Digitally synthesized beat frequency multiplexing for sub-millisecond fluorescence microscopy

Eric D. Diebold1,2*, Brandon W. Buckley1,2,3, Daniel R. Gossett4 and Bahram Jalali1,2,4,5

Fluorescence imaging is the most widely used method for unveiling the molecular composition of biological specimens. However, the weak optical emission of fluorescent probes and the trade-off between imaging speed and sensitivity are problematic for acquiring blur-free images of fast phenomena, such as sub-millisecond biochemical dynamics in live cells and tissues, and cells flowing at high speed. Here, we report a technique that achieves real-time pixel readout rates that are one order of magnitude faster than a modern electron multiplier charge-coupled device—the gold standard in high-speed fluorescence imaging technology. Termed fluorescence imaging using radiofrequency-tagged emission (FIRE), this approach maps the image into the radiofrequency spectrum using the beating of digitally synthesized optical fields. We demonstrate diffraction-limited confocal fluorescence imaging of stationary cells at a frame rate of 4.4 kHz, and fluorescence microscopy in flow at a velocity of 1 m s⁻¹, corresponding to a throughput of approximately 50,000 cells per second.

The spatial resolution of modern fluorescence microscopy has been improved to a point such that sub-diffraction-limited resolution is routinely possible. However, the demand for continuous, sub-millisecond time resolution using fluorescence microscopy remains largely unsatisfied. Such a real-time fluorescence microscope would enable the resolution of dynamic biochemical phenomena such as calcium and metabolic waves in live cells, action potential sequences in large groups of neurons, calcium release correlations and signalling in cardiac tissue. High-speed microscopy is also invaluable for imaging biological cells in flow. Flow imaging can quickly perform high-throughput morphometry, translocation and cell signalling analysis on large populations of cells. However, as fluorescence imaging frame rates increase towards the kilohertz range, the small number of photoelectrons generated during each exposure drops below the noise floor of conventional image sensors, such as the charge-coupled device (CCD).

The desire to perform high-speed, low-photon-number imaging has been the primary driving force behind the development of the electron multiplier CCD (EMCCD) camera. EMCCDs use on-chip electronic gain to circumvent the high-speed imaging signal-to-noise ratio (SNR) problem. However, although EMCCDs can exhibit 1,000-fold gain, the serial pixel readout strategy limits the full frame (512 × 512 pixels) rate to less than 100 Hz. A photomultiplier tube (PMT) can provide 1,000 times higher gain, 10 times lower dark noise and 50 times higher bandwidth than EMCCDs, but they are not typically manufactured in a large-array format. This limits the utility of PMTs in fluorescence microscopy to point-scanning applications, in which the speed of serial beam scanning limits the overall frame rate.

Because of their parallel readout architecture, scientific complementary metal–oxide–semiconductor (sCMOS) cameras exhibit low readout noise and pixel readout rates up to 50 times higher than EMCCDs, including line scan readout rates of ∼100 kHz. However, the internal gain of EMCCDs ultimately makes them more attractive than sCMOS cameras for the lowest light fluorescence imaging applications. Both technologies are widely used in high-speed fluorescence microscopy, but neither has a sufficient line readout rate to perform blur-free imaging of cells at the metre per second flow velocities typical of flow cytometry.

Here, we present fluorescence imaging using radiofrequency-tagged emission (FIRE) microscopy, a radiofrequency communications approach to high-speed fluorescence microscopy, which combines the benefits of PMT sensitivity and speed with frequency-domain signal multiplexing, radiofrequency spectrum digital synthesis, and digital lock-in amplification to enable fluorescence imaging at kilohertz frame rates. FIRE uses radiofrequency multiplexing techniques from the field of communication to address the speed limitations of fluorescence imaging, much in the same spirit as stretch-time encoded amplified microscopy (STEAM) makes use of time- and wavelength-division multiplexing to enable high-speed bright-field imaging.

