mirror device (DMD) or a liquid-crystal-on-silicon SLM (LCOS-SLM) – is used for projection of the illumination patterns [16–18]. The SLM provides full spatial and temporal control over the illumination intensity. Although technically SLM also allows the projection of grayscale patterns, only binary SLM patterns have been used in SIM until now. Here we demonstrate how grayscale patterns can be used for reduction of the illumination intensity in DMD-based SIM microscopes.

The idea to reduce photobleaching and phototoxicity by locally adjusting the illumination intensity dose based on the sample structure was first implemented in confocal microscopy. An acoustic optical modulator was introduced into the illumination path of the confocal microscope to reduce the exposure time (and, hence, the illumination intensity) in the background and bright foreground areas of the sample [19,20]. Another implementation was made in the Programmable Array Microscope (PAM) [21], a microscope which is equipped with an SLM in its primary image plane. PAM has a double-pass optical configuration, which means that both excitation and emission light pass through the SLM [22,23]. Multiple SLM illumination patterns are projected onto the sample during one camera exposure time. By applying binary masks to the projected SLM patterns, one can reduce the exposure time in certain areas of the sample. Both confocal and PAM implementations provide optically sectioned images with reduced photobleaching. Additionally, variations of adaptive illumination were used for improving the sensitivity in two-photon microscopy [24] and for photobleaching reduction in STED microscopy [25].

We bring the idea of reducing the photobleaching by locally adjusting the illumination intensity one step further and apply it to the resolution-doubling SIM microscopy. In our custom-built DMD-based microscope multi-spot illumination patterns are used together with a maximum-likelihood estimation reconstruction [26]. In order to regulate the illumination intensity dose, the multi-spot illumination patterns are weighed with a grayscale mask, which is calculated from an initial widefield image. We describe two grayscale mask designs and demonstrate experimentally the corresponding reduction in photobleaching. Our proof-of-principle experiments were conducted on fixed cells as a first step towards live-cell SIM imaging with reduced photobleaching.

## 2. Principles of adaptive SIM

The problem of photobleaching in fluorescence microscopy can be addressed from two sides. At the stage of sample preparation, the robustness of fluorophores can be improved by suppressing their photobleaching pathways. This approach is fluorophore-specific and requires the understanding of photobleaching kinetics, which can easily become a rather complex problem [27]. Another way to address the issue is to reduce the overall illumination dose received by the sample. This approach is a universal measure that can be applied to all types of samples, regardless of the fluorescent dye and its photobleaching pathways. In this work we do not attempt to study
the photobleaching behavior of the fluorophores, but seek to improve SIM by making it less aggressive for biological samples in terms of the amount of illumination light.

In order to reduce the overall illumination dose one can locally adjust either the exposure time or the illumination intensity. In our implementation the exposure time remains uniform over the whole field of view, and the illumination intensity is adjusted by applying grayscale masks to the illumination patterns. Technically, this is performed by projecting grayscale images with the DMD. The intensities of the illumination patterns should have an inverse dependency on the sample brightness: the brighter the sample area, the lower the illumination intensity that will be applied to this area. Such an approach results in a more uniform signal-to-noise ratio (SNR) across the whole image (except for the background areas, where SNR = 0). In other words, SNR in bright regions of the image is sacrificed for the possibility to image the sample longer. SIM images with adaptive grayscale illumination patterns are acquired in the following way. First, a widefield snapshot is taken at low intensity. A grayscale mask is calculated from this widefield image according to one of the schemes that will be described in section 2.1. The standard binary multi-spot illumination patterns are weighed with this mask image, and the SIM data is acquired with the resulting grayscale multi-spot illumination. The final SIM image is reconstructed using the pattern-illuminated Fourier Ptychography (piFP) algorithm, which is equivalent to non-regularized linear least-squares [26, 28]. For each time point in time-lapse SIM imaging a new widefield image is taken and a new mask is calculated, in order to adapt the mask for possible changes and movements in the sample. A block diagram describing time-lapse adaptive SIM is shown in Fig. 1.

