Super-resolution imaging of multiple cells by optimized flat-field epi-illumination

Kyle M. Douglass*, Christian Sieben, Anna Archetti, Ambroise Lambert and Suziana Manley*

Biological processes are inherently multi-scale, and supramolecular complexes at the nanoscale determine changes at the cellular scale and beyond. Single-molecule localization microscopy (SMLM)1–3 techniques have been established as important tools for studying cellular features with resolutions of the order of around 10 nm. However, in their current form these modalities are limited by a highly constrained field of view (FOV) and field-dependent image resolution. Here, we develop a low-cost microlens array (MLA)-based epi-illumination system—flat illumination for field-independent imaging (FIFI)—that can efficiently and homogeneously perform simultaneous imaging of multiple cells with nanoscale resolution. The optical principle of FIFI, which is an extension of the Köhler integrator, is further elucidated and modelled with a new, free simulation package. We demonstrate FIFI’s capabilities by imaging multiple COS-7 and bacteria cells in 100 × 100 µm² SMLM images—more than quadrupling the size of a typical FOV and producing near-gigapixel-sized images of uniformly high quality.

The development of super-resolution fluorescence techniques heralded a dramatic improvement over traditional wide-field and confocal microscopies by enabling resolutions that surpass the diffraction limit. When considering how excitation light is delivered to the sample in these techniques, two main strategies emerge. The first, which is applied in structured illumination microscopy (SIM)4,5, stimulated emission depletion (STED)6 and reversible saturable optical linear fluorescence transitions (RESOLFT)7,8, uses spatially patterned excitation light to control the photophysical states of fluorophores in a targeted way across a FOV. Alternatively, SMLM methods such as stochastic optical reconstruction microscopy (STORM) and (fluorescence) photoactivated localization microscopy ((f)PALM)9–11, typically use a simpler strategy: wide-field laser epi-illumination. In this geometry, the sample is illuminated with a laser beam that possesses a Gaussian-like irradiance profile of a few tens of micrometres in diameter. SMLM relies on the stochastic on-and-off switching of fluorophores, generating pointillist-like images of labelled structures with resolutions that depend on the density of the detected fluorophore positions—commonly referred to as localizations—and on the corresponding precision of the position estimates9. To achieve temporal separation of single-molecule emissions, such as those from commonly used synthetic dyes, relatively large laser irradiances are delivered to the sample to cycle fluorophores from emitting to long-lived, non-emitting states10.

In practice, the size and quality of an SMLM image is severely limited by several factors that relate to the illumination. Wide-field epi-illumination is achieved by focusing the Gaussian excitation beam to the centre of the objective’s back focal plane (BFP) (Supplementary Fig. 1). The resulting size of the illuminated spot on the sample is proportional to the numerical aperture (NA) of the input beam, which is restricted by apertures in the microscope’s mechanical housing. Additionally, SMLM image quality drops substantially and artefacts appear in the periphery of the illuminated region where the minimum irradiance requirement of 1–10 kW cm⁻² is not satisfied (Fig. 1a,b)11–13. This is because the density of the emitting molecules is too high to allow for precise and accurate localization in these regions, therefore violating a basic assumption of SMLM reconstruction algorithms (Supplementary Section 4). On the other hand, in regions where the irradiance is too high, the acquisition time becomes longer than necessary because too few molecules are emitting at any given time. The result is a field-dependent density of detected localizations (Fig. 1b,c), localization precisions and goodness of fit (Supplementary Figs 2 and 3). The illumination is therefore critical in determining the quality and final resolution of a large-FOV SMLM image (Supplementary Sections 4 and 5).

By contrast, a flat irradiance profile on the sample can produce a uniform localization density, precision and single-molecule fit quality across the FOV (Fig. 1c,d and Supplementary Fig. 2). Conceptually, this occurs because the optical power in the regions where the irradiance is too high in the Gaussian beam is redistributed to the periphery, where it is too low. Additionally, the optimum irradiance required to perform SMLM imaging is ultimately set by the highest local density of fluorophores on the structures to be imaged12 (Supplementary Sections 4 and 5). A flat profile that is adjusted to this irradiance will always span a larger area than a Gaussian beam of the same power (Fig. 1e), thereby producing larger images and increasing the throughput of the measurement.

