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# Photon-counting Raman spectroscopy at a MHz spectral rate for biochemical imaging of an entire organism

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Raman spectroscopy, which probes fine molecular vibrations, is crucial for interpreting covalent bonds, chemical compositions, and other molecular dynamics in mixtures via their vibrational fingerprint signatures. However, over the past few decades, longstanding barriers have been encountered in both the sensitivity and speed of Raman spectroscopy, limiting its ability to be extended to broader biochemical applications. Here, we introduce a versatile analytical workhorse, the fiber-array Raman engine (termed FIRE). In FIRE, a distinctive fiber array bundle delays the Raman shifts at a scale of 3-960 ns, and a highly dynamic single-channel photon-counting detector achieves spectral measurements that outperform the best commercial confocal Raman microscope. Crucially, FIRE features a major advantage of nonrepetitive singleshot spectra measurement at a MHz repetition rate with a full Raman span (-300-4300 cm<sup>-1</sup>) covering the fingerprint, silent, C–H, and O–H regions and therefore represents a major step toward overall improving of sensitivity, speed, and spectral span. We demonstrate full Raman spectral imaging of the metabolic activity of intact Caenorhabditis elegans. FIRE exhibits superior performance to a Raman microscope in all aspects, including autofluorescence suppression, and will elucidate a variety of biochemical applications.

Raman spectroscopy, which provides decisive spectral measurements of vibrational transitions of molecules, has become an indispensable analytical tool for deciphering molecular structures and their alternations during unknown physical and chemical phenomena<sup>1-3</sup> and for fingerprinting metabolic biomarkers and chemical species in complex biological systems with molecular specificity<sup>4-7</sup>. However, Raman spectroscopy has long been known for its weak signal and low throughput due to the extremely small scattering cross-sections (typical  $\sigma$ :  $10^{-30}$  cm<sup>2</sup>sr<sup>-1</sup>, which is ten orders of magnitude smaller than that for fluorescence). Over the past few decades, tremendous endeavors have been made to increase the sensitivity and speed of Raman spectroscopy. To detect precious and rarely scattered Raman photons, state-of-the-art Raman spectrometers routinely deploy deep-cooling (in liquid N<sub>2</sub>) charge-coupled devices (CCDs), with the dark

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noise reduced to a negligible level. However, they suffer from readout noise (RON) in the photon-to-electron conversion and charge-tovoltage amplification stages. In particular, intensified CCD (ICCD) and electron-multiplying CCD (EMCCD) cameras, in which ultrahigh gain magnification is applied to suppress RON, have become the prime alternatives that outperform CCDs. However, they are only suited for low-light applications, and the dynamic range of single-frame exposure is restricted to one photon. The noise floor has been reduced to a negligible level for the latest arrays of photomultiplier tubes (PMTs) and single-photon avalanche diodes (SPADs)<sup>8</sup>, which mostly work in photon-counting and gating mode. However, these photon-counting detectors respond to only one photon per laser shot and become blinded because of the intrinsic dead time. Recently, by using chromatic chirping within a long single-mode dispersive optical fiber, the emerging time-stretch dispersive Fourier transform (TS-DFT) has enabled up to MHz Raman-band measurements<sup>9-14</sup>. However, all these innovations employed a single-mode fiber to implement the spectral delay, which limited their dispersive capability on the ns scale. Moreover, because of the high coupling loss of single-mode fibers, these methods are not suitable for performing full-spectrum Raman imaging of highly scattering biological tissues. Many other new concepts, including coherent Raman spectroscopy<sup>15-21</sup>, Raman spectroscopy with laser frequency combs<sup>22</sup>, and light sheet Raman imaging<sup>23</sup>, have been extensively explored, but their progress in terms of practicability and wide application has been limited.

We propose the concept of ideal CCD-free Raman microspectroscopy, which simultaneously possesses all the following key features: (1) superior photon-counting sensitivity, in which all the precious Raman photons are collected and counted; (2) an ultimate Raman spectral speed, which enables uninterrupted MHz spectra updating; (3) a full-spectral span, covering the fingerprint, silent, C–H and O–H regions (–300 to 4300 cm<sup>-1</sup>, free of spectral tuning); (4) immunity from dark noise, RON and other types of noise, enabling low-light applications; (5) a dead-time-free enabled high dynamic range, which adapts to sudden strong light input; (6) a high collection efficiency with a multimode fiber; and (7) fluorescence suppression and separation.

To ensure that all the above features are simultaneously achieved, we develop a fiber-array-based Raman engine (termed FIRE). The core idea is that we excite the sample with a 1 MHz nanosecond pulsed laser, and the excited Raman spectra are unfolded and measured in the temporal interval between excitation pulses. Conceptually, we deploy a multimode fiber array assembled with different lengths, instead of the pixel arrays in traditional CCD detectors, to convert transient Raman spectra into temporal waveforms. A highly dynamic singlechannel photon-counting detector and a real-time digitizer count all the incoming Raman photons in spectral and temporal sequence after each laser shot at 1 MHz. In addition, the new Littrow architecture of the FIRE spectrometer ensures a full-spectral span and simplicity, both of which have long been desired. To validate the performance, we demonstrate continuous 1 MHz full-band Raman spectra updating and ultrafast Raman imaging, in which the full spectrum of various biological tissues and model organisms is acquired every microsecond. FIRE substantially relieves the tradeoffs among sensitivity, speed and spectral range and will enable many profound applications in extreme conditions in the future.