![Block diagram of time-lapse adaptive SIM](image)

### 2.1. Calculation of the illumination mask

Various schemes can be used to generate grayscale masks for the illumination patterns. One of the most straightforward ways is to use a linear relationship between the intensities in the widefield image $I$ and the weight of the grayscale mask $w$, as shown in Fig. 2(a). The areas of the widefield image with intensities lower than $I_{min}$ are identified as background, and the corresponding areas in the sample are not illuminated, i.e. weight of zero is applied in these areas. At the same time, lower limit for the weight $w_{min}$ has to be set in order to avoid insufficient illumination of the brightest areas of the sample, corresponding to areas in the widefield image with intensities higher than $I_{max}$. The upper and lower boundary values for the intensity and weight have to be set empirically for a given sample.

In reality the relationship between the illumination intensity and the photobleaching rate is often nonlinear. An improved method for calculating the grayscale mask takes this fact into account by establishing inverse proportionality between the intensities in the widefield image and the weight of the grayscale mask as shown in Fig. 2(b).
3. Materials and methods

3.1. Experimental setup

The key element of our home-built SIM microscope is the DMD (Vialux, Germany). The DMD supports projection of 8-bit grayscale images at 290 Hz frame rate. The device is placed in the secondary image plane of an inverted widefield Olympus IX71 microscope. Structured illumination is generated by projecting illumination patterns of the DMD onto the sample via a demagnifying optical relay. Details on the design and layout of the optical setup can be found in our earlier work [29]. Experiments were performed using a 60 × /0.7 air objective and 488/520 nm excitation and emission wavelengths. The sizes of the DMD pixel and the camera pixel back-projected to the sample plane are 137 nm and 108 nm, respectively.

3.2. Reconstruction algorithm

The illumination pattern at the DMD is generated as an array of spots arranged in a square grid with periodicity of 10 DMD pixels. This multi-spot pattern is translated with one DMD pixel per step and a camera frame is taken at each position of the pattern. The final SIM image is reconstructed from a dataset of 100 raw frames using the piFP algorithm, which is implemented in MATLAB (Mathworks, USA) in the same way as described in [26, 28]. The piFP algorithm retrieves the object by minimizing the difference between the unprocessed acquisitions and modeled images given the illumination patterns. The reconstructed object is therefore independent of the overall mask pattern since both the raw images and the modeled images scale with the mask pattern (up to a convolution with the microscope PSF). Therefore, no additional rescaling of the intensities in the reconstructed image is required, i.e. the flow of the original piFP algorithm does not change when the overall illumination is non-uniform. However, we have observed that the speed of convergence of the piFP algorithm decreases when non-uniform overall illumination is applied. Also, it may be anticipated that the local noise level in the reconstruction will vary with the mask pattern.

3.3. Mapping the DMD onto the camera

For adaptive SIM it is essential to establish precise mapping between the DMD chip and the camera chip. This mapping is done by projecting a multi-spot illumination pattern onto a thin (∼110 nm), spatially homogeneous fluorescent layer, which is produced as described in Ref. [30].
The resulting camera image contains an array of spots, each corresponding to a single DMD pixel. By extracting the coordinates of spots centers in the camera image we find the correspondence between DMD pixels and their image positions in the camera chip plane [29].

3.4. Sample preparation

Human Embryonic Kidney 293T (HEK 293T) cells were cultured on 100-mm plates (SARST-EDT) at 37 °C with 5% CO2 in Dulbecco’s modified Eagles medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO). At about 80% confluency the cells were washed once with phosphate-buffered saline (PBS, pH 7.4, GIBCO), treated with 0.25% trypsin-EDTA (Gibco) and collected in 1 mL of growth medium.