The central, defining feature of FIRE is its ability to excite fluorescence in each individual point of the sample at a distinct radiofrequency. Digitally synthesized radiofrequency ‘tagging’ of pixels’ fluorescence emission occurs at the beat frequency between two interfering frequency-shifted laser beams. Similar to frequency-domain multiplexing in wireless communication systems, each pixel in a row of a FIRE image is assigned its own radiofrequency. In a two-dimensional FIRE image, pixels are analogous to points on a time–frequency Gabor lattice. A single-element photodetector simultaneously detects fluorescence from multiple pixels, and an image is reconstructed from the frequency components of the detector output, which are resolved using parallel lock-in amplification in the digital domain. A diagram of the experimental implementation of the FIRE microscope is shown in Fig. 1. A detailed description is included in the Methods.

Figure 2 shows the typical output of the FIRE microscope. The detected time-domain signal (Fig. 2a) is a Fourier superposition of the radiofrequency-tagged emission from a row of pixels. The time-resolved frequency spectrum, calculated using a short-time Fourier transform, (Fig. 2b), reveals the frequency components associated with the positions of the sample within the row. The vertical locations of the sample are recovered from the reference output of the 2.2 kHz resonant scan mirror, and the final image is formed (Fig. 2c).

To demonstrate FIRE microscopy on biological samples, we imaged adherent cells stained with various fluorophores at a...
velocity using a frame transfer EMCCD in single exposure mode and gain of the EMCCD together yield a reasonable SNR image, the camera’s minimum exposure time of 10 µs and its frame transfer nature create significant blur at these flow velocities. In contrast, the FIRE line scan shutter speed of 1.25 µs yields blur-free images with comparable SNR.

Other approaches to frequency-domain fluorescence imaging have been reported previously\(^1\). However, the kilohertz-bandwidth, mechanical modulation schemes used in these works do not provide the sufficiently high pixel readout rates required for sub-millisecond imaging. This implementation of FIRE features pixel readout rates in the 100 MHz range, but this rate can be directly extended to more than 1 GHz through the use of wider-bandwidth acousto-optic deflectors\(^2\). FIRE’s maximum modulation frequency, and thus maximum pixel readout rate, is intrinsically limited by the sample’s fluorescence lifetime. If the excitation frequency is less than 1/τ, where τ is the fluorescence lifetime of the sample, the emitted fluorescence will oscillate at the excitation frequency with appreciable modulation\(^1\). Furthermore, beat-frequency modulation is critical to the speed of FIRE; the beating of two coherent, frequency-shifted optical waves produces a single radiofrequency tone, without any harmonics that can introduce pixel crosstalk and reduce the usable bandwidth. Because the FIRE system is designed so that every pixel is spatially resolved at the defraction limit, beating of the excitation light from two adjacent pixels generates a fluorescence signal at the comb spacing frequency. However, this frequency lies out of the image band and does not produce pixel crosstalk. With respect to blur-free imaging of fast phenomena in samples such as living cells, the speed of the fastest dynamic event that can be imaged is determined by the maximum allowable sideband frequency (half the comb spacing). In the FIRE system reported here, the fastest line scan shutter speed is 1.25 µs (800 kHz comb spacing), which allows for the capture of pixel dynamics with frequency content up to 400 kHz (see Supplementary Information).

The flexibility afforded by digitally synthesizing the amplitude and phase of the radiofrequency spectrum provides complete, real-time control over the number of pixels, pixel frequency spacing, pixel non-uniformity and field of view. Because PMTs inherently have smaller dynamic range than CCD or CMOS technologies, maximizing this quantity per pixel is critical to the performance of FIRE. Specifically, phase-engineering the excitation
frequency comb enables the dynamic range of each pixel to scale as $D/\sqrt{M}$, where $D$ is the dynamic range of the PMT, and $M$ is the pixel-multiplexing factor. This is in contrast to the case where all excitation frequencies’ initial phases are locked, which yields images with a dynamic range of $D/M$. Although FIRE fundamentally presents a tradeoff in dynamic range for speed, it improves in sensitivity when compared to single point scanning fluorescence microscopy, as multiplexing the sample excitation by a factor of $M$ yields an $M$-fold increase in the dwell time of each pixel. However, owing to the parallel nature of detection, FIRE shares shot noise across all pixels in a row. This causes the shot-noise-limited uncertainty at each pixel to scale with the square root of the total number of photons collected from all pixels in a line scan. The extent to which this effect degrades the SNR at each pixel depends inversely on the sparsity of the sample (see Supplementary Information).