#### Results

#### Concept and schematic of FIRE

Figure 1 illustrates the strategy of the FIRE concept. Specifically, a nanosecond pulsed laser (e.g., 532 nm) is deployed to excite the sample in less than 0.5 ns at a repetition rate of 1 MHz. For every laser shot, the spontaneous Raman spectrum of the molecules is excited; thus, we purposely assign the time slot between laser pulses, ~999.5 ns, to performing ultrafast single-shot spectral measurements. Thus, both the laser power and time for spectral measurement are reduced. To fully utilize the temporal capacity, the Raman photons of different colors are spatially separated by a grating and coupled into 160 multimode fibers in a fiber array bundle (FAB) system. Essentially, the lengths of the 160 multimode fibers are equally increased by 0.6 meters in arithmetic progression (i.e., 0.6, 1.2, 1.8, 2.4 m...), and thus, the Raman photons of different colors can be separated by approximately 3 ns (0.6 n/c; c: speed of light; n: reflective index of silica, ~1.5) in the time domain. Therefore, the attainable spectral range spans  $160 \times 3 = 480$  ns for a single channel, and two channels covering two Raman bands will spectrally span 960 ns, occupying almost the full 1 µs time slot. At the combined end of all the fibers, a single-channel photon-counting silicon photomultiplier (SiPM) detects all incoming photons, and a real-time GHz high-speed acquisition card (HAC) records all photon events in real time. Significantly, the 4774 SPAD microcells (with outputs terminating in a shared readout) in the SiPM naturally disperse the photon events into different cells, thus avoiding the influence of dead time and saturation existing in other singlechannel detectors<sup>24</sup>. The photon counting histograms (spectrum of the photon flux) shown in the inset of Fig. 1 indicate the dynamic range of the SiPM at low and high fluxes of light input. In this way, the complete time history of all Raman photons is recorded for every laser pulse, regardless of the incoming flux of photons.





fibers with lengths from 0.6 to 96 m, resulting in a constant time delay of 3 ns among the colors. Insets: Ability of the SiPM to simultaneously detect multiphoton events; histograms of the detected photons under low and high light inputs. Ex., excitation; S. R., spontaneous Raman spectrum; T.D. time delay.



**Fig. 2** | **Schematic of the FIRE system. a** Architecture of FIRE. PBS, polarization beam splitter; DM, dichroic mirror; GMS, galvanometer scanner; MFI, multimode fiber I; FA, fiber array; AC, achromatic lens; G, grating; M, mirror; FAB, fiber array bundle; AMP, amplifier; AFG, arbitrary function generator; NI, National Instrument acquisition card; HAC, high-speed acquisition card; and SiPM, silicon photo-multiplier. b Paraxial Littrow spectrometer of FIRE. In the real system, the two input

Figure 2a shows a schematic of the FIRE system, which is based on a custom-built laser scanning confocal Raman microscope (see Methods for more details). The core of the FIRE system lies in the detection part. Two multimode fibers (MF I and MF II) with a large inner core of 100 um and a numeric aperture (NA) of 0.22 efficiently collect the scattered Raman photons, which are separated into bands I and II by a dichroic mirror after de-scanning of the galvanometer scanner (GMS). Furthermore, in a custom-built compact paraxial Littrow spectrometer, the input Raman photons are collimated by an achromatic lens (AC) and then retro-diffracted by a reflective grating. Essentially, the spatially dispersed Raman photons of different colors are refocused into a 160-ch optical fiber array (FA) that is located beside the input fibers (Fig. 2b for clarity). In the following FAB, the Raman photons of different colors travel for different times. A single-channel SiPM detector counts the sequentially incoming photons and achieves single-shot Raman spectra measurement. To accomplish Raman imaging, an arbitrary function generator (AFG) synchronizes the laser pulses, two-axis laser scanning, and GHz analog acquisition card to the same clock.

#### Full Raman spectra measurements at 1 MHz

Since the Raman spectra in FIRE are unfolded in the time domain, we can acquire full Raman spectra by fully occupying the temporal interval between two consecutive laser shots. We arranged two multimode fibers to collect the first Raman band covering the fingerprint and silent regions (Band I: -300 to 2000 cm<sup>-1</sup>, MF I in Fig. 2a) and the second Raman band covering the silent, C–H and O–H regions (Band II: 2000 to 4300 cm<sup>-1</sup>, MF II). Intriguingly, when we space the output ends of MF II and MF I at a distance of an FA, the spectral span can be doubled. Importantly, Raman bands I and II can share the same FA, Littrow spectrometer, FAB and photon-counting system. In this case, the length of MF II should be set to delay the band II photons such that they take the second 500 ns slot. As a result, our current FIRE system is free of spectral tuning and achieves full Raman spectral coverage of the fingerprint, silent, C–H and O–H regions (-300 to 4300 cm<sup>-1</sup>) at a

fibers are located on two sides of an FA to achieve paraxial projection. **c** FIREmeasured full-band Raman spectrum ( $-300-4300 \text{ cm}^{-1}$ ) of a mixed sample (H<sub>2</sub>O:D<sub>2</sub>O:DMSO = 2:1:1 in volumetric ratio) at 100 ms. **d**, Raman spectrum of pure DMSO obtained within 1 ms. The spectrum in red shows the dark noise generated within 1 ms when the excitation laser is off.

