Multi-MHz laser-scanning single-cell fluorescence microscopy by spatiotemporally encoded virtual source array

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Abstract: Apart from the spatial resolution enhancement, scaling of temporal resolution, equivalently the imaging throughput, of fluorescence microscopy is of equal importance in advancing cell biology and clinical diagnostics. Yet, this attribute has mostly been overlooked because of the inherent speed limitation of existing imaging strategies. To address the challenge, we employ an all-optical laser-scanning mechanism, enabled by an array of reconfigurable spatiotemporally-encoded virtual sources, to demonstrate ultrafast fluorescence microscopy at line-scan rate as high as 8 MHz. We show that this technique enables high-throughput single-cell microfluidic fluorescence imaging at 75,000 cells/second and high-speed cellular 2D dynamical imaging at 3,000 frames per second, outperforming the state-of-the-art high-speed cameras and the gold-standard laser scanning strategies. Together with its wide compatibility to the existing imaging modalities, this technology could empower new forms of high-throughput and high-speed biological fluorescence microscopy that was once challenged.

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References and links

1. Introduction

Fluorescence microscopy is a versatile tool for scientific research and biomedical applications. In contrast to strenuous effort on enhancing the spatial resolution of fluorescence microscopy [1–3], advancement in scaling the imaging speed has yet been incremental. The challenge stems from the inherent speed limitations in the common image capture schemes. For instance, the speed-versus-sensitivity trade-off in the typical image sensor arrays (e.g., charge-coupled device (CCD), or complementary metal-oxide semiconductor (CMOS) cameras) hinders fast dynamical fluorescence microscopy with a time resolution of millisecond or below, especially under the photon-budget-limited scenario [4]. On the other hand, the inherent mechanical inertia in laser raster-scanning strategies (e.g., using galvanometric, resonant mirrors, and rotating polygonal mirrors) limits the laser scanning rate up to 10^5 kHz [5]. Although faster laser scanning up to hundreds of kHz can be achieved by acousto-optic deflectors (AODs) and electro-optic deflectors (EODs), these scanners suffer from the limited angular beam steering range (i.e., imaging field of view (FOV)) and the number of resolvable scanned points (and thus the resolution) [6]. In addition, AOD and EOD are intrinsically the dispersive elements which disperse (and distort) the...
broadband beams – making it non-trivial to be used in laser-scanning multi-photon imaging unless careful dispersion compensation is implemented [7].

Various novel schemes have been developed to increase the fluorescence imaging speed (e.g. frequency multiplexed excitation [8, 9], temporal pixel multiplexing [10], and multifoci microscopy [11]), but at the cost of FOV, resolution, and dynamic range of the image captured [12]. As a result, the ability to perform fluorescence microscopy at a line-scan rate above MHz or two-dimensional (2D) frame rate beyond 1,000 frames per second (fps) remains obscured. Yet, this unmet feature could critically address the widespread need for speed in real-time monitoring of fast dynamical processes and high-throughput screening. Recently, we have developed a new technique of all-optical laser scanning, called free-space angular-chirp-enhanced delay (FACED) [13] that bypasses the use of the mechanical scanning mirrors and their speed limitation to achieve ultrafast laser-scanning microscopy with a scan rate beyond multi-MHz. Unlike laser-scanning based on AOD and EOD, the free-space operation in FACED makes it insensitive to the dispersive effect and is thus particularly useful for ultrafast broadband pulsed beam scanning. However, its compatibility to fluorescence imaging and thus the applicability in biological imaging have not been fully exploited. To this end, here we report a FACED imaging system tailored for laser-scanning fluorescence microscopy (at a scan rate of 8 MHz) and characterize its imaging performance in terms of the imaging sensitivity and resolution. To highlight its versatile potential in the applications where high-speed and high-throughput measurements are critical, yet challenging, we present two demonstrations of FACED fluorescence imaging, namely high-throughput imaging flow cytometry at a fluorescence imaging throughput of 75,000 cells/second, and high-speed dynamical single-cell imaging at a 2D frame rate as high as 3,000 fps.