We developed FIFI to efficiently achieve a large and uniform illumination pattern in laser fluorescence microscopes. FIFI is an analogue beam-shaping epi-illumination system and extends the Köhler integrator14–16 to SMLM (Fig. 2). In this system, a collimated beam is launched into a telescope whose lenses are spaced by the sum of their focal lengths. A high-efficiency diffuser with average grain size on the order of 10 × 10 µm² that is rotating at approximately 2,000 revolutions per minute is placed at an offset distance Δr from their common focal planes. This offset determines the size of the beam on the diffuser and therefore the spatial coherence of the source. The beam is collected by the second telescope lens (focal length f₂) before encountering the primary components of the system: two MLAs consisting of identical spherical lenslets. The first MLA splits the different field angles in the incoming partially coherent laser beam into independently propagating beamlets that travel parallel to one another through the system. The lenslets in the second MLA, which is located exactly one lenslet focal length after the first, cancel the quadratic phase curvature imparted by the first MLA17. The lenslets in the second MLA also image each lenslet in the first array to infinity. The beamlets are finally focused through the infinity-corrected objective lens onto the sample plane where the images of the individual lenslets in the first array are overlapped. The ultimate effect is to illuminate the sample with a uniform irradiance pattern by averaging out heterogeneities in the input laser beam. Compared...
with other beam homogenizers, FIFI is easy to align, efficient, wavelength-insensitive and features a continuously variable illumination size (Supplementary Section 3).

A geometrical optics design is sufficient to implement the system into the laser epi-illumination path of a low magnification system such as a flow cytometer. However, the stringent requirements imposed by SMLM and the number of free parameters (Fig. 2) make the design of a FIFI system for super-resolution non-trivial. To overcome this difficulty, we developed a wave optics simulation platform that extends previous applications of scalar diffraction theory applied to MLAs and specifically accounts for the partial coherence and optical path through a high magnification fluorescence.

Figure 1 | Wide-field epi-illumination is not optimized for SMLM imaging of large FOVs. a, Gaussian-illuminated SMLM image of microtubules in COS-7 cells with antibodies against α-tubulin (AlexaFluor 647) displays a non-uniform image quality. b, Highlighted regions in a show variation in the localization density and artefacts in the periphery. c, Number of detected fluorescent molecules under Gaussian and flattened illumination patterns of the same power. Data are from ten combined FOVs, each of COS-7 microtubules spanning the full FOV. d, Radially averaged densities of detected molecules for the images in c. e, Profiles of three different Gaussian illumination patterns (the full widths at half maximum are 46, 56 and 80 μm for the pink, orange and green curves, respectively) and the diagonal of a 100 × 100 μm² flat-top pattern, all carrying 100 mW of power. The flat-top pattern produces the largest SMLM images without field-dependent fluorophore blinking. The black arrow denotes the approximate minimum required irradiance for performing SMLM. Scale bars, 20 μm (a,c) and 1 μm (b).

Figure 2 | Layout and design parameters for FIFI. An input Gaussian beam is launched into a telescope with focusing lens F₁ and collimating lens F_c. A rotating diffuser (RD) is offset from their shared focal plane, creating an extended source whose size depends on the offset Δr. Light from F_c propagates a distance L₁ to identical microlens arrays (MLAs) with periodicities p separated by one lenslet focal length f_{MLA}. The MLAs create independent ray bundles that propagate another distance L₂ and are redirected by the objective (OBJ) such that they overlap at the sample plane (S). Each illuminated point in the sample plane receives light from every point and from a range of solid angles in the source plane (solid versus dashed rays). Scalar fields u calculated in the simulations are marked with black dashed lines. f₁, f_c and f_{OBJ}, focal lengths of corresponding thin lenses; D_{MLA}, D_{BFP}, aperture sizes.
Figure 3 | Simulated 1D sample plane irradiance profiles through the centre of the illuminated region demonstrate the strong dependence of the illumination homogeneity on the design parameters. a, The offset of the rotating diffuser $\Delta r$ has an optimum position that reduces the modulation in the grating pattern and prevents overfilling the MLAs. b, The objective lens focal length $f_{OBJ}$ largely determines the size of the sample plane irradiance. c, The focal length of the collimating lens $f_c$ for the partially coherent source controls both the field homogeneity and the spatial coherence of the source, as evidenced by the modulation depth of the grating fringes. d, The distance between the second MLA and the objective ($L_2$) must be small to prevent overfilling of the objective.