Methanol fixation and DNA staining were performed in the following way. Glass coverslips (#1.5, Menzel Glaser) were sterilized in 70% ethanol, washed with milli-Q water and dried. The coverslips were then incubated with 0.01% Poly-L-Lysine (PLL, Sigma Aldrich) in PBS for 5 minutes at room temperature. PLL-coated coverslips were placed at the bottom of a six-well plate and 50 µL of the HEK 293T cell suspension were added. After 24 hours culture, the growth medium was removed, the cells were washed once with PBS and incubated for 10 minutes at room temperature with 100% methanol. After methanol fixation, the solvent was removed and the cells were washed once with PBS. Fixed cells were then incubated for 30 minutes at 37 °C with 0.2 U/µL RNase ONE (Promega) to avoid background signal from labelled RNA and washed with PBS and Milli-Q water. DNA staining was performed by further incubating the cells at room temperature with PBS containing 0.1 µM SYTOX Green Nucleic Acid Stain (Thermofisher Scientific) for 20 minutes in darkness. The cells were finally rinsed with PBS and Milli-Q water. Fixed samples were glued overnight to microscope slides (76 x 26 mm, Menzel-Glaser) with aqueous mounting medium (Sigma Aldrich) at room temperature in darkness. Samples were stored protected from light for up to two weeks at 4 °C.

4. Experimental results

4.1. Photobleaching behavior of the fluorophore

In order to demonstrate the photobleaching behavior of the SYTOX stain in HEK 293T cells sample, we have acquired photobleaching curves in widefield mode at different illumination intensities. The number of acquisitions scaled inversely proportional to the applied illumination intensity, so that each curve corresponded to an equal cumulative illumination dose received by the sample. As can be seen from Fig. 3, the photobleaching rate of the SYTOX stain depends non-linearly on the illumination intensity. However, photobleaching curves corresponding to illumination intensities \( I < 10 \text{ W/cm}^2 \) largely coincide with each other, which indicates that the photobleaching rate scales linearly with the illumination intensity in this range.

We have performed standard non-adaptive multi-spot SIM using an average intensity of approximately 0.08 W/cm² per multi-spot pattern. The average intensity used in adaptive SIM varies between 0.025 – 0.05 W/cm². Hence, we have operated in the region of linear photobleaching behavior of the fluorophore.

4.2. Illumination mask examples

For the generation of the grayscale masks we have employed the designs described in section 2.1. Here we present three examples in the decreasing order of the illumination dose that they impose on the sample. The linear mask shown in Fig. 4(b) is calculated using the intensity normalized widefield image and the boundary values of \( I_{\text{min}} = 10\% \) and \( I_{\text{max}} = 85\% \). A lower illumination dose can be achieved with the same boundary values if the linear grayscale mask calculation is based on a normalized widefield image to which 15% percentile clipping is applied (Fig. 4(c)). Finally, the nonlinear grayscale mask shown in Fig. 4(d) results in the most light saving regime.
Photobleaching curves were acquired in a widefield mode at different illumination intensities on a sample containing HEK 293T cells in which the DNA was labeled with SYTOX green nucleic acid stain. In the region of illumination intensities $0 - 10 \text{ W/cm}^2$, photobleaching rate depends linearly on the illumination intensity. Additionally, a small self-quenching effect is observable at initial times due to the dense DNA labeling.

This mask is calculated according to the $\sim 1/I$ scheme, using an intensity normalized widefield image and $I_{\text{min}} = 10\%$ and $I_{\text{max}} = 85\%$ boundary values. The integrated sample irradiance in adaptive SIM amounts to only $\sim 8\%$ (for linear mask), $\sim 7\%$ (for linear mask with 15\% clipping), and $\sim 5\%$ (for nonlinear mask) of the integrated sample irradiance in the non-adaptive SIM.

Performances of the three masks were compared in the time-lapse imaging. In standard SIM the integrated intensity of the image is decreased by 64\% after 30 minutes of time-lapse imaging. When adaptive SIM is used, the integrated intensity is decreased by only 46\%, 35\% and 28\% for the masks shown in Fig. 4(b-d) respectively. As expected, a lower total illumination dose results in slower photobleaching of the sample. For our further experiments we have used the linear mask shown in Fig. 4(c) and the nonlinear mask shown in Fig. 4(d).

4.3. Image quality in adaptive SIM

In order to test the feasibility of the proposed method, we have compared adaptive SIM images to standard SIM images acquired under equal imaging conditions. The linear grayscale mask shown in Fig. 4(c) was used as a weight for the multi-spot illumination patterns. The peak intensity of the illumination is equal in adaptive and non-adaptive SIM.

