

Wide-field bond-selective fluorescence imaging: from single-molecule to cellular imaging beyond video rate

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Wide-field (WF) imaging is pivotal for observing dynamic biological events. While WF chemical microscopy offers high molecular specificity, it lacks the sensitivity for single-molecule detection. In contrast, WF fluorescence microscopy provides live-cell dynamic mapping but fails to leverage the rich chemical information necessary for functional interpretations. To address these limitations, we introduce wide-field bond-selective fluorescence-detected infrared-excited (WF-BonFIRE) spectro-microscopy. This technique combines rationally optimized imaging speed and field of view (FOV) to achieve single-molecule sensitivity with bond-selective contrast. We demonstrate WF-BonFIRE's capabilities in imaging single molecules, cells, astrocytes, and live neurons, capturing single FOVs up to $50\ \mu\text{m} \times 50\ \mu\text{m}$, with further expansion via multi-FOV mosaicking. Additionally, we have implemented a temporal-delay modulation scheme that allows real-time kilohertz WF-BonFIRE imaging with speeds up to 1500 Hz. We showcase the millisecond temporal resolution through monitoring the random motion of live *Escherichia coli*. Leveraging its ability to distinguish molecules through distinct narrow-band BonFIRE signals, we further demonstrate multicolor real-time *E. coli* tracking. WF-BonFIRE should significantly broaden the boundary for chemical imaging, enabling high-speed observations at unparalleled sensitivity levels. © 2025 Optica Publishing Group under the terms of the [Optica Open Access Publishing Agreement](#)

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1. INTRODUCTION

Biological processes are inherently heterogeneous and dynamic. Consequently, the ability to image subcellular live-cell activities with high-sensitivity and fast speed has revolutionized our understanding of fundamental biology [1,2]. To this end, point-scanning microscopy configurations face fundamental constraints to achieve high temporal resolution due to serial pixel-by-pixel acquisition [1,3,4]. In contrast, wide-field (WF) microscopy, with down to single-molecule sensitivity, enables the capture of dynamic biomolecular processes, such as those involving RNA, proteins, and metabolites, at video-rate speeds simultaneously across the entire field of view (FOV). This capability is particularly valuable for applications like imaging and tracing neuronal action potentials that occur at millisecond timescales across entire neurons and neighboring cells [2,5]. Furthermore, leveraging the high-sensitivity capabilities of wide-field imaging, advanced functional fluorescence microscopy techniques have been developed, including single-molecule localization microscopy (SMLM), single-molecule fluorescence *in situ* hybridization (smFISH) microscopy, and structured illumination microscopy (SIM), which have significantly advanced our understanding of complex and dynamic biological phenomena to unprecedented levels [6–9].

Over the past two decades, chemical imaging techniques have dramatically advanced, offering high molecular specificity and detailed bond-selective information crucial for biological research [10]. Despite these advances, achieving single-molecule sensitivity in WF chemical imaging remains a formidable challenge. Raman scattering suffers from inherently small cross-sections of vibrational transitions, ranging from 10^{-30} to $10^{-28}\ \text{cm}^2$, more than 10 orders of magnitude smaller than the visible absorption cross-sections in fluorescence spectroscopy [11]. Nonlinear Raman techniques, such as stimulated Raman scattering (SRS), addresses this limitation with up to 10^8 stimulated emission amplification with two simultaneous pulsed lasers [12]. However, the reliance on tightly focused nonlinear excitation limits the scope of WF biological imaging, even with the most recent advances of electronic pre-resonance SRS (epr-SRS) and stimulated Raman-excited fluorescence (SREF) [13–16]. Conversely, mid-infrared (MIR) spectroscopy, with its substantially larger linear MIR absorption cross-sections (10^{-22} – $10^{-17}\ \text{cm}^2$), enhances the feasibility of high-speed WF imaging [17–19]. Recently, MIR photothermal microscopy has achieved significant advances, in both laser-scanning and wide-field imaging modes [20–22]. To enhance sensitivity, wide-field fluorescence-detected photothermal microscopy (WF-F-MIP) was developed [23]. It measures the modulation in fluorescent quantum yield of the reporter

fluorophores, induced by local thermal changes from the MIR vibrational excitation of nearby target non-fluorescent molecules. This scheme increased the sensitivity by up to 100-fold. However, these photothermal-based methods still remain considerably far from single-molecule sensitivity for universal probing of low-copy biological targets.

To advance toward single-molecule detectability, a seemingly similar but fundamentally different approach has proven to be effective. Building on the pioneering work of Kaiser and co-workers in 1975 [24], recent efforts focused on exploiting a nonlinear double-resonance fluorescence detection scheme mediated by MIR-excited vibrational states, through the adoption of various laser excitation and detection strategies [25–28]. The successfully achieved single-molecule detection and spectroscopy is expected to open the door for many new applications from biological imaging to single-molecule catalysis [25,26]. However, current demonstrations remain challenging for wide-field imaging with down to single-molecule sensitivity and up to video rate while maintaining superb biocompatibility [27,28].

Implemented with picosecond (ps) excitation pulses, we recently demonstrated bond-selective fluorescence-detected infrared-excited (BonFIRE) microscopy [26]. BonFIRE achieves single-molecule sensitivity while capturing mid-infrared chemical information for live biological imaging, representing a significant leap in bio-imaging capabilities [26,29]. Employing a 2-ps

pair of MIR and near-infrared (NIR) lasers for bond-selective double-resonance excitation, BonFIRE transfers the rich MIR-excited vibrational information into fluorescence signals for highly sensitive detection [Fig. 1(a)]. The initial design of BonFIRE utilized a point-scanning (PS) mode with tightly focused beams of both the MIR and the NIR lasers to ensure single-molecule detectability across the wide fingerprint and cell-silent regions ($1300 - 2400 \text{ cm}^{-1}$) for double- and triple-bond vibrational modes [26]. However, the serial-acquisition PS-BonFIRE is limited by its temporal resolution, making it challenging to capture rapid dynamic processes.

Here, we report wide-field (WF)-BonFIRE [Fig. 1(b)]. Through carefully balanced design and simulations of sensitivity against laser power, we push the imaging speed and the FOV limits of WF-BonFIRE to its maximum while achieving single-molecule sensitivity (Supplement 1, Table S1). Such a design facilitates up to 10,000-fold faster frame acquisition compared to PS-BonFIRE, under the same number of pixels and SNR. In addition, we demonstrate exceptional WF-BonFIRE imaging performance in cells, astrocytes, and in live neurons, capturing intricate structural details and networks with robust signal-to-noise ratios (SNRs). To further achieve kilohertz frame rate, we implement a new temporal-delay modulation scheme that obtains up to 1500 frames per second (FPS) for WF-BonFIRE. We showcase the performance of temporal-delay modulation by tracking

