TECHNICAL COMMENT

ADVANCED IMAGING

Comment on "Extended-resolution structured illumination imaging of endocytic and cytoskeletal dynamics"

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Li *et al*. (Research Articles, 28 August 2015, aab3500) purport to present solutions to long-standing challenges in live-cell microscopy, reporting relatively fast acquisition times in conjunction with improved image resolution. We question the methods' reliability to visualize specimen features at sub–100-nanometer scales, because the mandatory mathematical processing of the recorded data leads to artifacts that are either difficult or impossible to disentangle from real features. We are also concerned about the chosen approach of subjectively comparing images from different super-resolution methods, as opposed to using quantitative measures.

Li *et al.* (*1*) extend structured illumination microscopy (SIM) (*2*, *3*) by exploiting a higher numerical aperture (NA) lens and using standing light waves to switch reversibly switchable fluorescent proteins (RSFPs). The patterned on-switching of RSFPs yields a spatially controlled distribution of fluorophores in the fluorescent "on" state, adding spatial frequencies of higher order to the fluorescence emission. These higher-order frequencies improve SIM resolution, provided that they can be disentangled from the low-frequency signals. According to Li *et al.*, even moderate illumination intensities lead to higher frequencies of enhanced amplitudes, making their method more applicable to living cells than other super-resolution approaches. Employing Skylan-NS, an apparently quite photostable—but undisclosed—RSFP, recordings of a few tens of images were made. The resolution was reportedly improved to nominally 84 nm by SIM with an NA 1.7 lens and improved to nominally 62 nm when performing the RSFP switching with sinusoidal on-switching and readout patterns. By saturating the on-switching of the fluorescent proteins, Li *et al.* extended the resolution to nominally 45 nm.

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In this latter approach, which Li *et al.* call saturated (S) patterned-activation (PA) nonlinear (NL) SIM, fluorophores are first transferred from an inactive ("off") to an active ("on") state, preparing them in the inactive state at a set of positions defined by the minima of the activation light. This is followed by saturated readout (*4*)—first patterned, then uniform—which returns the active fluorophores to the nonfluorescent "off" state. Both the activation and the readout steps thus spatially confine a defined state by exploiting a reversible state transition as described within the reversible saturable/switchable optical (fluorescence) transitions (RESOLFT) concept (*5*–*11*). In a new twist, the activation and readout light patterns employed by Li *et al.* feature minima at the same positions. For much of their work, Li *et al.* moved to nonsaturated activation and only moderately saturated readout in an acquisition sequence termed PA NL-SIM.

Although not new, the application of standing wave illumination for reversible photo-switching (*5*–*11*) is a promising direction, which had been previously pursued also by Gustafsson (*12*). In these methods (*1*, *12*), making images requires mathematical reconstruction from the raw data in the presence of noise. Such reconstructions introduce artifacts that were not acknowledged by Li *et al.* This is of particular concern when imaging small, intricate structures located at or near extended objects generating a strong low-frequency signal. Compare Fig. 1A and B (data from figures S31 and 3A of Li *et al.*), where filaments of actin in dimmer image regions appear to be periodically fragmented. Structures imaged by Li *et al.* with (S)PA NL-SIM appear continuous in large-scale overviews. Yet the periodic artifacts may manifest as punctate or thin-segment features sticking out of or showing up parallel to a genuine structure. This hampers the interpretation of details. For example, a characterization of genuinely periodic features would be difficult. Zooming in on a further actin image example (Fig. 1C; taken from Fig. 3B of Li *et al.*) uncovers problems at the very spatial scale for which super-resolution methods are intended. The effects are less easily noticeable for brighter features. Even there, however, they frequently create the illusion of periodic patterns (Fig. 1D; data from figure S17 of Li *et al.*).

The raw data generated by (S)PA NL-SIM contains a dominant low-resolution component that superposes the weak contributions of aliased higher-resolution components. Due to noise from all components, we deem it challenging to differentiate and reassemble them reliably, despite the beneficial two-step readout in (S)PA NL-SIM. The recovery of the high-resolution information therefore requires high signal-to-noise ratios (SNRs) in the raw images and a sophisticated analysis.