The reconstructed adaptive SIM image bears a strong visual resemblance to the standard SIM image, as can be seen in Fig. 5(a). This visual resemblance is confirmed numerically by the structural similarity index $ssim$ [31]. The $ssim$ index between the standard and adaptive SIM equals 0.97, whereas the $ssim$ index between two repeated standard SIM images equals 0.99. A decrease of 0.02 in $ssim$ index roughly corresponds to adding Gaussian noise with a standard deviation of 0.5\% of the maximum pixel value to the image.

The sum of all acquired images in the standard SIM dataset results in the widefield image, which confirms the uniformity of the overall illumination; whereas the sum of all acquired images in adaptive SIM dataset results in an image with a more uniform signal level, revealing the non-uniform overall illumination in this case. Moreover, the sum of all the acquired images is substantially less bright in adaptive SIM compared to the standard SIM, which indicates the reduction in overall illumination intensity specific to the adaptive SIM (see Fig. 5(b)). Examples of standard and adaptive illumination patterns are given in Fig. 5(c) for comparison.
4.4. Photobleaching studies

The reduction in photobleaching was quantitatively assessed in time-lapse experiments. For each of the three imaging methods (standard SIM, adaptive SIM with linear mask and adaptive SIM with nonlinear mask) five separate sample areas were imaged over 30 minutes. During this time 60 SIM acquisitions were taken, with each SIM acquisition consisting out of 100 raw frames corresponding to the 100 multi-spot illumination patterns. The integrated image intensity per SIM acquisition is decreasing with time due to photobleaching of the fluorophores. The resulting photobleaching curves for the three methods are shown in Fig. 6 to demonstrate the improvement in photobleaching reduction achieved by applying adaptive grayscale illumination patterns. In standard SIM the integrated image intensity is reduced by 35% after 20 acquisitions, whereas in adaptive SIM with the linear mask design it takes 60 acquisitions to induce the same bleaching. Application of the nonlinear mask results in further improvements. Please note that the really dark areas of the cells bleach at the same rate in both SIM modes, because in our implementation illumination patterns for adaptive and non-adaptive SIM have equal maximum intensity value. The average intensity for standard non-adaptive multi-spot SIM was $\sim 0.08 \text{ W/cm}^2$ per multi-spot pattern and the average intensity used in adaptive SIM varied between 0.025 - 0.05 W/cm².

The reduced illumination dose in first instance is a local effect due to the spatially dependent mask and does influence bright and dark areas differently. In applications where only a small part of the fluorescent image is relevant for analysis, the overall improvement of photobleaching might not be the appropriate measure. For imaging of cellular structure and shape analysis, however,
Fig. 5. Comparison of standard and adaptive SIM imaging modalities. (a) Visual similarity of the adaptive and standard SIM images acquired under equal imaging conditions. Since the peak intensity of the illumination in adaptive and non-adaptive SIM is the same, adaptive SIM reconstruction has a similar range of intensities as the standard SIM reconstruction. (b) The sum of 100 raw images in standard SIM results in a widefield image, whereas the sum of 100 raw images in adaptive SIM results in an image with a more unified signal level. (c) Examples of the illumination patterns for the standard and adaptive SIM. Illumination patterns for the adaptive SIM are produced by multiplying the standard SIM illumination patterns with the mask shown in Fig. 4 (c).

the integrated intensity determines is a useful measure for the merit of adaptive illumination.

Fig. 6. Comparison of photobleaching in standard and adaptive SIM. Each curve shows the average of 5 measurements on separate sample areas and the error bars indicate the standard deviations over 5 measurements. Adaptive SIM enables at least three times longer imaging than the standard SIM.

Examples of the reconstructed images for the three considered methods are given in Fig. 7.
Time-lapse reconstructions for adaptive SIM show only small reduction in the image brightness, unlike the reconstructions for standard SIM, in which the brightness is progressively reduced during the 30 minutes imaging time. The SIM images were reconstructed using 6 iterations of the piFP algorithm in the case of standard SIM and 40 iterations of the piFP algorithm in the case of adaptive SIM. Since the comparison of photobleaching in adaptive and non-adaptive SIM was performed on the fixed cells, we have taken only one widefield image per time-lapse series. For dynamic samples that change sufficiently slow the SIM reconstruction at a certain time point could be used to generate the mask pattern for the subsequent set of acquisitions.