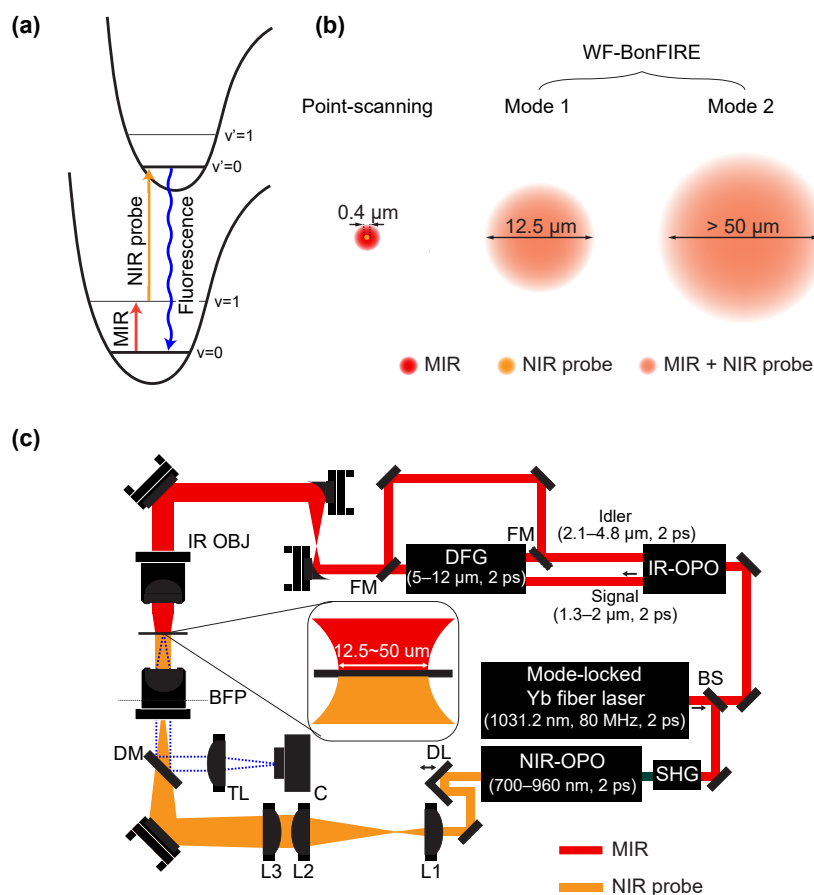


Fig. 1. Principle and design of WF-BonFIRE. (a) Energy diagram of BonFIRE spectroscopy. v and v' represent the vibrational states at ground and excited electronic states, respectively. (b) Two implementation modes of WF-BonFIRE (Mode 1 and Mode 2), in comparison to the point-scanning mode. Not drawn to scale. (c) WF-BonFIRE experimental scheme. Dotted blue line indicates fluorescence detection path. OBJ, objective; BFP, back focal plane; DM, dichroic mirror; TL, tube lens; C, camera; L, lens; DL, delay line; BS, beam splitter; DFG, difference frequency generation; OPO, optical parametric oscillator; SHG, sum frequency generation; FM, flip mirror.

the random motion of live *Escherichia coli* (*E. coli*). Furthermore, by distinguishing fluorophores with overlapping electronic spectra but distinct bond-selective MIR peaks, WF-BonFIRE enables precise multicolor differentiation in real time. We anticipate that WF-BonFIRE will significantly push the boundaries of both chemical imaging and fluorescence imaging, facilitating high-speed and high-throughput imaging at the single-molecule level [Fig. 1(c)].

2. RESULTS

A. Rational Design and Simulation of WF-BonFIRE

We first rationalize the feasibility of WF-BonFIRE especially in the single-molecule regime. Contrary to conventional virtual-state mediated two-photon imaging techniques such as two-photon fluorescence and SRS microscopy, which face challenges in high-sensitivity WF implementation due to high photon-flux requirements, BonFIRE is a real vibrational-state-mediated non-degenerate two-photon excitation process. Employing 2 ps laser excitation achieves a balance between bond-selectivity and efficient vibrational excitation, which competes with the picosecond vibrational relaxation lifetime (Supplement 1, Fig. S1). The up-conversion step is also highly efficient due to the large electronic absorption cross-section. These efficient excitation steps hence alleviate the high photon-flux requirement, thus allowing picosecond laser pulses to spread over a wider focal area. In addition, in PS-BonFIRE [Fig. 1(b), Point-scanning], the diffraction-limited MIR spot is significantly larger than that of the NIR probe laser, which results in more than 50% of MIR photons not being utilized for signal generation. Given the excess power of the NIR probe laser, the most straightforward design of WF-BonFIRE is to expand the NIR probe beam to match with the diffraction-limited spot of the MIR beam, thereby optimizing the utilization of MIR photons with opportunities for additional area expansion.

To quantitatively model the optimal FOVs, we next calculated the achievable signals and SNRs as a function of FOV using a high-sensitivity sCMOS camera (Supplement 1, Fig. S2, Eqs. S1–S3). At a 12.5 μm FOV, the achievable WF-BonFIRE signal from a single Rhodamine 800 (Rh800) molecule is estimated to reach 1 photon/ms with a SNR of three, under a short camera exposure time of 25 ms. In situations where single-molecule sensitivity is not essential, the FOV can be further expanded. Hence, we investigated the maximum FOV for higher concentration samples. To this end, we introduced a pixel rate ratio, R [Eqs. (S4) and (S5)], for comparing speeds between WF-BonFIRE and PS-BonFIRE. At the upper limit of $R = 1$ where WF-BonFIRE theoretically matches the speed of PS-BonFIRE, FOV of WF-BonFIRE extends to 620 μm . However, reaching such large FOV results in reduced detection sensitivity, due to the inverse quadratic relationship between laser intensity and the illumination size for each beam (Supplement 1, Fig. S3).

Based on the above simulation, we implemented WF-BonFIRE in two modes, each providing optimal combination of sensitivity, speed, and FOV for different biological applications. In Mode 1 [Fig. 1(b)], we expanded and matched the sizes of the NIR probe and the MIR beams to a diameter of 12.5 μm for fast single-molecule imaging applications, paving the way toward WF-BonFIRE SMLM. In Mode 2 [Fig. 1(b)], both beams are expanded and matched to 50 μm , sufficient to cover an entire mammalian cell while achieving an optimal balance between SNR and speed.

At 50 μm FOV, the imaging speed of WF-BonFIRE is estimated to be over 150 times faster than PS-BonFIRE (Supplement 1, Fig. S3, $R > 150$). The smaller pixel rate ratio R at 50 μm FOV (Mode 2) compared to that at 12.5 μm FOV (Mode 1) is due to the quadratic reduction in photon flux across both MIR and NIR beams (Supplement 1, Fig. S3). When a larger FOV is needed, a parallel mosaicking approach is applied by precisely moving the piezo stage. Experimentally, we focused the probe beam onto the back focal plane of the objective to ensure uniform illumination [Fig. 1(c)]. Simultaneously, the MIR beam underwent expansion using a lower-numerical-aperture (NA) MIR objective to align its spot size with that of the probe beam. System magnification was carefully determined to meet the requirements of the Nyquist theorem (Supplement 1, Fig. S4).