2. Materials and methods

2.1 Experimental setup

Figure 1 shows the schematic of the all-optical laser scanning microscope based on FACED. The laser source is a Ti:Sapphire laser centered at 710 nm (Maitai BB, Spectra-Physics) with a repetition rate of 80 MHz and pulse width of 150 fs. Using an acoustic optical modulator (MT250-A0.12-800, AA Opto-Electronic) as a pulse picker, we generate a pulse train at 8 MHz that defines the imaging line-scan rate. A cylindrical lens focuses the laser beam to the entrance \( O \) of the FACED device that consists of a pair of highly-reflective and quasi-parallel mirrors (reflectivity > 99.5% and a misaligned angle \( \alpha < 1 \) mrad) (ios Optics). The focused laser beam then is transformed into a set of beamlets, each of which follows a unique double-passed, spatially-chirped zig-zag path and has a substantial temporal delay with respect to the adjacent beamlets. In essence, the returning beamlets from the mirror pair manifest themselves as being emerged from an array of spatiotemporally encoded virtual sources (as explained in detail later).

With two sets of 4-f imaging optics (L3-L4 and L5-L6), the array of virtual sources is projected onto the focal plane of an excitation objective lens (40 ×, numerical aperture (NA) = 0.75) as an ultrafast line scanning beam, with a scanning FOV of 40 μm (along the fast-axis) at line-scan rate of 8 MHz. The fluorescence signal is detected by a photomultiplier tube (PMT) (H10721, Hamamatsu Photonics) which has a full width at half maximum (FWHM) of instrument response function of ~2 ns. The transmitted light is detected by a fast GaAs photodiode with an electrical bandwidth of ~9 GHz (ET-4000A, Electro-Optics Technology). Both signals are digitized by a real time oscilloscope (DSO-X 91604A, Keysight Technologies) with a bandwidth of 16 GHz and a sampling rate of 80 GS/s. Note that the system is configured as an inverted epi-fluorescence microscope.

Two imaging scenarios are demonstrated in this work: (I) 1D line-scan imaging of single cell flowing in microfluidic channel (cross-sectional dimensions: 60 μm × 30 μm) with a flow speed of 1.5 m/s; and (II) 2D dynamical imaging of single cells. In case (I), the microfluidic
flow is orthogonal to the major axis of the line-scan beam and thus naturally provides the slow-axis image scan of the cells in order to form the 2D image. In case (II), an additional galvanometric mirror (6210H, Cambridge Technology) is added to provide the slow-axis (y-axis) scan (Fig. 1), with a FOV of 40 μm at a scan frequency of 1,500 Hz, resulting in a 2D frame rate of 3,000 fps. This scan mirror is spatially conjugated to the entrance \( O \) and the rear pupil of the excitation objective lens. Note that in case (I), the scan mirror is deactivated. In both cases, the final 2D images are obtained by digitally aligning and stacking the line-scans along slow-axis.

In case (II), we specifically observe the dynamical change in cellular morphology subject to photothermal perturbations created by an additional modulated near infrared (NIR) laser (center wavelength at 1,440 nm; 100% modulation depth; 50% duty cycle; \( \leq 1,000 \text{ Hz} \)). The output laser beam is focused onto the cell with an average intensity of \( \sim 1 \text{ MW/cm}^2 \).

The working principle of FACED can accurately be explained by classical ray optics [13]. Briefly, the focusing beam entering the FACED device can be viewed as a collection of converging light rays (or beamlets) to the entrance \( O \) (see the highlighted three rays in Fig. 1). Among them, there is a discrete set of spatially-chirped zig-zag paths, called cardinal rays, that hits one of the two mirrors at normal incidence and reverses along the identical paths.
Because of the difference in path length in the FACED device, adjacent cardinal rays return at entrance \( O \) at different arrival times. For those light rays deviated from any cardinal ray can still be routed back, but are slightly shifted away from the cardinal rays at the entrance \( O \). In effect, for each returning cardinal ray, there is an accompanying “light fan” as if it is emerged from a virtual source with a beam diverging angle of \( \alpha \). Therefore, FACED effectively transforms the pulsed beam into an array of spatiotemporally-encoded virtual sources, i.e. the pulsed virtual source is spatially separated and each of them has a temporal delay with respect to each other. As a result, different virtual sources can be projected onto different positions on the imaging plane at different arrival times – resembling an action of a laser-scanning beam. This is done all-optically without involving any active beam steering elements, such as scanning mirrors.