Figure 4 | Uniform, large FOV STORM imaging of multiple eukaryotic cells or hundreds of bacterial cells in a single image. a, Dual-colour STORM image of COS-7 cells labelled with antibodies against $\alpha$-tubulin (AlexaFluor 647, green) and TOM20 (AlexaFluor 750, red). b, STORM image of C. crescentus labelled with wheat germ agglutinin (AlexaFluor 647). c, d, Magnified views of the regions enclosed by coloured boxes in a, b. e, f, Pixel intensities through marked lines in c, d showing the uniform resolution. Red curves are fits to two Gaussian curves. Scale bars, 10 $\mu$m (a, b) and 1 $\mu$m (c, d).
microscope. The simulations enabled us to identify the combination of design parameters that maximizes the two primary requirements of SMLM: the illumination uniformity and power throughput of the system (Fig. 3 and Supplementary Figs 4 and 5). For example, the diffuser must be offset from the telescope focal plane to reduce non-uniformities due to grating diffraction from the MLAs, but moving it too far from the focal plane results in overfilling of the objective back aperture and in a loss of both uniformity (Fig. 3a) and power (Supplementary Fig. 5a). The objective focal length and BFP diameter also have considerable impacts on the sample space illumination area, uniformity and transmitted power (Fig. 3b and Supplementary Fig. 5b). Additionally, \( f \) has an optimal value (Fig. 3c and Supplementary Fig. 5c) and the distance between the second MLA and the objective must be made as small as possible while still allowing for the insertion of dichroic filters (Fig. 3d and Supplementary Fig. 5d). Our simulation platform is therefore a non-trivial and important tool for navigating this rich parameter space.

We built a FIFI system into a STORM microscope with an scientific complementary metal-oxide semiconductor (scCMOS) camera (Supplementary Fig. 6 and Methods). The fluorescence profiles excited by the three lasers in a concentrated dye solution demonstrate the good uniformity, the large size of the illuminated area and the accuracy of the simulations (Supplementary Figs 7–9). Curvature in the dichroic and/or the introduction of a weak defocusing lens can help smooth any residual non-uniformities, better enabling wide-field measurements that require uniform illumination (Supplementary Figs 10–12). In practice, a variation of a few per cent in the illumination has no measurable effect on the STORM data when the diffuser is optimally placed (Fig. 1c, d and 4). Furthermore, the illumination can be continuously resized simply by adjusting the MLA spacing (Supplementary Fig. 13). The transmitted power at the objective’s BFP is about 70% for both lasers, with the diffuser and the MLAs each accounting for between 5% and 10% of the losses (Supplementary Table 2). (The remaining losses are due to the dichroics, mirrors and lenses.) The FOV, which corresponds to the area of homogeneous illumination onto the sample, is 100 \( \times \) 100 \( \mu \)m\(^2\), which is approximately 5–10 times that of traditional wide-field SMLM. The throughput is further improved when combined with the high frame rates of scCMOS cameras.

The system is capable of performing STORM on multiple eukaryotic cells (1–3 COS-7 cells) or on a large number of bacteria (\( \sim 400 \)) in a single image (Fig. 4 and Supplementary Figs 14–17). The uniformly high quality is verified by line profiles of the pixel intensities, which show double-walled microtubules and bacterial stalk appendages of sizes that are consistent with reported values. For bacteria, the fast acquisition time and large FOV (3.3 min per FOV) mean that variability in the cell shape or protein localization can now be studied simultaneously at both the nanometre and population scales.