B. Characterization of WF-BonFIRE (Mode 1) Spectroscopy and Imaging Performance

We first validated WF-BonFIRE (Mode 1) by targeting the C = C bond (1598 cm^{-1}) of Rh800, a NIR fluorescent molecule with an absorption peak at 696 nm (Supplement 1, Fig. S5). We tuned the NIR probe wavelength to 788 nm to achieve a sum frequency of NIR and MIR lasers that matches the absorption peak maximum (Supplement 1, Fig. S5). The WF-BonFIRE signal of Rh800-embedded spin-coated polymer film was generated by subtracting an image captured with a 20 ps temporal delay between MIR and NIR pulses from that with overlapping pulses ($t_D = 0\text{ ps}$) [Figs. 2(a) and 2(b)]. Minimal photobleaching was observed over 60 consecutive frames, as indicated by the flat baseline. The background from temporally separated laser pulses is attributed to anti-Stokes fluorescence and photothermal signal, resulting from local temperature increases from the environment, both of which are constant across the temporal profile (Supplement 1, Fig. S6) and exhibit no vibrational characteristics from the target molecules [26]. The authenticity of the BonFIRE signal was further verified by the WF-BonFIRE spectrum, obtained by scanning the MIR laser wavelength, which closely aligns with the FTIR spectra from Rh800 in solution [Fig. 2(c)] featuring prominent peaks at 1505 and 1598 cm^{-1} , and by the linear power dependence on both MIR and NIR laser powers (Supplement 1, Fig. S7). The difference in relative amplitude between FTIR and BonFIRE is due to the extra Franck-Condon factor involved in the BonFIRE vibronic excitation step. Slight spectral broadening observed in the WF-BonFIRE spectrum is primarily due to the laser broadening with a MIR pulse bandwidth of 10 cm^{-1} . An additional difference in peak shift may have resulted from the molecular aggregation effect due to high concentrations (millimolar) required for FTIR measurements but not in BonFIRE, which can perform spectral measurement with nM solution concentration.

To demonstrate the high sensitivity of WF-BonFIRE (Mode 1), single molecules of Rh800 were imaged with robust SNRs (>48). This was achieved under an exposure time of 200 ms [Fig. 2(d), Supplement 1, Fig. S8] and even as short as 20 ms [Figs. 2(g) and 2(h)], which is consistent with the predictions in Supplement 1, Fig. S2. For single-molecule measurements, the imaging speed of WF-BonFIRE exceeds that of PS-BonFIRE by over 200 times, underscoring its potential use in advanced super-resolution microscopy techniques like SMLM. Standard single-step photobleaching and blinking curves were observed throughout, indicating the single-molecule nature of the sample

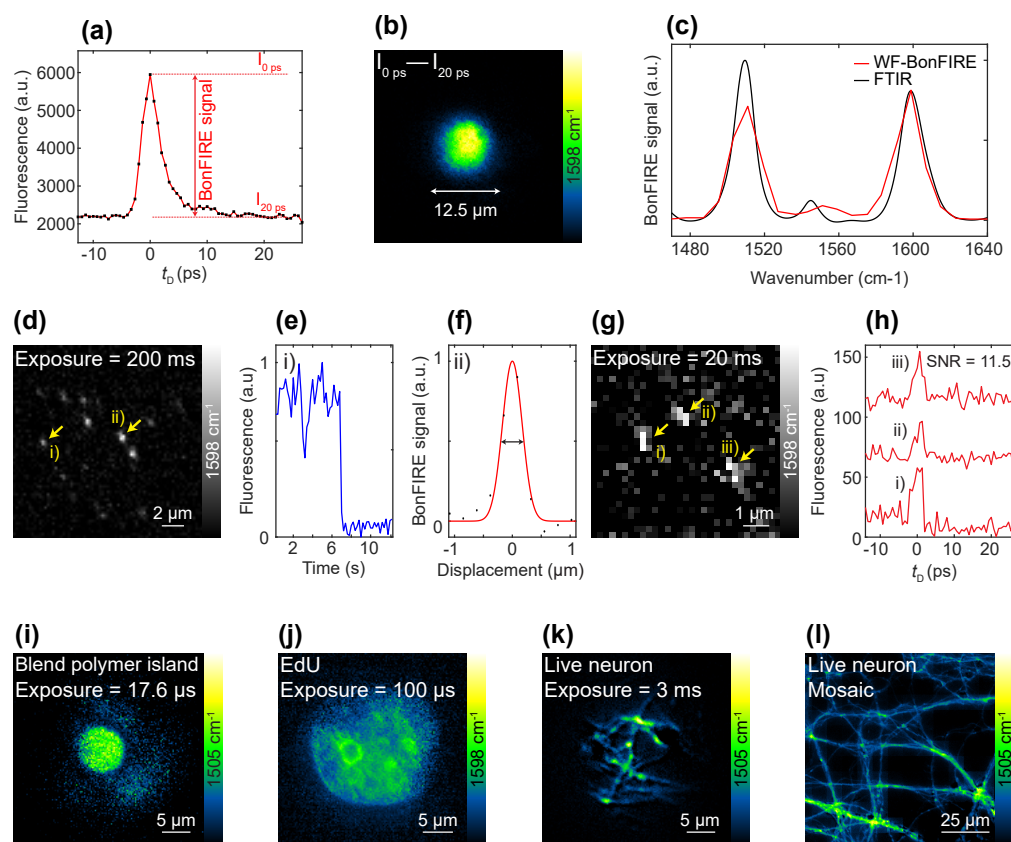


Fig. 2. Characterization of WF-BonFIRE (Mode 1) spectroscopy and imaging performance. (a) WF-BonFIRE signal of Rh800 polymer film as a function of temporal delay (t_D). (b) WF-BonFIRE image of Rh800 polymer film generated by subtracting two images acquired at temporal delays 0 ps and 20 ps [$I_{0\text{ ps}}$ and $I_{20\text{ ps}}$ shown in (a)]. (c) Overlay of WF-BonFIRE (red) and FTIR (black) spectra of Rh800. (d) Single-molecule WF-BonFIRE image of Rh800 at 1598 cm^{-1} . Exposure time: 200 ms. (e) Exemplary single-step photobleaching curve of a single molecule (i) in (d) for confirming single-molecule detection. (f) Cross-section profile of a single molecule (ii) in (d). (g, h) Short-exposure (20 ms) WF-BonFIRE image (g) of single-molecule Rh800, with corresponding temporal profiles (h) for single molecules of (i), (ii), and (iii) in (g). SNR = 11.5 ($N = 3$). (i) WF-BonFIRE images of Rh800 blend polymer island at on-resonance (1505 cm^{-1}). Exposure time: $17.6\text{ }\mu\text{s}$. (j) WF-BonFIRE image targeting C = C vibration in ATTO680-click-labelled EdU in the nuclei of HeLa cells at on-resonance (1598 cm^{-1}). (k, l) Single FOV (k) and mosaic (l) WF-BonFIRE images of Rh800-labelled mitochondria in live mouse neuronal cultures acquired at on-resonance (1505 cm^{-1}). Exposure time: 3 ms. Acquisition area in (l): $100 \times 100\text{ }\mu\text{m}^2$.

[Fig. 2(e), Supplement 1, Fig. S8] [30–32]. Here our single-molecule BonFIRE reaches a high signal-to-background ratio of five. By utilizing a single molecule as a point source, the spatial resolution of our WF-BonFIRE was next characterized to be 400 nm [Fig. 2(f)], confirming its diffraction-limited performance.