According to a conjugate-mirror ray-tracing model [13], the number of virtual sources, \( M \), generated by a FACED device is given by:

\[
M = \frac{\Delta \theta}{\alpha} < \frac{\theta_{\text{max}}}{\alpha} = N_{\text{max}},
\]

in which \( \Delta \theta \) is the input cone angle of the line-focusing beam. Also, \( \sin \left( \frac{\theta_{\text{max}}}{2} \right) \) is essentially the NA of the FACED device, i.e. \( \theta_{\text{max}} \) is the maximum acceptance cone angle within which the beamlets can be double-passed back to the entrance \( O \). It is governed entirely by the geometry of the devices, e.g. mirror separation \( S \) and misaligned angle \( \alpha \). \( N_{\text{max}} \) is thus the maximum number of virtual sources supported by the device. As the final FOV is generally determined by the objective lens of the microscope, the density of virtual sources, i.e. the number of scanned points per unit length, at the focal plane of the microscope can be flexibly reconfigurable by the mirror separation \( S \) and misaligned angle \( \alpha \), according to Eq. (1). In this work, by tuning \( \alpha \), an array of ~60 virtual sources is generated, which is coupled into the epi-fluorescence microscope, forming the all-optical line scanning beam on the focal plane within the FOV of 40 \( \mu \)m (Fig. 2). On the other hand, \( S \) also determines the temporal delay between the adjacent virtual sources, \( \tau \), given by [13]:

\[
\tau = \frac{2S}{c},
\]

in which \( c \) is the speed of light. In the present work which focuses on fluorescence imaging, \( \tau \) is optimized such that it is short enough to ensure ultrafast laser-scanning operation and should also be sufficiently long to minimize crosstalk between the scan points. This compromise derives from that the dwell time of each scan point depends not only on the scan speed, but also the fluorescence lifetime and bandwidth of the detector [14, 15] – a unique laser-scanning fluorescence imaging scenario brought by FACED owing to its ultrafast scanning action. To fully utilize the bandwidth of the PMT, \( \tau \) is optimized to be 2 ns with \( S \) fixed at 300 mm (Fig. 2(c)), enabling a multi-MHz laser-scanning fluorescence imaging that obviates the classical speed limitation associated with the active beam steering approaches (e.g. acousto-optic, electro-optic, or simply mechanical). We also note that the current FACED imaging system can be reconfigured to perform line-confocal fluorescence imaging by simply inserting a slit (line-aperture) right before the PMT that is on the conjugate plane of the sample plane.
2.2 Sensitivity and resolution tests

The sensitivity of the ultrafast FACED fluorescence microscopy is tested by measuring the signal-to-noise ratio (SNR) with a fluorescent dye solution, which is made of the CF750-conjugated donkey anti-goat antibody (SAB4600444, Sigma-Aldrich) and is diluted by phosphate buffered saline (PBS) to various concentrations (2, 4, 6, 8, 10 µg/mL). The solution is injected into a microfluidic channel (cross-sectional dimensions: 60 µm × 13 µm) with a flow speed of 100 mm/s across the laser beam. A sequence of 2,000 line-scans was recorded. The SNR is calculated from the mean-to-standard-deviation ratio of fluorescence intensities recorded from each virtual source across the line-scan.

The resolution of the ultrafast fluorescence microscopy is calibrated by imaging fluorescent microbeads fixed on a microscope slide. The goat anti-mouse microbeads (0.37 µm, MPB-03-5, Spherotech) are incubated with the CF750-conjugated donkey anti-goat antibody (10 µg/mL) for 60 minutes. The microbeads are then rinsed 3 times with 1 × PBS before the experiment.