We have demonstrated that the optimized FIFI system enables uniform, high-quality wide-field super-resolution fluorescence imaging. Importantly, we increased the throughput of SMLM by capturing multiple eukaryotic cells or hundreds of bacteria in each FOV, simultaneously resolving features that are smaller than the optical diffraction limit. The key design requirements are a uniform illumination and sufficient power delivered to the sample to induce fluorophore switching over a large area. Beyond imaging a larger FOV, FIFI improves the quality of the images by substantially reducing the position-dependent photophysics that result in a non-uniform resolution. FIFI is robust to misalignment and vibrations, and therefore serves as a simple, low-cost solution for fully exploiting the large sensor formats of new scCMOS cameras for SMLM.

**Methods**

Methods and any associated references are available in the online version of the paper.
The localizations from these images were combined across all ten FOVs, yielding exposure times and 1,000 mW of laser light at a wavelength of 642 nm, and care was taken (AC254-250-B, Thorlabs). For each FOV 25,000 frames were recorded at 10 ms against mouse (AlexaFluor 647-conjugate, A21237, Life Technologies) and rabbit PBS supplemented with 0.2% BSA and 0.1% Triton X-100. Secondary antibodies (see section ‘Preparation of labelled antibodies’) were diluted in blocking buffer to a final concentration of 4–10 µg ml⁻¹. The sample was incubated with mixed secondary antibodies for 2 h in the dark, then washed three times for 10 min in PBS supplemented with 0.2% BSA and 0.1% Triton X-100 and post-fixed for 10 min in PBS with 4% PFA, 0.1% GA. Standard chemicals were purchased from Sigma Aldrich unless otherwise stated.

Chin wild-type Caulobacter crescentus were grown in liquid M2G medium in a mid-exponential phase for 12–16 h. Bacteria were fixed with 2.5% PFA in a PIB solution for 10 min then immediately resuspended in a permeabilization buffer (0.1% Triton X-100 in PBS) for 10 min. The bacteria were then resuspended in 100 µg ml⁻¹ of the green secondary antibody (AlexaFluor 488-conjugate, W32466, Life Technologies) solution for 5 h at room temperature then washed three times in PBS solution and post-fixed for 10 min in PBS with 2.5% PFA.

Preparation of labelled antibodies. Unconjugated goat anti-rabbit antibody (31239, Thermofisher) was diluted to 0.6 mg ml⁻¹ in 100 µl PBS (supplemented with 50 mM NaHCO₃). AlexaFluor 750 NHS ester (A20011, Life Technologies) was added at a final concentration of (150 µM) and the solution was incubated for 30 min at room temperature. 100 µl PBS was added and the solution was applied to a NAP-5 size exclusion column (GE Healthcare) pre-equilibrated with PBS. 300 µl fractions were collected and analysed by ultraviolet-visible spectrophotometry (PFA, Nanodrop2000, Thermofisher). Peak fractions were collected and the degree of labelling calculated. The labelled antibody fractions were stored at 4 °C until further use.

Field-dependent blotting statistics. The field dependence of the SMLM imaging was assessed by performing direct STORM⁵ imaging of AlexaFluor 647-labelled α-tubulin in microtubes from COS-7 cells. Ten FOVs each were taken with microtubes filling the whole FOV for both flat-field illumination using FIFII and for standard wide-field illumination using an achromatic doublet as the field lens (AC254-250-B, Thorlabs). For each FOV 25,000 frames were recorded at 10 ms exposure times against mouse (AlexaFluor 647-conjugate, W32466, Life Technologies) solution for 5 h at room temperature then washed three times in PBS solution and post-fixed for 10 min in PBS with 2.5% PFA.

Imaging was performed using an optimized buffer as described previously 30. The MLA simulation code was verified by reproducing fig. 7bc in ref. 15. The partial coherence code was verified against fig. 9.11 in ref. 27.