We then applied WF-BonFIRE (Mode 1) to rapidly image various samples ranging from blend polymer films to live neurons. An exposure time of $17.6\text{ }\mu\text{s}$ was achieved when imaging a Rh800-labeled blend polymer film, consisting of phase-separated polystyrene and polymethylmethacrylate segments [Fig. 2(i)] with high bond-selectivity (Supplement 1, Fig. S9a). The imaging speed was indeed 10,000 times faster than PS-BonFIRE, closely aligning well with our earlier predictions (Supplement 1, Fig. S3). In these experiments, a single polymer island was captured, which was approximately the size of small mammalian cells ($\sim 10\text{ }\mu\text{m}$). Additionally, we imaged a single nucleus of a HeLa cell using microsecond-level exposure time [Fig. 2(j) and Supplement 1, Fig. S9b, $100\text{ }\mu\text{s}$], using ATTO680 click-labeled 5-ethynyl-2'-deoxyuridine (EdU) in newly synthesized DNA. The short exposure time demonstrates the high sensitivity of WF-BonFIRE, which is critical for enabling potential biological imaging at kilohertz speeds in temporally modulated systems, as

illustrated later in Fig. 4, a feat challenging for existing chemical-selective imaging modalities. Leveraging its high sensitivity and speed, WF-BonFIRE further demonstrated live neuron imaging at an exposure time of 3 ms, revealing distributions of mitochondria labeled with Rh800 [Figs. 2(k) and 2(l), and Supplement 1, Fig. S9c]. Such speeds fulfill the necessary requirements for resolving fast dynamics in cells, such as voltage imaging in neurons. Additionally, by mosaicking single FOV WF-BonFIRE images, a network of live neurons was captured within $\sim 30\text{ s}$, enabling extensive imaging of a large area ($100 \times 100\text{ }\mu\text{m}^2$) of live cells [Fig. 2(l)].

C. Large FOV WF-BonFIRE Imaging (Mode 2)

We then explored the expanded FOV capabilities of WF-BonFIRE (Mode 2), which more effectively captures a larger area for biological samples at $50\text{ }\mu\text{m}$. Imaging of a Rh800 blend polymer sample is achieved at an exposure time of $500\text{ }\mu\text{s}$ [Fig. 3(a)]. Additionally, a $200 \times 200\text{ }\mu\text{m}^2$ area was acquired within 1.3 s using mosaicking [Fig. 3(b)]. Although Mode 2 lacks single-molecule sensitivity, it effectively imaged a wide variety of biological targets, including low-abundance proteins at micromolar concentrations and below [Figs. 3(c)–3(h)]. Using ATTO680-immuno-labeled GFAP, a

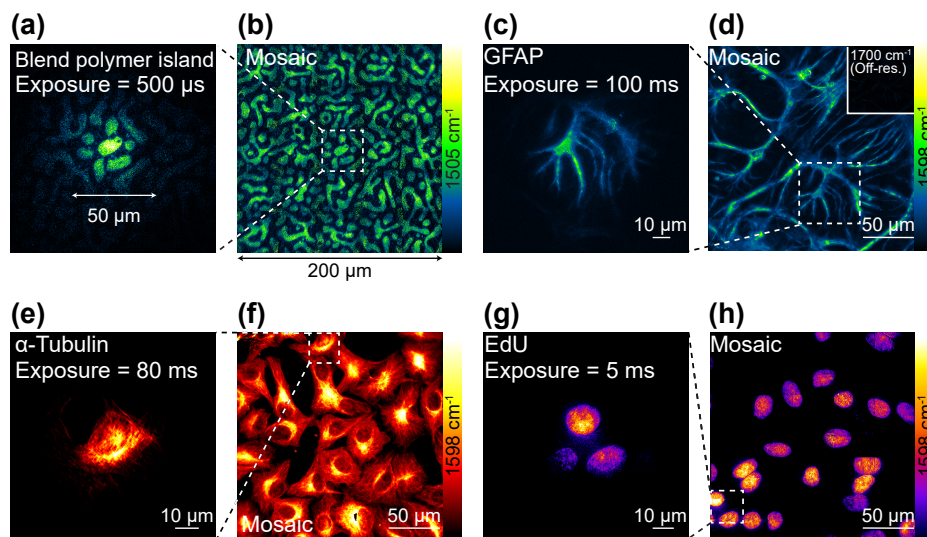


Fig. 3. Large FOV WF-BonFIRE imaging (Mode 2). (a), (b) Single FOV (a) and mosaic (b) WF-BonFIRE images of Rh800 blend polymer film acquired at 1505 cm^{-1} . Exposure time: $500\text{ }\mu\text{s}$. Acquisition area in (b): $200 \times 200\text{ }\mu\text{m}^2$. (c)–(h) Single FOV (c), (e), (g) and mosaic (d), (f), (h) WF-BonFIRE images targeting $\text{C}=\text{C}$ vibration (1598 cm^{-1}) for ATTO680-immunolabeled GFAP in mouse neuronal co-cultures [(c) and (d); exposure time: 100 ms ; acquisition area in (d): $200 \times 200\text{ }\mu\text{m}^2$]; ATTO680-immunolabelled α -tubulin in HeLa cells [(e) and (f); exposure time: 80 ms ; average: five frames; acquisition area in (f): $200 \times 200\text{ }\mu\text{m}^2$]; and ATTO680-click-labelled EdU in the nuclei of HeLa cells [(g) and (h); exposure time: 5 ms ; average: 16 frames; acquisition area in (h): $200 \times 200\text{ }\mu\text{m}^2$].

key marker protein for astrocytes, we obtained an exposure time of 100 ms for single-FOV WF-BonFIRE (Mode 2) imaging that targeted the 1598 cm^{-1} of the $\text{C}=\text{C}$ vibration [Figs. 3(c) and 3(d)]. This enabled capturing a $200 \times 200\text{ }\mu\text{m}^2$ area within 10 s [Fig. 3(d), Mosaic], with high bond-selectivity [Fig. 3(d), inset]. Similarly, ATTO680-labeled α -tubulin [Figs. 3(e) and 3(f)] and EdU [Figs. 3(g) and 3(h)] were imaged with WF-BonFIRE (Mode 2). The high SNR enabled resolving clear tubulin [Fig. 3(e)] and nuclei [Fig. 3(g)] structures, providing detailed insights into their spatial organization. Here, a single-FOV image successfully captured an entire HeLa cell [Fig. 3(e)] and multiple nuclei [Fig. 3(g)].

D. Kilohertz WF-BonFIRE Imaging with Newly Developed Temporal-Delay Modulation

A major advantage of WF microscopy is its ability to perform live-cell dynamic imaging at kilohertz frame rates surpassing video-rate FPS, which is valuable for applications such as tracking bacterial movement or mapping the neuronal action potentials. Although WF-BonFIRE achieved microsecond acquisition speed for a single image, a bottleneck to reaching kilohertz frame rate is the requirement to subtract subsequent images between the temporal on ($t_D = 0\text{ ps}$) and off states ($t_D = 20\text{ ps}$) to effectively remove the flat photothermal background, which accumulates over a slower timescale [26]. The adjustment of the temporal delay involves tuning a delay stage, which requires an additional 0.2 s mechanical settling (Supplement 1, Fig. S10), thereby hindering the achievement of real-time dynamic imaging. High-frequency modulation of the MIR proved to be an effective approach in removing photothermal background in PS-BonFIRE [26]. However, such approach is challenging with high-sensitivity wide-field camera detection with such high-frequency modulation (beyond 1 MHz).

To address this challenge, we developed a temporal-delay modulation scheme that allows instantaneous modulation of pulse

delays without relying on the physical position of the mechanical delay line [Figs. 4(a) and 4(b)]. To achieve this, the NIR probe beam was split into two pulse trains of equal intensity using a beam splitter [Figs. 4(a) and 4(b), BS1]. The equal intensity for each beam arm was carefully calibrated using a continuously variable neutral density (ND) filter. One path was deliberately lengthened by Δl relative to the other [Fig. 4(b)], introducing an additional delay (t_D) of about 20 ps compared to the pulse trains from the shorter path with $t_D = 0\text{ ps}$ [Fig. 4(a)]. To synchronize imaging, a chopper rotating at half the frequency of camera frame rate was employed to alternately allow pulse trains from only one path to reach the sample at a time, while blocking the other [Figs. 4(a) and 4(b), Chopper]. These two beams were then recombined using a second beam splitter [Figs. 4(a) and 4(b), BS2] and directed towards the sample, producing pulse trains with alternating temporal delays [Fig. 4(c), Recombined at BS2]. The chopper was precisely synchronized with the camera, ensuring that one image was captured with only the first pulse train at $t_D = 0\text{ ps}$, followed by another image with the second pulse train at $t_D = 20\text{ ps}$, [Figs. 4(d) and 4(e)].

