Instant FLIM enables 4D *in vivo* lifetime imaging of intact and injured zebrafish and mouse brains: supplement

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Instant FLIM enables 4D in vivo lifetime imaging of intact and injured zebrafish and mouse brains: supplemental document

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Section S1. Principle of instant FLIM

We first assume the fluorescence sample has a single-exponential decay with an impulse response function \( f(t) = \exp(-t/\tau) / \tau \), where \( \tau \) is the fluorescence lifetime and the function’s integral on the time domain \((t \geq 0)\) is normalized to one. A mode-locked Ti:sapphire laser which generates femtosecond pulses at 80 MHz is used as the excitation source. Since the femtosecond pulses \((100 \, \text{fs})\) are orders of magnitude shorter than the 80 MHz modulation period \((12.5 \, \text{ns})\), we can consider these pulses as a Dirac comb with a period of \( T = 12.4 \, \text{ns} \).

Mathematically, the excitation irradiance can be denoted as \( I(t) = P \delta(T) \), where \( P \) is a coefficient related to the average power of the laser beam. Based on the quadratic nature of two-photon excitation, the fluorescence \( F(t) \) is proportional to the convolution of \( I^2(t) \) and \( f(t) \),

\[
F(t) = cI^2(t) \ast f(t) = \frac{P^2c}{\tau} \delta_T(t) \ast \exp\left(-\frac{t}{\tau}\right),
\]

(S1)

where \( c \) represents the fluorophore concentration. Note that the instrument response function (IRF) is ignored here to simplify the mathematical derivations because the IRF’s full width at half maximum of our PMT (Hamamatsu H7422PA-40) is below 350 ps [1], which is much shorter than the lifetime values of most fluorophores we consider in this work. Note that simply ignoring the PMT’s IRF would affect the accuracy for short lifetime measurements and could lead to biased results. Therefore, in instant FLIM, we calibrated the system using a fluorescence sample with a known lifetime (see Section S2), so the bias effect of the PMT’s response function to lifetime measurements was compensated. This was further confirmed with the fact that we could measure the four fluorescence lifetime standards to the expected values (Fig. 1C).

Since the fluorescence signal \( F(t) \) is periodic with an angular frequency of \( \omega = 2\pi / T \), it can be written as a Fourier series,

\[
F(t) = \sum_{k=-\infty}^{\infty} q_k \exp(ik\omega t),
\]

(S2)

where the Fourier coefficients \( q_k \) are

\[
q_k = \frac{1}{T} \int_0^T F(t) \exp(-ik\omega t) dt = \frac{P^2c}{T} \frac{1}{1+ik\omega}.
\]

(S3)
We separate the fluorescence signal $F(t)$ detected by a PMT into DC and RF parts using a bias tee. The DC part is linearly related to $q_0$ and can be written as

$$V_{\text{DC}} = B q_0 + O_B = \frac{B P_c^2}{T} + O_B.$$  

\hspace{1cm} (S4)

where $B$ is the conversion loss from the bias tee's RF&DC to DC ports, and $O_B$ is the offset that is invariant to the PMT signal variations. The RF part, on the other hand, is $\sum_{k \neq 0} q_k \exp(ik\omega t)$. Since $q_k = q_{-k}^*$, it can also be written as

$$v_{\text{RF}}(t) = 2 \sum_{k \in Z} \Re \{ q_k \exp(ik\omega t) \} = \frac{2P_c^2}{T} \sum_{k \in Z} \frac{1}{1 + (k\omega)^2} \left[ \cos(k\omega t) + k\omega \sin(k\omega t) \right].$$  

\hspace{1cm} (S5)

We use a homodyne detection method to extract the lifetime information from the first harmonic ($\omega$) of $v_{\text{RF}}(t)$. Specifically, we use RF mixers to mix the fluorescence signal with the 80 MHz reference signal from the Ti:sapphire laser. The reference signal is low-pass filtered and amplified such that it only contains the fundamental harmonic ($\omega$). For both the fluorescence and reference signals, a 4-way power splitter is used to split the signal to four paths. In instant FLIM, the four paths are operated independently and simultaneously during imaging acquisition. For each reference signal path, a phase shifter is utilized to introduce a voltage-controlled phase shift of $\phi$ (from 0 to $2\pi$) to the signal. The reference signals are sent to the local oscillator (LO) ports of the mixers and can be denoted as

$$v_{\text{LO}}(t, \phi) = \sin(\omega t + \phi).$$  

\hspace{1cm} (S6)