1MHz spectral rate. As shown in Fig. 2c, we measured the full-band Raman spectra of mixed H<sub>2</sub>O, D<sub>2</sub>O, and dimethyl sulfoxide (DMSO) at a volume ratio of 2:1:1 (10<sup>5</sup> spectra acquired within 100 ms). The Raman bands in the fingerprint, silent, C-H and O-H regions reflect the molecular vibrations of DMSO, D<sub>2</sub>O, and water. Figure 2d shows the full Raman spectrum of pure DMSO obtained with an integration time of only 1 ms, reflecting very high sensitivity and minimum fluorescence counts. The beneath red curve presents the actual noise of the 1 dark count within the 1 ms acquisition time, which is completely negligible. The FIRE spectra of typical chemicals, including ethanol, glyceryl trioleate, and phenylbromoethyne are shown in Supplementary Fig. 1. In particular, the fluorescence background appearing for phenylbromoethyne can be significantly suppressed by time gating in the software, in which the Raman signals within the first 1 ns after the laser pulse are picked up by each fiber channel. For the photons arriving immediately at 0 ns every 3 ns, we count them as Raman photons and ignore the photons arriving in the following 2 ns (dashed curves in Supplementary Fig. 1c), which are not related to the Raman signal. This means that the Raman photons within the first 1 ns after the laser pulse are picked up by all the fiber channels. As a result, the fluorescence background can be largely suppressed by gating.

#### Performance characterization of the FIRE system

The FIRE system possesses the unique capability of ultrafast Raman spectra fingerprinting up to MHz with single-photon sensitivity. As shown in Fig. 3a, we recorded 1 ms photon events of the Raman spectra of pure DMSO and the equivalent dark noise (in the absence of laser excitation). Within 1 ms, the photon-counting detector, which was water cooled to -70 °C, recorded 1000 Raman spectra of DMSO, whereas only -0.6 dark counts were observed. Within 1 s, approximately 650 dark counts were recorded, approximately 19% of which were crosstalk counts (CTCs) with 2–4 simultaneous counts (Fig. 3b). Figure 3c clearly presents the electrical profiles of quantum jumps of a single photon and six photons simultaneously, which result in approximately 20 mV per SPAD event and a 120 mV signal,



Fig. 3 | Performance characterization of the FIRE system. a Dark count versus photon count of the measured Raman spectrum of DMSO. b Statistical analysis of dark noise and crosstalk. CTC, crosstalk count. c, Electrical profiles of 1 and 6 photons indicated in a with an FWHM of 4 ns. d Recorded Raman spectrum of pure DMSO within 100 ms. e, Raw data of 19 continuously recorded Raman spectra of

DMSO within 19  $\mu$ s, truncated from (**a**). **f** Verification of the shot-noise-limited sensitivity. Error bars: SD (*n* = 2500 measurements). **g** Comparison of the sensitivity between FIRE and a Horiba HR800. The O–H background from water was removed for both spectra.

respectively, with a full width at half maximum (FWHM) of 4 ns. Figure 3d shows the Raman spectrum of pure DMSO obtained with a 100 ms integration time. Figure 3e shows the consecutively acquired 19 DMSO spectra produced by 19 laser shots within 19  $\mu$ s, which proves the ultrahigh speed of Raman spectra updating. We also validated the linearity of the Raman signal with the excitation laser power (Fig. 3f), and the photon-counting FIRE exhibits a shot-noise-limited sensitivity.