In Fig. 4(d), we present a wiring diagram for control signals that govern the camera and lock-in synchronization within the system. The reference output from the chopper ($y(f)$) is doubled in frequency by a lock-in amplifier, and this digital output [$y_{\text{trigger}}(2f, \theta)$] serves as the trigger for the sCMOS camera's exposure [Figs. 4(d) and 4(e), $y_{\text{exposure}}(2f, \theta, T)$]. The critical elements for achieving precise synchronization are the camera's exposure timing output and the recombined alternating pulse train [Fig. 4(e)]. To ensure seamless coordination, adjustments are made to the lock-in phase (θ) to account for the chopper's rise and fall times, during which the beam partially obstructs. Moreover, the exposure duration (T) is set to approximately 60% of the reciprocal of the doubled chopper frequency ($1/2f$).

Utilizing the temporal-delay modulation scheme, we performed real-time WF-BonFIRE imaging on a Rh800 blend

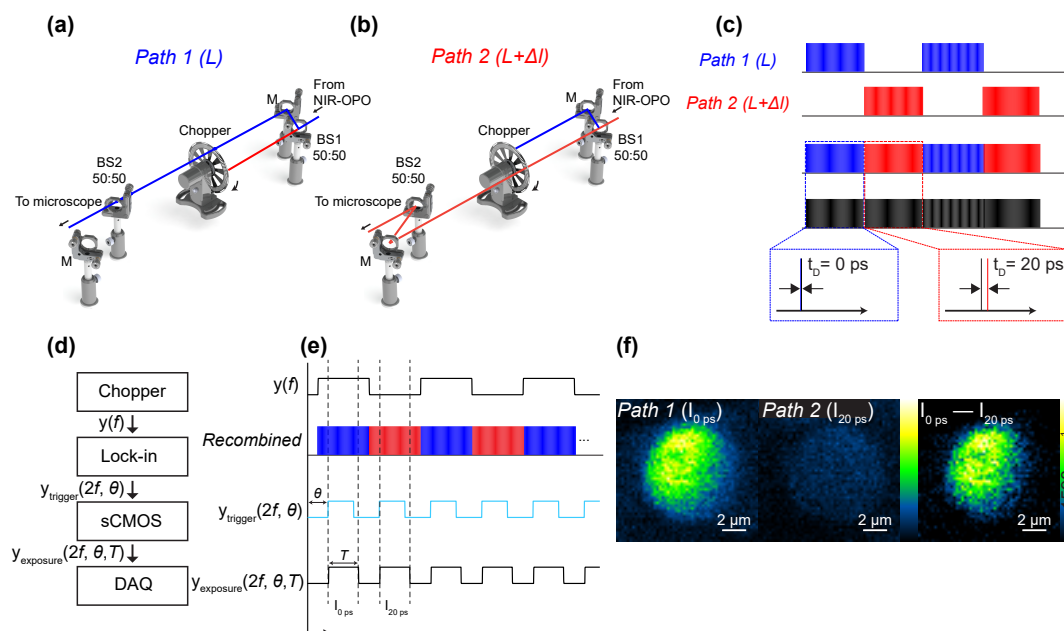


Fig. 4. Kilohertz WF-BonFIRE imaging using temporal-delay modulation using Mode 1 (FOV = 12.5 μm). (a)–(c) Experimental setup of temporal-delay modulation scheme. NIR laser pulses, divided into Path 1 [(a), blue] and Path 2 [(b), red], which have the exact same intensity but with different path lengths (Δl), introduce different temporal delays (0 and 20 ps) relative to the pulse trains of the MIR pulses after recombination at BS2 (c). A chopper is precisely aligned and synchronized to ensure that only one path of NIR pulse trains reaches the sample at any given time. BS: beam splitter. (d) Wiring diagram for camera-chopper synchronization. (e) Synchronization timing chart. (f) Kilohertz (FPS, 1500 Hz) WF-BonFIRE image ($I_{0\text{ ps}} - I_{20\text{ ps}}$) acquired using temporal-delay modulation, through subtraction of subsequent camera frame images, where one image is from Path 1 pulse trains [Path 1 ($I_{0\text{ ps}}$)] and the other image is from Path 2 pulse trains [Path 2 ($I_{20\text{ ps}}$)].

polymer sample [Fig. 4(f)]. Subsequent camera frames corresponding to Path 1 ($I_{0\text{ ps}}$) and Path 2 ($I_{20\text{ ps}}$) delays were captured according to the chopper's modulation frequency [Fig. 4(e)]. Consequently, a background-free WF-BonFIRE image [Fig. 4(f), $I_{0\text{ ps}} - I_{20\text{ ps}}$] was directly obtained through subtraction. As a control, dark images were consistently confirmed by setting the time delays of Path 1 and Path 2 to the temporal-off position, indicating the absence of artifacts or power difference between the two paths (Supplement 1, Fig. S11). Using the temporal-delay modulation, we achieved kilohertz real-time WF-BonFIRE imaging at 1500 FPS [Fig. 4(f)]. To demonstrate WF-BonFIRE's dynamic imaging capabilities beyond video rate, we applied it to track the random motion of Rh800-stained *E. coli*—a critical aspect of microbial behavior in liquid environments. The diffusion coefficient of *E. coli* has been experimentally measured to range from 10 to 100 $\mu\text{m}^2/\text{s}$ in aqueous buffers and polymeric solutions [33]. Furthermore, *E. coli* exhibit rapid rotational and tumbling motions [34]. To effectively capture and analyze this rapid microscopic motion, high-speed microscopy with frame rates exceeding several tens of frames per second is required. We acquired WF-BonFIRE images at 150 FPS. In Fig. 5(a), eight consecutive frames within 50 ms are displayed, confirming that WF-BonFIRE can precisely track the rapid movement of *E. coli* without motion artifacts [Figs. 5(a) and 5(b), Visualization 1].