2.3 Cell culture and preparation

A breast cell line (MCF-7) is employed in the experiments of high-throughput imaging flow cytometry and high-speed 2D dynamical imaging. The cells are cultured in 5% CO₂ incubator at 37 °C using culturing medium formulated with 90% Dulbecco’s modified eagle medium, 10% fetal bovine serum, and 1% penicillin-streptomycin. The cultured sample is trypsinized for 3 minutes, followed by centrifugation to re-suspend the MCF-7 cells in a freshly prepared culturing medium. The extracted MCF-7 cells are first incubated with goat anti-epithelial cell adhesion molecule (EpCAM) antibody (AF960, R&D Systems) (10 µg/mL) for 30 minutes and then rinsed 3 times with 1 × PBS. To detect EpCAM through fluorescence microscopy, the cells are further incubated with CF750-conjugated donkey anti-goat antibody (10 µg/mL) for 30 minutes. The labeled MCF-7 cells are then rinsed 3 times with 1 × PBS for the experiment.

The peripheral blood mononuclear cell (PBMC) used in the high-throughput imaging flow cytometry is extracted from 2 mL of human buffy coat provided by Hong Kong Red Cross. All the blood donors have given written consent for clinical care and research purposes. The research protocol is approved by Institutional Review Board at the University of Hong Kong. Human buffy coat is carefully pipetted on top of 2 mL Ficoll in centrifugal tubes, which is used to separate PBMCs from buffy coat. Centrifugation is performed under 400 × g (gravitational constant) for 20 minutes at 20 °C. The mononuclear cell layer is carefully aspirated and transferred to 1 × PBS. Followed by centrifugation with 200 × g for 5 minutes at 20 °C, the extracted PBMC is resuspended in 1 × PBS for the experiment.
2.4 Microfluidic channel fabrication

The microfluidic channels used for the high-throughput imaging flow cytometry are fabricated following the standard casting technique using polydimethylsiloxane (PDMS). The channel design based on the principle of inertial focusing [16, 17] is first transferred to a silicon wafer by maskless soft lithography prior to channel fabrication. Then, PDMS is mixed with the corresponding curing agent (10:1) before pouring onto the silicon wafer. The PDMS thickness is controlled by spin coating (200 rpm for 1 minute) to achieve a final height of 0.2 mm. After the curing step for the thin PDMS layer, a thick, pre-cured PDMS layer with no pattern is physically attached to the inlet and outlet regions with un-cured PDMS glue added to strengthen the bonding. The channels are then cured in an oven at 65 °C before demolding. A pair of holes is punched to allow plastic tubing insertion to the inlet and outlet of the microfluidic channel. The channel is then treated with oxygen plasma before being bonded to a glass cover slip. Lastly, plastic tubing is inserted into punched holes inside the thick PDMS layer for fluid transportation with epoxy glue to seal the gap.

3. Results and discussion

3.1 Imaging sensitivity and resolution

We characterize the detection sensitivity of FACED fluorescence microscopy, especially at its ultrafast laser-scanning rate. The detection limit (SNR = 1) at the line-scan rate as high as 8-MHz is measured to be ~12 nM (Fig. 3(a)). It corresponds to ~150 fluorescent molecules in the focal volume (~20 μm³). Such sensitivity is adequate for many typical biological imaging applications. For example, it was reported that the typical surface antigen density in cancer cells and blood cells is around 10⁴-10⁵ per cell [18, 19]. Further supported by the continuing advances in brightness enhancement of fluorophores as well as signal amplification strategies in immunolabeling, our technique could widely be adopted in live-cell dynamical imaging and imaging flow cytometry as demonstrated in this work (See Sections 3.2 and 3.3). Furthermore, we note that a trivial step for improving SNR is to perform multiple line-scans averaging. We measured that the SNR is proportional to the square root of the number of line-scans (inset of Fig. 3(a)), confirming the system is shot-noise limited. Hence, the sensitivity drops with higher effective imaging speed, as expected. Nevertheless, as the scan rate of FACED imaging can readily be configured well beyond 1 MHz, which is 2-3 orders-of-magnitude faster than the standard galvanometric mirrors, such a “speed gap” allows FACED fluorescence microscopy to offer a sufficient room to tailor adequate SNR while maximizing the imaging speed (especially in the underexploited sub-MHz to MHz-scan-rate regime) for specific applications.