For regular confocal Raman imaging, a CCD can rarely work with shot-noise-limited sensitivity at a high spectral rate of 10-1000 Hz due to the lack of sufficient Raman photons to compete with the intrinsic RON. However, the CCD becomes a shot-noise-limited detector by accumulating sufficient photons under longer exposure times (e.g., 10 s). In such cases, the CCD will surprisingly outperform all other ultrasensitive detectors, including EM-CCDs, ICCDs, and EM-ICCDs, but long-term exposure is not realistic for CCD-based Raman imaging. In contrast, FIRE is free of RON and directly counts every photon collected. It does not require long-term exposure and naturally achieves shot-noise-limited detection even at a MHz spectral rate. To compare the direct sensitivity of FIRE with that of the most advanced commercial device, i.e., a Raman HR800 (Horiba), in its best scenario (10 s), we performed spectral measurements of 0.1% DMSO. Supplementary Fig. 2 presents the original raw spectra of 0.1% DMSO obtained from the HR800 and FIRE, both of which worked in photon-noise-dominant conditions. As observed, the spectral background obtained by the HR800 contained a large amount of systematic fluorescence (dashed yellow line in Supplementary Fig. 2). The Raman HR800 includes a slit at the entrance of the spectrometer, which can only partially reject outof-focus photons and allows much more background fluorescence. In contrast, to obtain sharp Raman images, the spatial resolution of FIRE imaging is guaranteed by the coupling fiber 100 µm in diameter, which results in a much lower background fluorescence. As shown in Fig. 3g, a signal-to-noise ratio (SNR) of 105 (signal-to-background ratio (SBR) ~ 0.25) was measured for the photon-counting FIRE, which was approximately 2.5 times better than that measured for the standard HR800 (SNR: 42.5: SBR ~ 1). This difference occurs because the 100 um fiber core in the FA is approximately 4 times the pixel size (26 µm) of the CCD and accommodates 16 times more photons, which should result in 4 times better sensitivity in FIRE, assuming that their photon throughputs are the same. This is also the reason why larger pixel sizes are always preferred for spectrometer CCDs. Moreover, taking the 4 times higher background in the CCD into account, we realized that FIRE is still missing 10 times more photons. Even so, the photoncounting FIRE has substantial advantages over CCD-based Raman spectrometers in performing fast Raman imaging.

#### High-speed chemical imaging by FIRE

Compared to conventional spontaneous Raman microscopes, FIRE provides substantially improved sensitivity and speed for chemical-



Fig. 4 | High-speed chemical imaging by FIRE. a Background dark noise in FIRE images with 1 and 100 ms exposure times (with the excitation laser off).
b Hyperspectral FIRE image of an adult *daf-2 C. elegans* obtained within only 40 min. c, d, Zoomed-in images showing the concentration maps of proteins (blue)

and lipids (green) in the tail and eggs, and corresponding Raman spectra. **e** Typical hyperspectral SRS image of an intact *daf-2 C. elegans* obtained within approximately the same time. The insets show the concentration maps of proteins and lipids and their SRS spectra. Scale bar, 100  $\mu$ m.

specific imaging of biological samples. As shown in Fig. 4a, the background noise of FIRE images is significantly reduced to the minimum, approximately 0.6 dark counts within 1 ms per image (25 ns/pixel, 200  $\times$  200 pixel, 1 ms/image) and ~60 counts within 100 ms (2.5  $\mu$ s/pixel). In Supplementary Fig. 3, we demonstrate fast FIRE imaging of pure DMSO with dwell times of 2  $\mu$ s, 5  $\mu$ s and 1 ms. As shown in Supplementary Fig. 4a, spectral imaging of the epidermal tissue of a mouse ear was performed within approximately 4 min, and Raman spectra were obtained at each image pixel. By applying the multivariate curve resolution (MCR) algorithm<sup>25</sup>, we clearly retrieved concentration maps of protein-rich hair and fat cells and their corresponding Raman spectra. To present an application demonstration for an intact organism, we performed diffraction-limited microscopic imaging of an adult daf-2 Caenorhabditis elegans (C. elegans) within only 40 min (Fig. 4b), which was the total time, including a 16 min exposure time and a 4 min data-saving time. Great details of lipid droplets were observed in the tails (Fig. 4c), eggs (Fig. 4d), lumen, intestinal cells, epidermis and head. In contrast, Fig. 4e shows a typical hyperspectral stimulated Raman scattering (SRS) image of intact daf-2 C. elegans, which took approximately the same amount of time as FIRE. FIRE can compete with SRS in all aspects of image quality, including sensitivity, spatial resolution, and spectral resolution. Supplementary Fig. 4b presents the transport and diffusion of DMSO in the hypodermis of the mouse ear. The concentration distributions of DMSO that penetrated between and inside the lipid cells are shown in Supplementary Fig. 4c. Other FIRE images of mouse brain tissues, fatty liver tissue and NIH 3T3 cells are shown in Supplementary Fig. 4d.