E. Multicolor Tracking of *E. coli* Using Temporal-Delay Modulation

Leveraging the unique advantage of high-speed (at 150 FPS) and chemically sensitive WF-BonFIRE imaging, we sought to achieve multicolor imaging at the same frame rate. To test this, we used

widely used fluorophores Rh800 and Cy5.5 [Fig. 5(c)]. Despite the nearly identical emission and absorption spectra of these dyes [Fig. 5(d)], which make them difficult to distinguish using fluorescence alone, their distinct chemical structures result in resolvable BonFIRE peaks [Fig. 5(e)], enabling clear differentiation of the two molecules in the BonFIRE channel. By employing temporal-delay modulation, we captured successive camera frames at Path 1 ($I_{0\text{ ps}}$) and Path 2 ($I_{20\text{ ps}}$) delays at 300 Hz, corresponding to a WF-BonFIRE frame rate of 150 FPS. At 1484.6 cm^{-1} , strong WF-BonFIRE signal was observed in single-color *E. coli* stained with Cy5.5, while no WF-BonFIRE signal was detected in those stained only by Rh800 (Supplement 1, Fig. S12, Visualization 2 and Visualization 3). We then mixed two populations of *E. coli*, each stained separately with Rh800 and Cy5.5, and imaged at 1484.6 cm^{-1} and 150 FPS. Although a single fluorescence channel showed no distinguishable differences between the three *E. coli* cells [Fig. 5(f), $I_{0\text{ ps}}$ and $I_{20\text{ ps}}$], our WF-BonFIRE channel [Fig. 5(f), $I_{0\text{ ps}} - I_{20\text{ ps}}$, 1484.6 cm^{-1}] clearly differentiated them: *E. coli* containing Cy5.5 exhibited a strong BonFIRE signal, while those with Rh800 showed essentially no signal at 1484.6 cm^{-1} , but a detected fluorescence at $I_{20\text{ ps}}$ [Fig. 5(g) and Supplement 1, Fig. S13]. As a control, tuning the MIR laser excitation to 1400 cm^{-1} , where both dyes are vibrationally off-resonant, resulted in no BonFIRE signal for any *E. coli* entering the field of view, monitored by fluorescence at $I_{20\text{ ps}}$ [Fig. 5(f), 1400 cm^{-1}]. The color-coded images [Fig. 5(h), Visualization 4 and Supplement 1, Fig. S14], based on chemically sensitive WF-BonFIRE signal [Fig. 5(g)], effectively captured the real-time movement of *E. coli*, enabling multicolor tracking at speeds far exceeding the standard video rate.

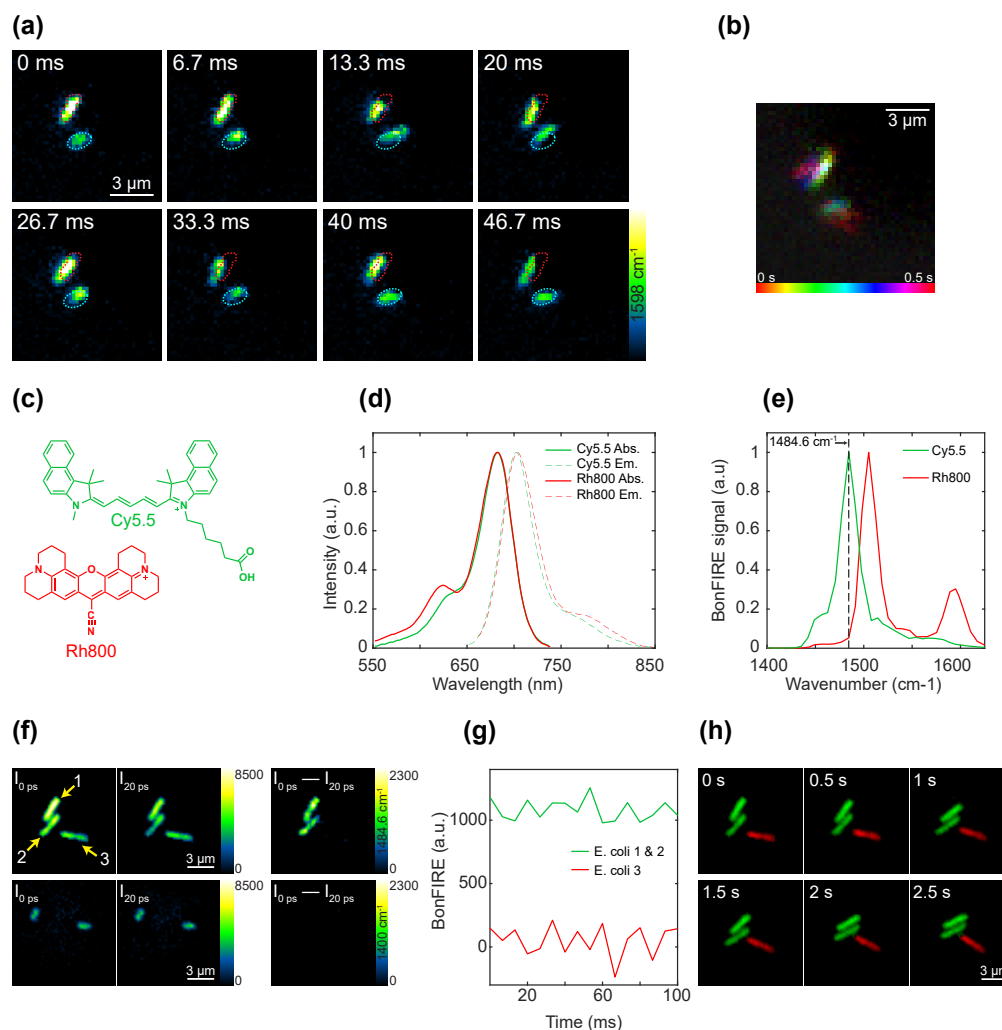


Fig. 5. High-speed WF-BonFIRE imaging with real-time and multicolor visualization of fluorophore-stained *E. coli* at 150 frames per second (FPS) using temporal-delay modulation. (a), (b) Single-color WF-BonFIRE imaging (a) of Rh800-stained *E. coli* at sequential time points. Red and blue dotted profiles indicate the positions of the Rh800-stained *E. coli* at $t = 0$ ms frame. FPS: 150 Hz. Temporal color-coded image (b) showing the moving trajectory of *E. coli* over a period of $t = 0$ to 0.5 s. (c)–(h) Multicolor WF-BonFIRE imaging at 150 FPS. Chemical structures (c), and absorption (solid) and emission (dashed) spectra (d) in PBS, and the BonFIRE spectra (e) of two dyes Cy5.5 (green) and Rh800 (red). WF-BonFIRE images [(f), $I_{0\text{ ps}} - I_{20\text{ ps}}$] of *E. coli* mixtures separately stained with Cy5.5 and Rh800 imaged at 1484.6 cm^{-1} (top row, 1484.6 cm^{-1}) and at off-resonance (bottom row, 1400 cm^{-1}) frequencies acquired using temporal-delay modulation. BonFIRE signal (g) of *E. coli* 1 and 2, and 3 indicated in (f) at 1484.6 cm^{-1} as a function of time. Color-coded images (h) at different representative time points (Cy5.5, *E. coli* 1 and 2, green and Rh800, *E. coli* 3, red), based on the chemically sensitive WF-BonFIRE signal (g).

3. DISCUSSION

In this manuscript, we present wide-field bond-selective fluorescence imaging that achieves high sensitivity and speed. This advancement is attributed to the efficient vibronic transitions of WF-BonFIRE, which reduce the typically high photon flux requirements of multiphoton microscopy. We demonstrate significant increases in imaging speed using WF-BonFIRE compared to PS-BonFIRE, for both polymer films at high concentrations and single-molecule samples. Key results include achieving a 20 ms exposure time for single-molecule WF-BonFIRE imaging with robust SNRs, and rapid WF-BonFIRE acquisition to capture fine structures in live neurons and cells at low concentrations. This method achieved large FOV imaging ($200 \times 200\text{ }\mu\text{m}^2$) within several seconds, exceeding point-scanning capabilities. Moreover, by introducing a temporal-delay modulation scheme, WF-BonFIRE achieved imaging kilohertz speed up to 1500 FPS, far exceeding

video rates. This development enables the multiplexed tracking of random motion in *E. coli*, which requires millisecond-level temporal resolution, and offers potential to capture other rapid dynamic processes such as neuronal firing.