We inferred by a spatial frequency analysis of the published data that the obvious artifacts are related to the five-phase/five-orientation PA NL-SIM raw data acquisition (compare the PA NL-SIM optical transfer function (OTF) shown in green in Fig. 1E). This acquisition sequence leaves its clear signature: The two-dimensional (2D) spatial frequency ($\boldsymbol{k}$) spectrum (Fig. 1F) taken from an image region (Fig. 1G, reconstructed image provided by Li *et al.*) reveals a 10-fold symmetric amplitude enhancement at a $|\boldsymbol{k}|$ of the first harmonic k_1 of PA NL-SIM. This reflects the presence of periodic patterns at spatial frequency ~k_1, which has no real-space match in the imaged actin filaments. Filtering the image by limiting the frequencies to $|\boldsymbol{k}| < 10\ \mu m^{-1}$ does not fully remove the artifacts (Fig. 1, H and I). Limiting to $|\boldsymbol{k}| < 5\ \mu m^{-1}$ avoids the spurious patterns, yet at the loss of high-resolution information (Fig. 1, J and K). Thus, we could link the patterns' source directly to the spectral region around k_1. We note that the sought-after high-resolution information is encoded in components with $|\boldsymbol{k}| > k_1$. Whether dedicated filtering or weighting in reciprocal space could attenuate the problematic frequency components—and still make use of the higher frequencies (i.e., of higher resolution)—should be a matter of further investigation. In any case, the peaked shape of the (S)PA NL-SIM OTF causes artifacts. Such reconstruction challenges still persist after many years of SIM development [see also the artifacts in (*12*)]. This is because the phase noise in the spatial frequency spectrum critically affects the reconstruction and is difficult to eliminate.

Unfortunately, all resolution values reported by Li *et al.* are asserted on the basis of frequency-space arguments, lacking any demonstration in real space. All resolutions stated are "theoretical" (figure S6 in Li *et al.*), and no evaluations are presented as to what extent the claims are supported by the data. Instead, inadequate comparisons with other investigators' work (e.g., figures S6 and S31 in Li *et al.*) are shown. We are led to speculate that the presence of high-frequency artifacts hampered the experimental verification of the resolution claims. Understandably, small details can be created in the reconstructions of Li *et al.*, but at the risk of misrepresentation.