**Microscopic imaging of glucose metabolism in intact** *C. elegans* Glucose metabolism is fundamental to physiological processes and plays a central role in maintaining cellular homeostasis and growth for all life. However, the existing microscopic imaging methods can visualize only part of the biological information. For SRS, simultaneously mapping glucose, lipids, proteins and other metabolic processes is difficult because of the limited spectral range<sup>26</sup>. The full-band FIRE microscope perfectly solves this problem. We first validated the Raman spectrum and detection limit of D7-glucose, which is a metabolic probe and glucose isotopologue with deuterium labeling on all the carbons (Fig. 5a, b). FIRE clearly revealed the characteristic C–D band at approximately 2150 cm<sup>-1</sup> in the cell-silent region and three Raman peaks in the fingerprint region (Fig. 5a), confirming the detectability of 10 mM D7-glucose (Fig. 5b), which is comparable to the sensitivity of SRS<sup>26</sup>. We further fed D7-glucose to wild-type and *daf-2* mutant C. elegans to visualize glucose metabolism. Figure 5c shows the selected full Raman spectra obtained at the daf-2 C. elegans locations indicated in Fig. 5d, in which the C-D, C-H (lipid), C-H (protein) and O-H bands are red, green, blue and black, respectively. In Supplementary Fig. 5. the color-overlaid image of an intact daf-2 C. elegans and four concentration maps corresponding to the fingerprints, D7glucose, C-H and water are shown. Supplementary Fig. 6 shows daf-2 worms at different stages. In the early stages of daf-2 worms (L4 in Fig. 5d, L2 in Fig. 5e), D7-glucose was mostly concentrated in the tissue near the intestine and was rarely involved in the formation of lipids on the skin. This result implies that the metabolic activity was stronger for energy production in daf-2 worms. Surprisingly, for L2 dauer, D7glucose was metabolized and turned into skin in the face of excessive fasting, and the priority to thicken the skin was crucial to resist the harsh environment (Fig. 5e and more contrast images in Supplementary Fig. 7).

We noticed that the precious Raman photons can be detected more efficiently by a SiPM with higher fill factor and photon detection efficiency (PDE). We further implemented a Broadcom-made SiPM with 35% PDE and eventually achieved a fivefold improvement in the photon throughput of FIRE (see more details in the Methods section). Figure 6a shows the FIRE imaging of a *daf-2 C. elegans* with 200 × 200 full-spectral pixels, and the overall time was only 55 s, including a 20 s exposure time and a 35 s extra time. The total time to image an intact *C. elegans* was approximately 5 min with a dwell time of 0.5 ms/pixel, and the spectral image showed great chemical differences throughout the entire worm (Fig. 6b, c). Undoubtedly, CCD-based Raman imaging will take hours. Two movies showing the real imaging process are provided to demonstrate the speed of FIRE (Supplementary Movies 1 & 2). We believe that such 1-min chemical imaging will move FIRE one step closer toward practicability in methods.

#### Discussion

When the Raman signal is limited to 5–6.25 photons under lightstarved conditions or at high spectral acquisition rates, the photon-



e C. elegans in stages of L2 and L2 dauer

a FIRE imaging of intact daf-2 C. elegans



**Fig. 5** | **Spectral imaging of D7-glucose metabolism in intact** *C. elegans.* **a** Chemical structure and FIRE spectrum of pure D7-glucose. **b** Sensitivity measurement of D7-glucose in 10 mM and 20 mM solutions with a 100 s integration time. **c** Full-band FIRE spectra obtained in the four regions of interest in **d**. The metabolites or chemicals in P1, 2, 3, and 4 are largely D7-glucose, proteins, lipids,



and water, respectively. d, FIRE imaging of *daf*-2L4 *C. elegans* fed D7-glucose. **e** FIRE imaging of *daf*-2L2 and L2 dauer *C. elegans* fed D7-glucose. Dauer presents particularly more D7-glucose metabolized on the skin than the L2 worm. Scale bar, 100 μm.



**Fig. 6** | **High-speed chemical imaging by FIRE. a** FIRE imaging of an intact *daf-2 C. elegans* within 5 min. **b** Full-band FIRE spectra obtained in the five regions of interest in (**a**). **c** In FIRE imaging, each 200 × 200 pixels image is obtained by

integrating 20 frames with a total dwell time of 0.5 ms/pixel. The total time for full-spectral imaging is 55 s, including a 20 s exposure time and a 35 s extra time. Scale bar, 50  $\mu$ m.

counting FIRE will surpass CCD-based Raman, because CCD suffers from the dominating reading noise and low sensitivity. As shown in Supplementary Movie 2, with only 10 photons/pixels (dwell time: 0.5 ms), photon-counting FIRE can obtain decent Raman spectra and image with SBR > 10. FIRE has the advantage of acquiring Raman spectrum and imaging with single digit number of photons. For CCDbased Raman, these 10 photons will be inevitably lost in the reading noise. Under condition of a photon flux above 1000 photons, both CCD and FIRE can work in shot noise limited sensitivity. Although single-frequency SRS has substantially higher sensitivity and imaging speed than FIRE, FIRE can compete with SRS when spectral imaging is required. Typically, single-frequency SRS imaging with a pump–Stokes frequency difference tuned to a specific C–H band produces very detailed vibrational images of biological samples in less than one

C 1-minute chemical imaging

second (e.g.,  $300 \times 300$  pixels,  $300 \times 300 \mu$ m, ~10 µs dwell time). To perform spectral SRS imaging and obtain SRS spectra at each pixel, the wavelength of the laser is tuned approximately one hundred times (typically frame by frame) in various hyperspectral or multiplex SRS techniques. Thus, obtaining an SRS image stack with 300 × 300 pixels and a spectral span of approximately 200 cm<sup>-1</sup> takes approximately 2-3 min. As shown in Fig. 6a, to map an intact adult C. elegans, 5 or more FIRE image stacks should be obtained. For each substack image, mapping 200 × 200 pixels with a dwell time of 0.5 ms takes approximately 1 min, corresponding to 40000 full Raman spectra (covering fingerprint, silence, C-H, and O-H bands). With respect to the spectral range, the typical hyperspectral SRS imaging modalities usually do not provide a large spectral span because of the limited wavelength tunability of the fs laser and supposedly do not simultaneously cover all Raman spectral regions (~4000 cm<sup>-1</sup>). From this point of view, we conclude that FIRE is comparable to hyperspectral SRS in chemical imaging.