Imaging speed is influenced by the SNR, which is primarily determined by the photon flux of MIR and NIR probe lasers where detection shot noise is the limiting factor (Supplement 1, Fig. S15 and Table S2). In scenarios where the available laser power exceeds necessity, increasing the FOV by a factor of M (in one dimension) improves imaging speed by M^2 . However, our system primarily operates in a source-limited regime where an increased FOV decreases photon flux and signal, resulting in a net decrease in overall imaging speed despite gains from spatial multiplexing. For this reason, we have implemented two distinct operational modes to accommodate various application needs. Mode 1 is utilized for situations that demand high photon flux, including

single-molecule imaging and fast dynamic imaging. Mode 2 is selected for simultaneous detection across larger areas, suited for relatively more abundant molecular targets. Additionally, using a larger FOV reduces the number of stage steps needed to cover a large acquisition area (e.g., $200 \times 200 \mu\text{m}^2$). As an outlook, adopting a higher-power laser could enable larger FOVs beyond $50 \mu\text{m}$. Achieving speeds beyond 1500 FPS is mostly limited by the current camera technology and frequency constraints of chopper rotation. For instance, the sCMOS camera used in our experiments is limited to a maximum speed of ~ 3500 FPS with external triggering, and the chopper's maximum speed is limited to 10 kHz. Nonetheless, employing electro-optic modulators (EOM) and quartz crystals could provide a solution by enabling temporal-delay modulation at significantly higher frequencies, potentially overcoming these limitations [35].

WF-BonFIRE offers unique advantages over conventional wide-field fluorescence imaging by integrating bond-selectivity with enhanced speed, enabling fast vibrational lifetime imaging in live cells. This makes local environment sensing more accessible, quantitative, and physically interpretable in biological systems. For example, WF-BonFIRE holds exciting potential for electric field sensing, a critical task in molecular biology for characterizing enzyme active sites and mapping hydration [36–38]. Recently, we demonstrated that nitrile vibrational lifetimes can detect local electric fields in a physically interpretable manner, a task challenging for fluorescence due to its complex response to applied fields [29].

In addition to local environment sensing, WF-BonFIRE enables high-speed multiplexed imaging by leveraging the narrow linewidths of vibrational peaks. This capability allows for simultaneous monitoring of multiple molecular targets, providing insights into microbial behavior, live-cell processes, and complex biological interactions in real time [39]. Moving forward, we aim to expand these multiplexing capabilities by targeting additional vibrational peaks in both the fingerprint and cell-silent regions, further broadening the palette of BonFIRE-compatible dyes for high-content imaging (Supplement 1, Fig. S16).

Furthermore, WF-BonFIRE presents significant opportunities for super-resolution bond-selective imaging, overcoming limitations faced by existing fluorescence-based methods. In chemical imaging, achieving sub-100 nm spatial resolution has been challenging due to limited SNR [40–43]. Combining chemical imaging with techniques such as STED and RESOLFT often results in photobleaching and limits multiplexing capabilities. In contrast, the sensitivity and speed of WF-BonFIRE offer considerable opportunities for super-resolution bond-selective imaging, especially using single-molecule localization microscopy (SMLM), which could substantially minimize photobleaching effects [44]. When combined with SMLM, WF-BonFIRE could enable highly multiplexed super-resolution microscopy with local environment sensing [29], addressing a challenge for current fluorescence techniques.

4. MATERIALS AND METHODS

A. Materials

ATTO dyes were purchased from ATTO-TEC. Rhodamine 800 was purchased from Sigma Aldrich. All dyes were aliquoted in DMSO as stock solutions (10 mM) upon receipt and stored at -20°C . The following primary antibodies were used: anti- α -tubulin in rabbit (ab18251, Abcam) and anti-GFAP in

mouse (3670S, Cell Signaling Technology). The following secondary antibodies were used: goat anti-mouse antibody (31160, Invitrogen) and goat anti-rabbit antibody (31210, Invitrogen).

B. Experimental Setup of WF-BonFIRE

The laser sources for WF-BonFIRE are identical to that of PS-BonFIRE1. A 1.8 ps, 80 MHz, 1031.2 nm mode-locked Yb fiber laser (aeroPULSE PS10, NKT Photonics, Copenhagen, Denmark) was used as a seed laser for both NIR and MIR optical parametric oscillators (OPOs), providing wide wavelength tunabilities. The frequency doubled beam was used to pump the NIR-OPO (picoEmerald, Applied Physics and Electronics), which tunes from 700 to 960 nm. IR-OPO (Levante IR, Applied Physics and Electronics, Berlin, Germany) generates an idler beam that tunes from 2.1 to $4.8 \mu\text{m}$ ($2083 - 4762 \text{ cm}^{-1}$). Differential frequency generation (HarmoniXX DFG, Applied Physics and Electronics) inputs signal and idler beams of the IR-OPO to generate MIR wavelengths from 5 to $12 \mu\text{m}$ ($833 - 2000 \text{ cm}^{-1}$). The bandwidth of NIR and MIR pulses is 10 cm^{-1} . NIR power of 1–50 mW and MIR power of 50 mW on sample were used for all Mode 1 and Mode 2 imaging.

For wide-field illumination, the NIR beam was focused onto the back focal plane of the NIR objective (XLPLN25XWMP2, Olympus) using a plano-convex lens to ensure homogeneous illumination. The average power density of the NIR beam focused at the back focal plane was $<2.5 \text{ kW/cm}^2$, below the manufacturer's general damage threshold guideline. The location of the back focal plane was confirmed by monitoring the collimation of the beam after the objective at varying lens positions. A 2000 mm (LA1258-B-ML, Thorlabs) and 500 mm (LA1908-B-ML, Thorlabs) focal length lens were used to achieve $12.5 \mu\text{m}$ (Mode 1) or $50 \mu\text{m}$ (Mode 2) FOVs, respectively. The MIR beam was loosely focused onto the sample plane using a 6.35 mm (39-469, Edmund optics) or a 20 mm (LA7733, Thorlabs) focal length lens to achieve $12.5 \mu\text{m}$ (Mode 1) and $50 \mu\text{m}$ (Mode 2) FOVs, respectively. The lenses used for MIR illumination in Mode 1 and Mode 2 are not optimized for large MIR wavelength ranges, and thus chromatic aberration needs to be considered. To assess the extent of chromatic aberration, we generated chromatic focal shift curves using Code V. These simulations revealed focus shifts of $0.11 \mu\text{m/cm}^{-1}$ and $0.27 \mu\text{m/cm}^{-1}$ for these lenses, respectively. To compensate for this, the z -position of the lens was optimized for each IR frequency using a focusing module. This ensures that the MIR density profile at the sample plane remains consistent, minimizing spectral distortion caused by chromatic aberration. For the imaging path, a sCMOS camera (C15440-20UP, Hamamatsu Photonics) was installed at the focal plane of the tube lens with focal length of 200 mm. The magnification of the imaging system was $200 \text{ mm}/7.2 \text{ mm} = 27.78\times$, confirmed by measuring the effective pixel size. Fluorescence was separated from excitation using a dichroic mirror (FF738-FDi01, AVR optics) and bandpass filter (FF01-665/150, AVR optics). A custom LabVIEW program was used for all data acquisition, including stage scan, delay line movement, and camera capture.