Beat frequency multiplexing is also applicable to other types of laser scanning microscopy, including two-photon excited fluorescence microscopy. Perhaps most notably, because emission from each pixel is tagged with a distinct radiofrequency, FIRE is inherently immune to pixel crosstalk arising from fluorescence emission scattering in the sample—the effect that typically limits the imaging

![Figure 2](image-url)

**Figure 2** | Illustration of the radiofrequency tagging of fluorescent emission in FIRE. **a**, Time-domain data output from the PMT. **b**, Short-time Fourier transforms (STFTs) of the signal in **a**, indicating the bead horizontal positions. **c**, A 256 x 256 pixel image of three immobilized fluorescent beads recorded using 256 excitation frequencies. The sample was imaged at a 4.4 kHz frame rate. The vertical axis in the image is oversampled to yield 256 pixels. Scale bar, 30 μm.

![Figure 3](image-url)

**Figure 3** | Comparison of FIRE microscopy and wide-field fluorescence imaging. Laser excitation at 488 nm was used for FIRE imaging (8.5 μW per pixel, measured before the objective), and mercury lamp excitation was used for wide-field imaging. All FIRE images use a radiofrequency comb frequency spacing of 400 kHz, and are composed of 200 x 92 pixels. Slight vignetting is observed in the FIRE images due to the mismatch of the Gaussian profile of the LO beam with the flat-top radiofrequency comb beam. This mismatch and the resulting vignetting can be eliminated using digital pre-equalization of the radiofrequency comb in the direct digital synthesis generator. The particular objective lens used is denoted in each FIRE image. Processing of the raw FIRE images is performed in Matlab (see Supplementary Information). **a–c**, C6 astrocytes stained with Syto16. Scale bars, 10 μm. **d**, S. cerevisiae yeast stained with Calcein AM. Scale bars, 5 μm. **e–f**, NIH 3T3 cells stained with Calcein AM. Scale bars, 20 μm.
depth in multifocal multiphoton microscopy\textsuperscript{26}. In combination with fast fluorophores\textsuperscript{27}, FIRE microscopy may ultimately become the technique of choice for the observation of nano- to microsecond timescale phenomena using fluorescence microscopy.

Methods
FIRE performs beat frequency excitation multiplexing by using acousto-optic devices in a Mach–Zehnder interferometer (MZI) configuration. As shown in Fig. 1, the light in one arm of the MZI is frequency shifted by a 100 MHz bandwidth AOD, driven by a comb of radiofrequencies, phase-engineered\textsuperscript{28} to minimize its peak-to-average power ratio. The AOD produces multiple deflected optical beams with a range of both output angles and frequency shifts\textsuperscript{24}. Light in the second arm of the interferometer passes through an acousto-optic frequency shifter, driven by a single radiofrequency tone, which provides a local oscillator (LO) beam. A cylindrical lens is used to match the LO beam’s angular divergence to that of the radiofrequency comb beams. At the MZI output, the two beams are combined and focused to a horizontal line on the sample, mapping frequency shift to space. Because fluorescent molecules in the sample function as square-law detectors of the total optical field, fluorescence is excited at the various beats defined by the difference frequencies of the two arms of the interferometer. Fluorescence emission from the sample is detected by a PMT in a confocal configuration, using a slit aperture to reject out-of-plane fluorescence emission. A resonant scan mirror performs high-speed scanning in the transverse direction for two-dimensional imaging. Given the finite frequency response of fluorophores, the LO beam frequency shift is chosen to heterodyne the beat frequency excitation spectrum to the baseband to maximize the usable modulation bandwidth. This is necessary because AODs typically operate over an upshifted, sub-octave passband to avoid harmonic interference\textsuperscript{24}. Direct digital synthesis (DDS) of the radiofrequency comb used to drive the AOD defines each pixel’s excitation by a specific radiofrequency and phase, resulting in phase coherence between the radiofrequency comb and the detected signal\textsuperscript{29}. This phase coherence enables image demultiplexing using a parallel array of phase-sensitive digital lock-in amplifiers, implemented in Matlab. FIRE’s parallel readout results in a maximum pixel rate equal to the bandwidth of the AOD.