Spontaneous Raman is fundamentally a stochastic and highly dynamic process of photon scattering. So far, all current knowledge and impression of Raman spectroscopy are based on long-time averaging of vibrational spectra of millions of molecules, which present identical or non-dynamic fingerprint features. It is because all CCDs are incapable to capture stochastic and spontaneous Raman dynamics at single molecule level at a time. FIRE obtains Raman spectra at 1 MHz, which is fast enough to divide Raman process in time domain at single molecule level. The isolated Raman scattering events of single molecule or even single vibrational bond can be observed separately at a time. Each single photons in Fig. 3e present isolated Raman scattering events of single molecules distinguished in time domain. Because at that moment of 1 µs, only one molecule emitted one photon at correct Raman shift, which reveal the physical nature of Raman that all Raman bands are exited stochastically. However, photon counting alone does not necessarily imply single-molecule detection. In the present method, each detected photon most likely originates from a different molecule, meaning that the method does not isolate signals from individual molecules. Instead, it merely subdivides a large number of stochastically scattered photons from an ensemble of molecules into individual photon detection events. This process seems very similar to the bi-analyte technique in surface-enhanced Raman spectroscopy (SERS)<sup>27</sup>, where only a single-molecule event can be observed at a low molecular concentration but with a very long integration time of 1s. More importantly, the measurements of single-molecule events in microsecond can be applied with SERS for reproducible quantification of molecular concentrations and understanding many unresolved fundamental questions related to SERS process<sup>28</sup>.

For the most advanced CCD-based Raman microscopes, such as those with multifocus illumination, line illumination, multiline illumination and structured line illumination<sup>29–31</sup>, the speed of Raman imaging has been increased to a new level by distributing the laser power on one laser line, multiple laser focuses, or multiple lines. However, owing to the limited CCD chip size, a tradeoff between the imaging speed and spectral span must be made.

In conclusion, we present a state-of-the-art method for ultrafast Raman spectral measurement and spectroscopic imaging of biological samples, which overcomes the long-standing limitations of Raman spectroscopy in terms of sensitivity, spectral speed, spectral span, and dynamic range. Specifically, compared with the available prevailing Raman spectrometers and microscopes, the most common module, i.e., the CCD chip, is abandoned and a single-channel photon-counting detector is utilized in FIRE to measure the sequentially incoming photons of Raman spectra unfolded in the temporal domain. The FIRE method substantially improves the sensitivity of Raman imaging to the single-photon level, pushes the limit of the spectral rate of Raman imaging to MHz, and achieves single-shot spectral measurements. Moreover, we built a compact FIRE spectrometer in a paraxial Littrow configuration and achieved -300-4300 cm<sup>-1</sup> full Raman band coverage without the need to tune gratings. The dynamic range of FIRE is guaranteed by the dead-time-free SiPM detector, which can adapt to low-light Raman photons and a sudden influx of fluorescence photons or ambient light without saturation. We demonstrate large-scale fullspectrum imaging of the metabolic activity of *C. elegans* fed D7glucose. FIRE, with superior performance in terms of sensitivity, spectral speed, spectral span and fluorescence suppression, is expected to contribute to a variety of research frontiers, including Ramanbased ultrafast transcriptome imaging, hundred-color Raman imaging, and tumor margin assessment in the clinical environment.

## Methods

#### The FIRE system

The experimental schematic is shown in Fig. 2a. A custom-built nanosecond pulsed laser outputs 0.5 ns (FWHM) laser pulses at a 1 MHz repetition rate. For the confocal Raman microscope, a two-axis galvanometer (8310 K, Cambridge Technology) is used for laser scanning imaging, and two ACs with f = 100 mm (GLH31-050B-100-VIS) and f = 200 mm (GLH31-050B-200-VIS, Hengyang Optics) are used as the scanning and tube lenses, respectively. The scattered Raman photons are collected by the objective and sent back through the galvanometer to achieve de-scanning. After a 550 nm short-pass dichroic mirror (DMSP550, Thorlabs), a 600 nm long-pass DM (HGLP-600-D25, Hengyang Optics) splits the Raman photons into Bands I and II, which are further coupled into two multimode fibers before they enter the FIRE spectrometer. Two notch filters (NF533-17, Thorlabs) are used to block the 532 nm laser. In the spectrometer, an FA integrates two MFs as inputs and 160 MFs as outputs. An AC collimates input light into a 1200/mm reflective holographic grating (GH50-12V, Thorlabs) in the visible range, which disperses the Raman photons of different wavelengths. The spatially dispersed photons are focused into 160 MFs, which are connected to the FAB. The photons of different wavelengths travel for different times in the FAB. The output fibers of the FAB are combined into a bundle, and all Raman photons are detected by a 3 × 3 mm SiPM (MICROFC-30035-SMT, ON Semiconductor). After an amplifier (AMP), the signals from the SiPM are acquired by a HAC with a GHz sampling rate.