Fig. 3. Imaging performance of the all-optical, ultrafast laser beam scanner. (a) SNR (at a line-scan rate of 8 MHz) measured at different fluorescent dye concentrations in solution; inset shows the SNR against the square root of the number of averaging line-scan. The lines show the linear fit to the data; error bars represent the standard deviations obtained from 18 measurements. (b) Intensity profiles of the microbead along slow and fast axis; The FWHM values represent the mean ± standard deviation obtained from 20 beads; inset shows the averaged microbead image.
We next measure the resolution of the FACED fluorescence microscope, which is estimated based on the FWHM of intensity profiles (i.e. point spread function (psf)) along the slow and fast axes in the microbead images (Fig. 3(b)). In the slow axis, the FWHM is measured to be $0.69 \pm 0.07 \mu m$, which is consistent with diffraction-limit resolution. To ensure the Nyquist sampling condition along the slow axis, the images are sampled at 5 line-scans/μm in the experiments of imaging flow cytometry at a flow speed of 1.5 m/s (i.e. case (I)), whereas 60 line-scans/μm for 2D imaging at 3,000 fps (i.e. case (II)). In the fast-axis, the FWHM of the psf is measured to be $1.41 \pm 0.19 \mu m$. We note that the resolution in this case is mainly governed by the maximum number of virtual sources $N_{max}$ allowed within the FOV (i.e. density of the virtual sources). In general, the geometry of the mirrors (e.g. mirror separation, mirror length, misalignment angle) defines the maximally achievable $N_{max}$ [13]. However, the scan period (or the scan rate) imposes a practical upper bound of $N_{max}$ beyond which cross-talk between adjacent line-scans occurs. In our current setup with a line-scan rate of 8 MHz and time delay of $\tau = 2$ ns between adjacent virtual sources, $N_{max}$ has to be limited to 60. Across the FOV of 40 μm, this implies a resolution of $\sim 1.33 \mu m$ along the fast axis according to the Nyquist sampling theorem. If we decrease the line-scan rate to, for example 2 MHz, we can further scale $N_{max}$ up to 240 with the same mirror geometry. Considering the same FOV, the resolution could then approach to the diffraction limit. In this work, the FOV is chosen to be 40 μm to cover the microfluidic channel for imaging flow cytometry.

3.2 High-throughput imaging flow cytometry beyond 10,000 cells/second

Circulating tumor cells (CTCs) could be indicative of the risk of metastatic progression and useful to guide therapy [20, 21]. However, current technologies run short of throughput and sensitivity to detect and analyse CTCs, which can be as rare as one in billions of blood cells. To this end, we employ our FACED imaging system integrated with microfluidic technology to demonstrate high-throughput imaging flow cytometry in the context of breast cancer cell (MCF-7) detection in blood. The FACED microscope is used to perform single-cell imaging in a mixed population of fluorescent-labeled (specific to the surface marker EpCAM) MCF-7 and blood cells flowing a microfluidic channel. Consider the ultrafast flow speed of 1.5 m/s and the cell-cell separation in the channel is $\sim 20 \mu m$, which are controlled by the inertial flow focusing condition [16, 17], the imaging throughput is as high as 75,000 cells/second – 1-2 orders-of-magnitude higher than existing imaging flow cytometers [22, 23]. EpCAM is employed as it is generally overexpressed in many cancer cell types except blood cells, and has been the gold-standard marker for CTC enrichment and detection from peripheral blood.
Fig. 4. Ultrafast dual-contrast flowing single-cell imaging by FACED microscopy. (a) Imagery of MCF-7 cells flowing at 1.5 m/s. Left and middle columns show the FACED bright-field and fluorescence images, and right column shows the overlaid images. (b) Flowing MCF-7 cells at 1.5 m/s and (c) static MCF-7 cells captured by a sCMOS camera with an exposure time of 5 ms. All scale bars are 10 μm.