Received 4 March 2013; accepted 15 August 2013; published online 22 September 2013

References
1. Lakowicz, J. R. \textit{Principles of Fluorescence Spectroscopy} 3rd edn (Springer, 2006).
2. Scanziani, M. & Hausser, M. Electrophysiology in the age of light. \textit{Nature} \textbf{461}, 930–939 (2009).
3. Elliott, G. S. Moving pictures: imaging flow cytometry for drug development. \textit{Comb. Chem. High. T. Scr.} \textbf{12}, 849–859 (2009).
4. Coates, C. \textit{New sCMOS vs. Current Microscopy Cameras}, Andor white paper (Andor, 2011).
5. Huang, B., Bates, M. & Zhuang, X. Super-resolution fluorescence microscopy. \textit{Annu. Rev. Biophys.} \textbf{78}, 993–1016 (2009).
6. Tsien, R. Y. Fluorescent indicators of ion concentrations. \textit{Methods Cell Biol.} \textbf{30}, 127–156 (1989).
7. Petty, H. R. Spatiotemporal chemical dynamics in living cells: from information trafficking to cell physiology. \textit{Biosystems} \textbf{83}, 217–224 (2006).
LETTERS

11. Shiferaw, Y., Aistrup, G. L. & Wasserstrom, J. A. Intracellular Ca2+

10. Yuste, R. & Denk, W. Dendritic spines as basic functional units of neuronal integration. Nature 375, 682–684 (1995).

9. Cheng, A., Goncalves, J. T., Golshani, P., Arisaka, K. & Portera-Cailliau, C. Optical recording of action potentials in mammalian neurons using a microbial rhodopsin. Nature Methods 9, 90–95 (2012).

8. Grinvald, A., Anglister, L., Freeman, J. A., Hildesheim, R. & Manker, A. Real-time optical imaging of naturally evoked electrical-activity in intact frog brain. Nature 308, 848–850 (1984).

7. Allard, W. J. Isolation of rare circulating tumour cells in cancer patients by high-throughput single-microparticle imaging flow analyzer. Proc. Natl Acad. Sci. USA 109, 11630–11635 (2012).

6. Nagrath, S. et al. Frequency division multiplexed multichannel high-speed fluorescence confocal microscope. Nature Photon. 7, 33–37 (2013).

5. Sanders, J. S., Driggers, R. G., Halford, C. E. & Griffin, S. T. Imaging with frequency-modulated reticles. Opt. Eng. 30, 1720–1724 (1991).

4. Futia, G., Schlup, P., Winters, D. G. & Bartels, R. A. Spatially-chirped modulation imaging of absorption and fluorescent objects on single-element optical detector. Opt. Express 19, 1626–1640 (2011).

3. Wu, F. et al. Frequency division multiplexed multichannel high-speed fluorescence confocal microscope. Biophys. J. 91, 2290–2296 (2006).

2. Deisseroth, K., Takahashi, K., Easterday, R. B. & Yuste, R. Optical recording of naturally-occurring fast dynamic phenomena in the mammalian visual system. Nature 375, 685–688 (1995).

1. Yuste, R. & Denk, W. Imaging and analyzing parameters of small moving objects such as cells. US patent 6,211,955 (2001).

Acknowledgements

The authors thank D. Di Carlo (UCLA) for use of his laboratory’s cell culture facilities. The authors acknowledge the Broad Stem Cell Research Center at UCLA 2012 Innovation Award for financial support. The authors also thank L. Bentolila for assistance with the EMCCD imaging, which was performed at the California NanoSystems Institute Advanced Light Microscopy/Spectroscopy Shared Facility at UCLA.

Author contributions

E.D.D. conceived of the beat frequency multiplexing approach, built the FIRE microscope, and collected the data. D.R.G. cultured and stained the biological samples, and fabricated the microchip technology. B.W.B. conceived of and implemented the demodulation algorithms, generated the phase engineered excitation frequency combs, and performed image processing. D.R.G. cultured and stained the biological samples, and fabricated microfluidic channels. B.J. conceived of and implemented the demodulation algorithms, generated the phase engineered excitation frequency combs, and performed image processing. D.R.G. cultured and stained the biological samples, and fabricated microfluidic channels. B.J. conceived of the use of DDS and other communication techniques for FIRE, and supervised the project. E.D.D. wrote the first draft of the manuscript, and all authors contributed to subsequent revisions.

Additional information

Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to E.D.D.

Competing financial interests

The authors declare no competing financial interests.

The authors declare no competing financial interests.