#### FA and FAB

For the FAB, the maximum optical throughput is guaranteed by multimode optical fibers with a purposely selected large inner core of 100 µm and an ultrathin cladding layer with a diameter of 110 µm. The 160 optical fibers are closely arranged shoulder by shoulder in a onedimensional array in the FA with negligible gaps (see Supplementary Fig. 8). We integrated 160 multimode fibers of different lengths in a coil rack and overcame the difficulty of ending all the fiber tips in a glass tube to fit the SiPM. The 3 ns time delay (corresponding to a 0.6 m fiber, n = 1.5) between adjacent fiber channels was purposely selected. Notably, a time delay of less than 3 ns will degrade the spectral resolution, and a longer time delay will lead to longer fiber bundles, eventually introducing severe optical loss.

#### SiPM

For the detection of sequentially incoming photons, a single PMT or SPAD will not work for obtaining the Raman spectrum because of the fixed blind period of approximately 20–200 ns after every photon event, called the dead time (i.e., only approximately 50 photons can be handled within a 999.5 ns detection time). Crucially, we chose a 3 × 3 mm photon-counting SiPM with 4,774 avalanche photodiode (APD) microcells pixel-arrayed together in two dimensions. This SiPM can potentially count 50 × 4774 photons in one microsecond, recording all incoming photons at a MHz spectral rate free of dead time and saturation. As illustrated in the inset of Fig. 1, the incoming photons from all 160 optical fibers are dispersed onto thousands of

different microcells of the SiPM, which possesses a sufficiently large sensing area to avoid coincidence of photon responses of the same microcells and thus significantly eliminates the dead time. The SiPM has a nearly two orders of magnitude higher dark count rate (DCR) than that of a single APD, which is only approximately 10 counts/s. To overcome the dark noise, we cooled the temperature of the SiPM down to -70 °C via an effective water-cooling system, and the actual DCR of the SiPM was dramatically reduced from 300 k to <1000 counts/s, which is key for the FIRE system (Fig. 3b).

## SiPM upgrade

The original SiPM from ON Semiconductor has a lower PDE of approximately 13% at 630 nm (corresponding to the C–H vibration at 2923 cm<sup>-1</sup>) but has a fast output with a total pulse width of approximately 4 ns. Therefore, we chose it for direct counting of single photons. For the results in Fig. 6, the new SiPM from Broadcom (model: AFBR-S4N44P014M) has 3 times higher PDE in theory and actually a 3.94 times higher signal measured in the experiment, but the afterpulse tail is as long as 200 ns, which is not our first option. To recover the photons and speed up the FIRE imaging, we managed to solve this issue.

### **FIRE spectrometer**

The FIRE spectrometer, which has a much-simplified Littrow configuration in a retroflection architecture, exploits an FA, combines input/ output fibers, and cannot be realized with traditional CCD-based spectrometers. As a result, the simple paraxial design with a single achromatic doublet and a reflective grating allows minimum spherical and chromatic aberrations. The spectral range can be doubled or tripled with the 160-ch FA by including two or three input fibers.

#### Optical settings for spectra and image acquisition

For all imaging experiments, the laser powers before the objective were measured (~70% transmission efficiency, 1.2 NA 60×, UPLSA-PO60XW, Olympus). For the results in Fig. 2c, d, a 40 mW laser power was applied to the DMSO solution. For those in Fig. 3d, the DMSO spectrum was measured without de-scanning, and the collecting fiber was positioned as close as possible to the objective, which could obtain eight times more Raman signal. For the results in Fig. 3a, d, e, a 40 mW laser was applied to the pure DMSO sample. In Fig. 3g, the background of water was subtracted from the spectra. The SNR refers to the ratio of the peak value of the signal to the noise. For FIRE, the laser power and exposure time used to obtain the results in Fig. 3g were 40 mW and 1s, respectively. The 0.1% DMSO spectra were further averaged 10 times under a 0.9 NA 40× objective (UPLSAPO, Olympus). For the HR800 (Horiba), a 532 nm laser with a 27.2 mW power was applied to 0.1% DMSO under a 0.9 NA 40× objective (UPLSAPO, Olympus). The exposure time was 10 s. Figure 4a shows the dark noise in an image frame with a dwell time of 25 ns and a total exposure time of 1 ms. In the lower image, for contrast, the dwell time was set to 2.5 µs, with a total exposure time of 100 ms. The field of view (FOV) was  $140 \times 140$  $\mu$ m<sup>2</sup>, with 200 × 200 pixels. For all FIRE imaging of *C. elegans*, the laser power was set to 20 mW. Four to ten images were stitched together to obtain a chemical map of intact C. elegans (L2-Adult). For the results in Fig. 6, we collected Raman photons with an f = 50 mm AC lens and 50 µm fibers. The input of the spectrometer was collimated with an f=75 mm AC lens. To recover the signal, the SiPM was switched to Broadcom (model: AFBR-S4N44P014M).