We first image a pure population of MCF-7 cells to verify the imaging performance. The current FACED microscope is capable of capturing both epi-fluorescence and transmitted bright-field images of single cells simultaneously in real-time at the line-scan rate of 8 MHz as shown in Fig. 4(a). Compared with the images captured by a scientific CMOS (sCMOS) camera, FACED fluorescence images not only are motion-blur-free (Fig. 4(a) vs. Fig. 4(b)) under a flow speed of 1.5 m/s, but also show image contrast and resolution comparable to the static cell images taken by the sCMOS camera (Fig. 4(a) vs. Fig. 4(c)). Notably, apoptotic cells with the characteristic blebbing features are clearly identified from the FACED images.

We note that there have been attempts to enhance the imaging speed in the context of imaging flow cytometry, notably spectrally-encoded imaging [24, 25] and time-stretch imaging [17]. Nevertheless, the spectral-encoding imaging throughput is still limited by either the frame rate of the cameras (~1,000 fps) [24] or the speed of mechanical polygonal scanning mirror (~10’s kHz) [25]. Also, in the context of fluorescence spectrally-encoded imaging, each spectrally-encoded excitation spot only shares a small fraction of the entire usable excitation spectrum. Thus, the fluorescence excitation efficiency and thus the imaging sensitivity could be comparatively lower than the classical (spectral-encoding-free) excitation scheme, including FACED imaging. Adopting the same spectrally-encoded illumination scheme, conventional time-stretch imaging also suffers from the same limitation, not to mention that fluorescence imaging in the visible spectrum by time-stretch is generally forbidden because of the prohibitively high fiber loss [13].

We then analyze the mixed population of MCF-7 cells and blood cells by their cell sizes (quantified from the bright-field images) as well as the peak SNR (PSNR) of the fluorescence images (Fig. 5(a)). We identify the major groups of blood cell types according to their negligible fluorescence signal and the characteristic sizes, i.e. ~3 μm for platelets, ~6 μm for red blood cells (see also the characteristic bi-concave shape captured in FACED images (inset)), and ~8 – 15 μm for PBMCs. As shown in Fig. 5(a), another major cluster, which
shows almost 2-orders-of-magnitude stronger fluorescence PSNR than that of the blood cell cluster, is identified as MCF-7 cells. They are generally larger, with a size of ~15 – 20 μm.

We further analyse the morphological information of the MCF-7 cells gated from the scatter plot shown in Fig. 5(a). We particularly investigate two morphological/texture parameters extracted from FACED fluorescence images (Fig. 5(b)); (1) circularity which is defined as $4\pi \times \text{cell area}/\text{perimeter}^2$ and (2) kurtosis, which is defined as the fourth-order statistical moment of the fluorescence intensity distribution of each image. The kurtosis measures the flatness of a distribution, i.e. the spatial heterogeneity of the fluorescent labels. While we observe the majority of the MCF-7 cells exhibits uniform fluorescence distribution (i.e. low kurtosis) and generally circular in shape (i.e. high circularity), we identify the outliers of the cluster plot show some interesting morphologies and features. For instances, we identify a sub-population of clustered MCF-7 cells (mostly in doublets) by show uniform fluorescent distribution but low circularity. We note that the ability to detect CTC clusters has been found to be of valuable to correlate with higher chance of metastasis initiation and an inferior prognosis [26]. We also observe other sub-groups of cells, such as apoptotic cells and fragments/debris which show high kurtosis and low circularity; cells with heterogeneous distribution of fluorescence signals (circular shape but high kurtosis), which could be related to the non-uniform EpCAM expression on the cell surface. Such expression heterogeneity has also been argued to have a diagnostic value for recognizing cancer types/state [27].