The simulated objective parameters were chosen from common commercial microscope objectives used in SMLM. From top to bottom for the objective parameters labelled with m and f, the objective lens (CFI60 PlanApo Lambda ×60/NA 1.4, Nikon) was injected into the telescope (f₁ = 100 mm, f₂ = 50 mm). The rotating diffuser (2.5° ± 0.25° FWHM at 650 nm, 24-00066, Süss MicroOptics SA) was adjusted at an offset of –5 mm from the shared focal planes of the telescope lenses. A series of mirrors was then used to align the beam to both the MLAs (500 µm pitch, 10 mm, 10 mm, f = 13.7 mm, square lenses, 18-00021, Süss MicroOptics SA) and the objective lenses (CFI60 PlanApo Lambda ×60/NA 1.4, Nikon). A custom dichroic (ZT405/561/642/750/850rpc, Chroma) reflected the laser light and transmitted fluorescence emission. Emitted light from the sample was collected by the same objective lens used through the pupil parameters imaged by a 40× objective lens (f₁ = 200 mm, M2A2096, Nikon) onto the SCMOS camera (Zyla 4.2, Andor). The width of a square camera pixel corresponds to 106 ± 2 µm on the sample. One of two emission filters (ET700/75M, Chroma and ET810/90M, Chroma) was combined with a short-pass filter (FF01-842/SP, Semrock) and inserted into the emission path between the tube lens and camera depending on the fluorophore being imaged. A separate, 850 nm laser (0.9 mW continuous wave (cw) circular beam, 85–238, Thorlabs) passed through a clean-up filter (L101-852, Semrock) and was reflected from the coverslip by total internal reflection. The reflected beam was directed through an 850 nm band-pass filter (86–090, Edmund Optics) and onto a linear light sensor (TLS1401CL, AML-TAOUS USA, Inc.). Changes in the beam position were read using the pgFocus open hardware microscope motorization at umassmed.edu/wiki/index.php/PgFocus. This information was used to send a feedback signal to the piezo z stage and lock the objective–coverslip distance within a standard deviation of 10 nm. Microscope control was orchestrated with Micro-Manager (Version 1.4.22, nightly build 2015-07-27)². The single-molecule localization analysis was performed using a sCMOS-specific maximum likelihood algorithm following a previously described calibration routine³.

The MLAs and rotating diffuser were chosen on the basis of the design specifications from the simulation package and the need to minimize losses in the transmitted laser power. Square lenses provide a complete fill factor and minimize scattering. The size of the lenslet inter- and intra-lenslet was chosen at a wavelength of 650 nm to optimize the pattern on the sample. A diffuser with a small divergence angle specification helps to maintain a minimally divergent laser beam profile through the system. Imaging was performed using an optimized buffer as described previously¹. Microtubes in Fig. 4a were imaged by acquiring 30,000 frames at 10 ms continuous exposure and approximately 100,000 frames at 50 ms exposure. Mitochondria were imaged with the same parameters but a BFP laser power of 350 mW at 750 nm. The bacteria in Fig. 4b were imaged by acquiring 20,000 frames at 10 ms continuous exposure and 1,400 mW of 642 nm laser light in the objective BFP.

Illumination profile measurement. The illumination profile in the sample plane just above the coverslip was measured using a highly concentrated fluorescent dye solution⁵. Briefly, 0.45 g ml⁻¹ of acid blue no. 9, also known as eriochrome disodium salt (3844–45–9, Sigma-Aldrich), was mixed with deionized water and vortexed for 5 min, followed by sonication for 1 h until the dye was completely dissolved. A 4 µl drop of the solution was then pipetted onto a coverslip at a wavelength of 450 nm, no. 1.5 coverslip and covered with a 12 mm diameter coverslip to create a thin, uniform layer. During the profile measurements, the laser powers were set to be as low as possible while still obtaining a good signal-to-noise ratio from the fluorescence signal, doing so ensured that out-of-focus fluorescence did not erroneously smooth out the measured profile and give a false sense of uniformity. The dye sample was used immediately after preparation and discarded after use.

When measuring the illumination profiles, only the emission filter was changed between profile measurements at different wavelengths. References

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