For temporal-delay modulation, a pair of pellicle beam splitters (BP145B2, Thorlabs) was used to divide the beam into two paths. To ensure equal power in both beams, a continuously variable neutral density (ND) filter (NDL-10C-2, Thorlabs) was placed in one of the paths and adjusted until the fluorescence signals measured using a photomultiplier tube (PMT1002, Thorlabs)

from both paths were identical. The XY translation stage of the chopper (MC2000B, Thorlabs) was then fine-tuned to make both beams equidistant from the chopper's center. The optimal position of the chopper was found by minimizing the demodulated anti-Stokes fluorescence signal at the chopper frequency. The reference output from the chopper was frequency-doubled using a lock-in amplifier (HF2LI, 50-MHz bandwidth, Zurich), which was then used to trigger the sCMOS camera. To achieve perfect synchronization between the chopper and the camera, the phase of the lock-in output was adjusted. This setup ensured that only the pulse trains from a single path reached the sample at any given time. For 1500 Hz WF-BonFIRE imaging, the camera was operated at 3000 FPS, capturing consecutive frames alternating between two temporal delays. Each frame consisted of 60×60 pixels, corresponding to a field of view (FOV) of $14 \mu\text{m} \times 14 \mu\text{m}$, sufficient to capture the FOV of Mode 1. The BonFIRE imaging frame rate, therefore, is effectively 1500 FPS. The camera speed is determined by the camera's operation mode and the number of rows acquired. Using external triggering and $\sim 60 \times 60$ pixels, we verified experimentally that the fastest speed that can be achieved by the sCMOS camera is 3480 FPS. This was done by counting the number of frames acquired within a known time window.

For mosaic acquisitions, the unevenness of the beam was addressed by performing a nonlinear background correction, which involved: (1) averaging the frames to generate a background image, (2) fitting the background image with a two-dimensional polynomial, and (3) dividing each frame by the fitted polynomial to correct for power variations.

C. Preparation of Single-Molecule and Polymer Samples

For single-molecule samples, PVA (363138, Sigma) solution of 2.7 mg/mL in H₂O was used to dilute Rh800 (83701, Sigma) solution in DMSO to 1 pM. Rh800-PVA solution was spin coated onto a CaF₂ window (CAFP10-0.35, Crystran) at 5000 RPM for 30 s. For blend polymer film samples, 12 mg/mL polystyrene (430102, Sigma) and 18 mg/mL PMMA (200336, Sigma) in toluene were mixed. After dissolving Rh800 with the mixture to reach a final concentration of 100 μM , Rh800-PS-PMMA solution was spin coated (BSC-100, MicroNano Tools) onto a CaF₂ window at 1200 RPM for 30 s.

D. Preparation of Fixed HeLa Cell Samples

HeLa-CCL2 (ATCC) cells were seeded onto 10 mm diameter 0.35 mm thick CaF₂ windows at a cell density of 105 cells/mL and were cultured in Dulbecco's modified Eagle medium (DMEM) at 37°C with 5% CO₂. The DMEM mixture was composed of 90% DMEM (11965, Invitrogen), 10% fetal bovine serum (FBS; 10082, Invitrogen), and $1 \times$ penicillin/streptomycin (15140, Invitrogen). For HeLa cells with EdU labeling, the medium was switched to FBS-free DMEM (Gibco) for 20–22 h to synchronize the cell cycle. After synchronization, the medium was reverted to the original DMEM and EdU (10 mM stock in H₂O) was added at a concentration of 200 μM for 20–24 h. The cells were then fixed with 4% paraformaldehyde (PFA) for 20 min, and the PFA was removed using Dulbecco's phosphate-buffered saline (DPBS). For click reaction labeling of EdU incorporated cells, the cells underwent permeabilization using 0.2% X-100 (T8787, Sigma) for 20 min. They were then washed with 2% BSA in PBS.

Subsequently, the cells were incubated in a reaction buffer containing 10 μM of ATTO680, prepared as specified in the click reaction buffer kit (Thermo Fisher, C10269). After a 30 min incubation at room temperature, the cells were washed again with 2% BSA in PBS prior to imaging.

E. Preparation of Live and Fixed Neuron Culture Samples

For neuron culture, primary hippocampal neurons were isolated from neonatal Sprague–Dawley rat pups using a Caltech-approved protocol (IA22-1835) by the Institutional Animal Care and Use Committee (IACUC). The brains were removed and immersed in ice-chilled Hanks' balanced salt solution (Gibco) in a 10 cm Petri dish. Under a dissection microscope, the hippocampi were separated, finely minced to approximately 0.5 mm pieces, and digested in 5 mL of 0.25% Trypsin-EDTA (Gibco) at 37°C in a 5% CO₂ incubator for 15 min. After aspiration of the Trypsin-EDTA, the tissue was quickly neutralized with 2 mL of DMEM containing 10% FBS. The tissue pieces were then gently moved into 2 mL of neuronal culture medium (Neurobasal A, B-27 and GlutaMAX supplements, Thermo Fisher, with $1 \times$ penicillin-streptomycin) to dissociate the cells. The resultant cell suspension was further diluted to a final density of 9×10^4 cells/mL using the same medium. Each well of a 24-well plate, containing pre-coated CaF₂ windows, received 0.7 mL of this suspension. The CaF₂ windows had been prepared by incubating them with 100 $\mu\text{g/mL}$ poly-d-lysine (Sigma) at 37°C and 5% CO₂ for 24 h, followed by a laminin mouse protein (Gibco) layer at 10 $\mu\text{g/mL}$, also at 37°C and 5% CO₂ overnight. After rinsing twice with ddH₂O and drying in a biosafety cabinet at room temperature, the neurons were maintained with a half-medium exchange every four days. At day 14 *in vitro* (DIV14), neurons were fixed with 4% paraformaldehyde (PFA) for 20 min, washed with DPBS, and could be stored in DPBS at 4°C for several days. For live neuron samples, the neurons were incubated in DMEM containing 5 μM Rh800 for 30 min at 37°C in a 5% CO₂. After incubation, the DMEM was replaced with D₂O-PBS before proceeding to imaging.

F. Immunolabeling of Fixed HeLa and Neurons

Fixed cells were first permeabilized using 0.1% Triton X-100 (T8787, Sigma) for 20 min. After blocking for 1–3 h in 10% goat serum/1% BSA/0.3 M glycine/0.1% PBST, the cells were incubated overnight at 4°C in 10 $\mu\text{g mL}^{-1}$ primary antibody in 3% BSA. After washing with PBS, the cells were blocked using 10% goat serum in 0.1% PBST for 1–3 h, followed by overnight incubation at 4°C in $\sim 10 \mu\text{g mL}^{-1}$ secondary antibody in 10% goat serum. The cells were blocked with 10% goat serum for 30 min and dried before imaging.

G. Preparation of Live *E. coli* Sample

E. coli BL21 was cultured in LB broth overnight at 37°C. The *E. coli* culture was centrifuged, and the supernatant was removed. The bacterial pellet was then incubated for 3–10 min in PBS containing 0.5 μM Cy5.5 or Rh800. After another centrifugation and removal of the supernatant, the cells were resuspended in D₂O before imaging. For two-color *E. coli* samples, the dye-stained *E. coli* solutions were mixed prior to imaging.

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Disclosures. The authors have filed a patent (18/645,178) based on this work.

Data availability. The authors declare that all data supporting the findings of the present study are available in the article and its supplementary figures and tables, or from the corresponding author upon request.

Supplemental document. See [Supplement 1](#) for supporting content.

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