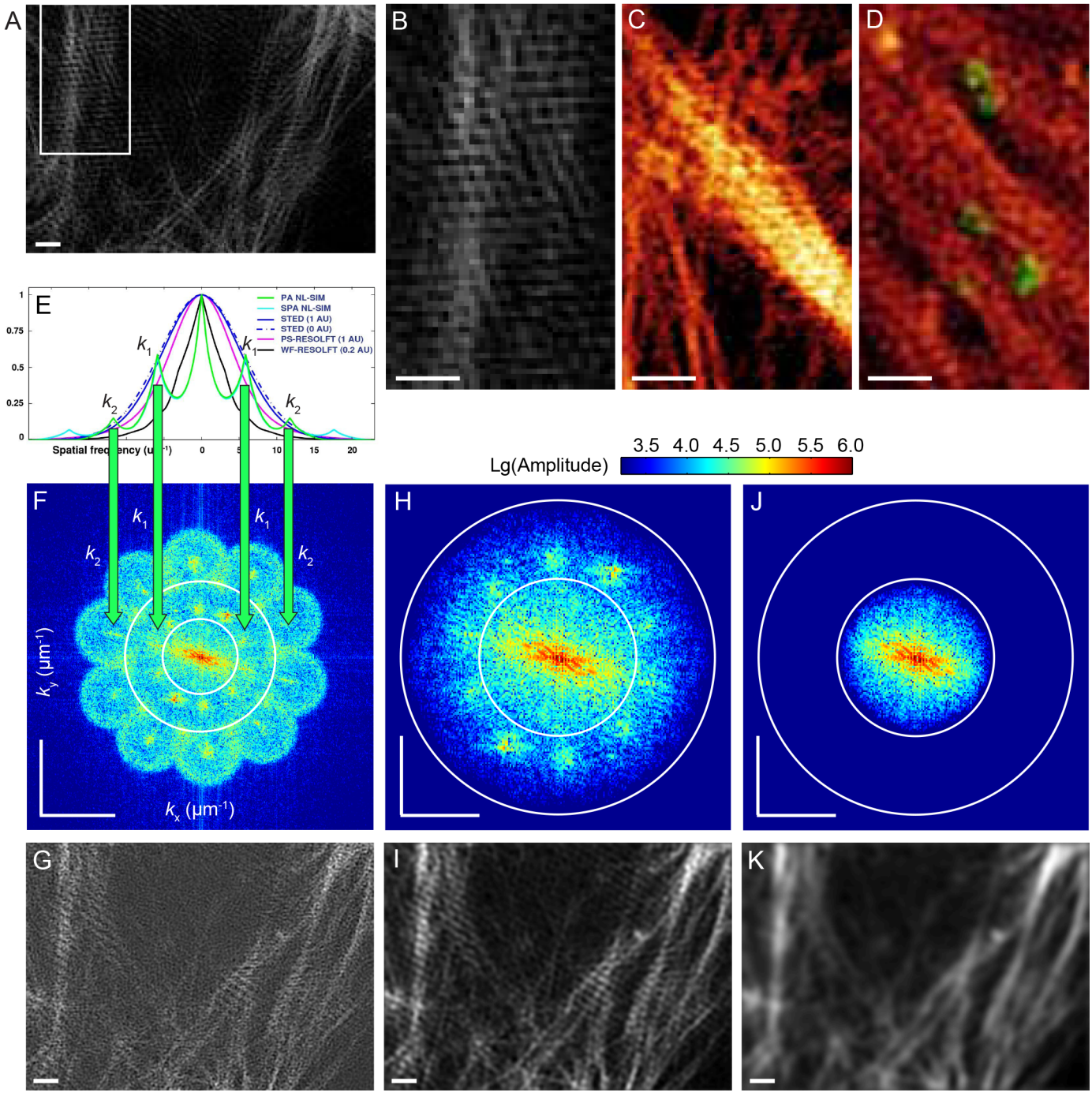

Fig. 1. Artifacts in (S)PA NL-SIM extended-resolution methods. Image details recorded by (S)PA NL-SIM exhibit conspicuous patterns, gaps, and other spurious spatial structures. These patterns do not originate from incomplete fluorescent labeling but are artifactual and inherent to the current implementations of the methods. (**A** to **D**) Magnified image regions from figures published by Li *et al*. (*1*). (A) Figure S31 (same region as in figure 3A). (B) Enlarged view of region in the white rectangle in (A). (C) Figure 3B. (D) Figure S17. (**E**) Optical transfer functions of several imaging methods, taken from figure S24 (Li *et al*.). (**F**) Amplitude of the 2D Fourier transform of the region shown in (A), which reveals enhancements of 10-fold symmetry in $\boldsymbol{k}$ space. This occurs at frequencies that correspond to the secondary and tertiary peaks of the OTF [green arrows from (E)]. (**G**) Original data of the region shown in (A) (unthresholded, linear colormap) used for the calculation of (F) (kindly provided by Li *et al*.). The insufficient attenuation of the problematic frequency components visible in (F) is directly responsible for artifactual spatial image patterns. This is illustrated by low-pass filtering the original image (G): leaving only the components below the second harmonics ($|\boldsymbol{k}| < 10\ \mu m^{-1}$), shown in (**H**), still contains the artifacts (**I**); further filtering to exclude the first harmonics ($|\boldsymbol{k}| < 5\ \mu m^{-1}$), shown in (**J**), removes the spurious patterns (**K**), but also comes with a loss of resolution. A Lanczos window with hard edges at the white circles was applied in the retransforms [(H) to (K)] to avoid the undesired introduction of additional high-frequency structure. Scale bars [(A) to (D) and (G), (I), and (K)]: 500 nm. Scale lines represent 10 μm^{-1} (F) and 5 μm^{-1} [(H) and (J)]. The circles' radii in (F), (H), and (J) correspond to 5 μm^{-1} and 10 μm^{-1}, respectively.