3.3 High-speed dynamical single-cell imaging at sub-millisecond resolution

To further show the versatile potential of FACED fluorescence microscopy in biological imaging, we also demonstrate high-speed dynamic imaging of single MCF-7 cells subject to photothermal perturbations from the frequency modulated NIR laser at 1440 nm. In contrast to the imaging flow cytometry experiments, an additional scanning mirror is added to provide the slow-axis scan (Fig. 1) at a scan frequency of 1,500 Hz, and thus a 2D frame rate as high as 3,000 fps. The perturbation is expected to create time-varying thermoelastic expansion of the cells that can be monitored in real-time by the ultrafast FACED fluorescence microscope (Visualization 1). We note that the photothermal effect is significant because of the high absorption coefficient (~25 cm$^{-1}$) of water at 1,440 nm [28].
Fig. 6. High-speed dynamic imaging of single live MCF-7 cells subject to photothermal perturbations (Visualization 1). (a) Cell size variation over time under different perturbation frequencies $F$. Top panel: the NIR laser is off. Other panels: the NIR laser is on from the 21st to 146th frame. Inset in top panel: images of the first and last frame with yellow curves showing the cell outlines (b) Frequency spectra of corresponding time signals in (a). Inset in top panel: galvanometric mirror scanning time sequence (O, odd frame; E, even frame) which shows the dead-time slots (~20 μs) results in the apparent 1,500 Hz scanning pattern.

From the high-speed fluorescence image sequence, we can notice that the cell size is modulated in response to the NIR laser (Fig. 6(a)). This is further ascertained by the frequency analysis of photothermal modulation of the cell size based on Lomb-Scargle periodograms (Fig. 6(b)), which show the modulation peaks at the corresponding perturbation frequencies $F$, from 400 Hz up to 1,000 Hz. The experiments were repeated for 10 different single cells. Note that the galvanometric mirror scanning time sequence (inset in Fig. 6(b)), which shows the dead-time slots (~20 μs), creates an apparent 1,500 Hz scanning signal on top of the photothermal-induced modulation signal. Specifically, the effect results in the frequency-shift of the baseband signal (i.e. modulation at $F$) by the 1,500-Hz mirror-scanning signal, i.e. $(1,500 - F)$ Hz. This explains the spurious peaks at 500 and 700 Hz in the 1,000-Hz and 800-Hz perturbations, respectively. This artifact disappeared in the case of 400, 500, and 600-Hz modulation, when frequency analysis is applied to the even or odd frames separately.

Such dynamical image analysis is otherwise impossible with the state-of-the-art sCMOS camera, which could achieve a 2D frame rate of ~1,000 fps, however, at the expense of insufficient pixel resolution because of the necessity of pixel binning at high frame rate; and image artifacts because of the rolling shutter exposure scheme [4]. In our current setup, the maximum 2D imaging frame rate is limited by the scanning speed of the galvanometric mirror (over sampled along slow-axis). If a faster scanner, such as a resonant scanning mirror and an acousto-optic deflector [6], is used to scan the slow-axis, frame rate beyond 3,000 fps is expected to be feasible and allows the study of fast dynamics with sub-millisecond resolution, e.g. real-time spatiotemporal studies of neuronal activities [29], rapid intracellular signaling events [30].
4. Conclusions

In conclusion, we have employed the concept of spatiotemporally encoded source array, enabled by FACED, tailored for all-optical ultrafast laser-scanning fluorescence microscopy at a scan rate of 8-MHz. Not only we have characterized its fluorescence imaging performance in terms of resolution and sensitivity, but also demonstrated its potential applications in high-throughput imaging flow cytometry and high-speed (sub-millisecond) single-cell dynamics imaging.

We note that FACED fluorescence imaging follows the classical direct laser-scanning strategy but in an all-optical, all-passive manner, resulting in straightforward image reconstruction and wider compatibility with other laser-scanning imaging modalities, including multiphoton microscopy. It is also readily scalable to multi-colour or three-dimensional imaging – unleashing a wider scope of applications where speed is critical.

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Disclosures

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