Fig. 7. Comparison of the photobleaching induced by 30 minutes of time-lapse imaging in standard and adaptive SIM on HEK 293T cells, in which DNA is labelled with SYTOX stain. (a) First acquisition, (b) 20\textsuperscript{th} acquisition (10 min), (c) 40\textsuperscript{th} acquisition (20 min), (d) 60\textsuperscript{th} acquisition (30 min). Adaptive SIM leads to deceleration of the photobleaching, enabling longer observation time of the sample. Intensities are comparable over all images.
4.5. The influence of out-of-focus light

The sample under study has a 3D structure, and the fluorophores located outside the focal plane can be excited when the illumination pattern is projected onto the sample. In order to investigate the impact of the photons emanating from the out-of-focus planes on the measured photobleaching curves, we have applied pinholing to the acquired raw image data. The pinholing is done by multiplying each of the raw frames of the SIM acquisition with the corresponding illumination pattern, and leads to suppression of out-of-focus light. The difference between the photobleaching curves obtained from the original and the pinholed data is below 2% of the original integrated intensity. Therefore, we conclude that the same reduction in photobleaching is achieved in and out of focus, since cumulative light dose is equal in and out of focus and the bleaching rate scales linearly with the illumination intensity for the used range of intensities.

5. Conclusion

We have demonstrated the use of grayscale illumination patterns of the DMD for lowering photobleaching in SIM. The overall illumination dose in SIM was reduced by adapting the grayscale illumination patterns to the sample structure.

A reduced illumination dose inevitably leads to a reduced SNR in the acquired images. However, the final reconstructions in adaptive SIM do not display significant degradation of the image quality compared to standard SIM. We have used the ssim measure in order to test their similarity. The degree of image deterioration in adaptive SIM is equal to adding Gaussian noise with a standard deviation of 0.5% of the maximum pixel value to the standard SIM reconstruction.

We considered two schemes for the generation of adaptive patterns: with a linear and a nonlinear dependence of the grayscale mask on the initial widefield intensity. The performance of adaptive SIM with both schemes was compared to standard SIM in time-lapse experiments on HEK 293T cells with SYTOX-stained DNA. The imaging time in adaptive SIM can be at least three times longer than in standard SIM because of the reduction in photobleaching. The nonlinear scheme for adaptive patterns generation provides a slightly bigger improvement than the linear scheme.

The degree of improvement achieved by adaptive SIM strongly depends on the sample structure and the fluorophore that is used. Adaptive SIM is expected to provide even bigger photobleaching reduction when applied in 3D imaging due to the generally higher sparsity of the sample in 3D than in 2D. A fast 3D mask could be computed in the following way without the need to make a slow 3D SIM scan. Acquisition of a stack of widefield images at different defocus levels, followed by a deconvolution will effectively remove out-of-focus blur and then a mask can be computed in analogy to 2D.

Moreover, the types of masks that are used to create adaptive illumination patterns may vary for different sample types. In order to support adaptive SIM imaging for a broad spectrum of sample types, various strategies for generation of the adaptive patterns have to be developed. For example, a mask based on a variance filter can be used to image samples with multiple nearly uniform objects or to study processes occurring at the border of such objects.

A next step along the lines of the currently proposed technique is the extension to classical line pattern based SIM. This would have the benefit of requiring a lower number of raw images for a single reconstruction. Although it is possible to generate modulated line patterns with a fine pitch using the DMD, the imaging step from DMD to object entails a certain degree of blurring that compromises the modulation depth of the line patterns. This could in principle be solved with a spatial filtering technique but this may prove to be incompatible with the modulation of the mask pattern.

Finally, one of the potential pitfalls of adaptive SIM is non-uniform bleaching of the sample. Since the overall illumination in adaptive SIM is not uniform, the areas of the sample that receive a higher intensity will bleach faster than the other areas. Hence, adaptive SIM has to be applied
cautiously in applications where precise quantitative analysis of the images is required.

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