RESOLFT microscopy had previously also been implemented with the intensity minima leaving emitters confined in the "off" state (*13*, *14*). However, in the works using photoswitchable proteins (*8*–*12*), the strategy was to spatially confine the "on" state rather than the "off" state, because the image is then directly obtained from the raw data in real space. If sufficient "on-off" state contrast is achieved, the signals are read out without much contribution from (off-state) fluorophores located outside the targeted feature. The image consists mainly of high-resolution signal. Therefore, there is no strict minimal signal required to maintain correct spatial assignment, even down to the regime of a few registered photons.

Li *et al.* state that RESOLFT and stimulated emission depletion (STED) nanoscopy are "ill-equipped to study live-cell dynamics noninvasively." However, fast imaging, to date mostly by STED nanoscopy, has been used to record various structures quickly and repeatedly in living cells and has, for example, recently revealed genuinely periodic actin in living neurons (*15*). Moreover, dendritic spines in living brain slices have been followed over hours by RESOLFT nanoscopy (*16*).

Several aspects of Li *et al.*'s analysis of RESOLFT nanoscopy do not reflect the state of the art. For typical RSFPs, the switching behavior between inactive and active states is not well modeled by the $1/(1 + I/I_s)$ scaling used by Li *et al.* (with I the applied intensity and I_s the characteristic switching intensity), but better described by a (multi) exponential dependence (*17*). Li *et al.* model the RESOLFT readout at saturated levels, which also unnecessarily implies high levels of illumination and is typically avoided to better preserve the contrast between "on" and "off" states (*17*). Li *et al.*'s assessment of RESOLFT and STED nanoscopy does not seem to vary the applied intensity with the desired resolution (compare figure S2 of Li *et al.*). Tuning of resolution by varying the intensity is, however, a key benefit of RESOLFT, STED, and similar methods.

Li *et al.* emphasize that they achieve the claimed resolution gains "even at low activation and excitation saturation factors," yet it remains unclear as to what extent this can be relied on in practice. Adjusting resolution gains by applying lower intensities is routinely done in RESOLFT and STED nanoscopy in favor of increasing the number of images taken before bleaching. A lower-end choice of intensity entails less photodamage and readily delivers resolution in the regime claimed to be achieved by Li *et al.*

Although lower light levels are desirable by definition, and Li *et al.* are correct in pointing out this issue, it is worth noting that microscopy illumination is virtually always at unnaturally high light levels. Light-induced effects are wavelength-dependent, and they are often acceptable as long as they do not distort the sought-after information. Furthermore, mere comparisons of absolute photon numbers and deduced "photon efficiency" are questionable without considering sample absorption and creation of phototoxic molecular species.

The potential of using switchable fluorescent proteins for realizing low-light-level super-resolution was already pointed out in 2003 (*5*–*7*) and fervently advocated since then, also for structured illumination (*5*–*7*, *9*). Since then, it has been clear that RSFPs can provide super-resolution at low photon fluxes (*5*–*11*) due to their long-lived active and inactive states. This has now been reconfirmed by Li *et al.* In fact, the identification of fluorophore states and the implementation of selected "on-off" state transitions have always been at least as essential for providing super-resolution as the optical system in use.

Although Li *et al.* have added a valid new twist to the RESOLFT concept, their work again shows that the actual limitations lie in the properties of the fluorophores. As enough factors already complicate the rigorous analysis of biological systems, new super-resolution variants should produce data that can be interpreted with high levels of confidence.

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