#### Hyperspectral SRS imaging

Hyperspectral SRS microscopy has been previously reported<sup>32</sup>. A dual-output femtosecond laser (InSight DeepSee, Spectra Physics, Newport) provides pump (800 nm, ~120 fs) and Stokes (1040 nm, ~220 fs) beams with an 80 MHz repetition rate. The intensity of the 1040 nm Stokes beam was modulated at 10.55 MHz by a customized

resonant electro-optical modulator (EO-AM-R-10.5-C2, Thorlabs). The pump and Stokes pulses were both chirped to ~3 ps by a 64 cm long SF57 glass rod and were spatially overlapped via a dichroic mirror (DMSP1000L, Thorlabs, US). The temporal overlap between the pump and Stokes beams was ensured by the time delay line of the Stokes beam. For SRS imaging, beams were scanned by a two-axis galvanometer (GVS002, Thorlabs). For signal detection, the Stokes beam was blocked by two short-pass filters (ET980sp, Chroma). The SRS signal was detected by a custom photodiode (PD) with a resonant amplifier and demodulated at 10.55 MHz by a lock-in amplifier (LIA, HF2LI, Zurich Instruments). For C. elegans imaging, a 60× objective (N.A. 1.2, UPLSAPO 60XW) was used, and the laser powers of the pump and Stokes beams were 50 and 120 mW, respectively. The laser powers before the galvanometer were measured. The dwell time was set at 10  $\mu$ s, the FOV was 150  $\times$  150  $\mu$ m<sup>2</sup>, with 200  $\times$  200 pixels, and the SRS spectra were acquired by adjusting the delay time of the Stokes laser.

#### Sample preparation

Droplets of DMSO (30072418, Sinopharm Chemical Reagent Co., Ltd), glyceryl trioleate (T7140-5G, Sigma–Aldrich) and phenylbromoethyne (S78797-250 mg, Shanghai Yuanye Bio-Technology Co., Ltd) were sealed between two cover glasses (48393-172, VWR) before imaging. The mixed solution whose spectrum is shown in Fig. 2c was prepared by diluting H<sub>2</sub>O, D<sub>2</sub>O (151882-100G, Sigma–Aldrich), and DMSO at a volume ratio of 2:1:1. For the result in Fig. 3g, DMSO solutions were prepared by diluting pure DMSO in ddH<sub>2</sub>O (sterile ultrapure water).

#### Preparation of tissue from mice

The animal studies were approved by the Institutional Animal Ethics Committee of Huazhong University of Science and Technology, and the procedures for all the animal experiments were conducted in accordance with the Experimental Animal Management Ordinance of Hubei Province, P. R. China. The brain and fatty liver tissues of a 4week-old BALB/c female mouse (Supplementary Fig. 4) were obtained after perfusion with phosphate-buffered saline (PBS) and fixed overnight in 4% paraformaldehyde. The tissues were sliced at a thickness of 100  $\mu$ m with a vibratome and washed once with PBS before being sealed between two coverslips for imaging.

#### Preparation of C. elegans

*Escherichia coli* strain OP50 was mixed with 10 mM D7-glucose and then applied to nematode growth medium (NGM) plates. After 24 h, eggs of *daf-2* mutant *C. elegans* were placed on these plates and grown to different stages at 20 °C. The *C. elegans* were then mixed with anesthetic agents (10 µg/ml levamisole) and 4% paraformaldehyde for imaging.

#### Statistics & reproducibility

Figure 4a was independently repeated over 100 times; Figs. 4b, e and 5d were repeated over 10 times; Figs. 5e and 6 were repeated 4 times, with similar results. The selection of all *C. elegans* is completely random. No statistical method was used to predetermine the sample size. No data were excluded from the analyses. The investigators were not blinded to allocation during experiments and outcome assessment.

#### **Reporting summary**

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

## **Data availability**

Source data in Fig. 3f are provided with this paper. The data that support the findings of this study are available from the corresponding authors upon request. Source data are provided with this paper.

## Article

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# **Author contributions**

S.C.L. and H.Z.L. constructed the FIRE system, and S.C.L., H.Z.L. and Y.R.L. performed the experiments. S.W., Q.Z., Q.P.Z. and H.Z.L. developed the electronic system. X.L., X.B.L. and S.Y. worked on the FAB. S.C.L., Y.G.C. and Y.R.L. prepared the biological samples. B.Z. and J.F.L. provided worms and mutants. S.C.L., Y.R.L., L.X., Z.W. and P.W. analyzed the data and wrote the manuscript with input from all the authors. P.W. and S.C.L. conceived the concept. W.J.L., A.G.S., J.F.L. and P.W. supervised all the experiments.

## **Competing interests**

X.B.L. and P.W. declare involvement with the company Earthome Technology. The other authors declare that they have no conflicts of interest.

# **Additional information**

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