In instant FLIM, the four mixers take the PMT and reference signals to their RF and LO ports, respectively. In theory, the output signal on the intermediate frequency (IF) ports can be regarded as the product of the signals on its RF and LO ports; in practice, however, a DC offset due to circuit imbalance also exists in the output [2]. We denote this DC offset as

$$O_M = O(P_{\text{RF}}) + d,$$  

\hspace{1cm} (S7)

where $O(P_{\text{RF}})$ is a function of the RF signal’s power, $P_{\text{RF}}$, and $d$ is a constant. The DC offset $O_M$ cannot be eliminated; it could be minimized by matching the powers of the RF and LO signals [2], but in our setup $P_{\text{RF}}$ is a variable related to the excitation power and the fluorophore concentration, so the powers of RF and LO do not match. Considering the existence of $O_M$, we model the output of the mixers as

$$v_w(t, \phi) = M v_{\text{LO}}(t, \phi)v_{\text{RF}}(t) + O_M,$$  

\hspace{1cm} (S8)

where $M$ is the conversion loss from the mixer’s RF to IF ports. The mixers’ outputs are then low-pass filtered to eliminate all the harmonics; therefore, the filtered signals are pure DC voltages, as

$$V_w(\phi) = \frac{1}{T} \int_0^T v_w(t, \phi) dt$$

$$= \frac{P^3 M c}{T} \frac{1}{1 + (\tau\omega)^2} \left[ \sin(\phi) + \tau\omega \cos(\phi) \right] + O_M$$  

\hspace{1cm} (S9)

$$= \frac{P^3 M c}{T} \frac{1}{\sqrt{1 + (\tau\omega)^2}} \sin \left[ \phi + \arctan(\tau\omega) \right] + O_M,$$

which are sampled by a DAQ card and used to calculate fluorescence lifetimes.

A calibration procedure described in Section S2 is required before measurement. When the calibration is complete, we can control the phase shifts $\phi$ by applying corresponding bias
voltages to the phase shifters; we will also have the knowledge of \( M/B \), a ratio between the conversion losses of the mixer and the bias tee, that will be used for lifetime and phasor calculations. The instant FLIM measurement is performed by applying four different phase shifts, 0, 0.5\( \pi \), \( \pi \) and 1.5\( \pi \), respectively, to the four phase shifters on the reference signal paths. Based on Eq. (S9), the measured mixer outputs are

\[
V_w(0) = \frac{P^2M_c}{T} \frac{\tau \omega}{1 + (\tau \omega)^2} + O_M,
\]

\[
V_w(0.5\pi) = \frac{P^2M_c}{T} \frac{1}{1 + (\tau \omega)^2} + O_M,
\]

\[
V_w(\pi) = \frac{P^2M_c}{T} - \frac{-\tau \omega}{1 + (\tau \omega)^2} + O_M,
\]

\[
V_w(1.5\pi) = \frac{P^2M_c}{T} - \frac{1}{1 + (\tau \omega)^2} + O_M.
\]

Then, by taking the differences among them, we have

\[
V_w(0) - V_w(\pi) = \frac{2P^2M_c}{T} \frac{\tau \omega}{1 + (\tau \omega)^2},
\]

\[
V_w(0.5\pi) - V_w(1.5\pi) = \frac{2P^2M_c}{T} \frac{1}{1 + (\tau \omega)^2},
\]

which are proportional to the imaginary and real parts of the first harmonic \((1\omega)\) Fourier coefficient of the fluorescence, \( q_1 \), in Eq. (S3). Based on the measurements in Eq. (S11), it is easy to extract the fluorescence lifetime by

\[
\tau = \frac{1}{\omega} \frac{V_w(0) - V_w(\pi)}{V_w(0.5\pi) - V_w(1.5\pi)}.
\]

Next, we show that the instant FLIM method is also applicable for fluorophores with multi-exponential decays by employing the phasor plot approach [3]. Considering that the fluorophore sample consists of multiple fluorophores with different lifetimes, \( \tau_i \), the impulse response function becomes

\[
f(t) = \sum_i a_i \frac{1}{\tau_i} \exp\left(-\frac{t}{\tau_i}\right),
\]

where \( a_i \) is the intensity weighted fractional contribution of the fluorophore with lifetime \( \tau_i \), and \( \sum_i a_i = 1 \). Therefore, Eq. (S3) becomes

\[
q_k = \frac{P^2c_e}{T} \sum_i a_i \frac{1}{1 + ik\tau_i \omega},
\]

and Eq. (S11) changes to

\[
V_w(0) - V_w(\pi) = \frac{2P^2M_c}{T} \sum_i a_i \frac{\tau \omega}{1 + (\tau \omega)^2},
\]

\[
V_w(0.5\pi) - V_w(1.5\pi) = \frac{2P^2M_c}{T} \sum_i a_i \frac{1}{1 + (\tau \omega)^2}.
\]
On the other hand, the DC signal from the bias tee, Eq. (S4), does not change when we assume a multi-exponential decay model. If we divide Eq. (S15) by \(2(V_{DC} - O_B)\) in Eq. (S4) and the calibration coefficient \(M / B\), we get

\[
g = \frac{V_{\phi}(0.5\pi) - V_{\phi}(1.5\pi)}{2(V_{DC} - O_B)(M / B)} = \sum_i a_i \frac{1}{1 + (\tau_i \omega)^2},
\]

\[
s = \frac{V_{\phi}(0) - V_{\phi}(\pi)}{2(V_{DC} - O_B)(M / B)} = \sum_i a_i \frac{\tau_i \omega}{1 + (\tau_i \omega)^2},
\]

where \(g\) and \(s\) are the horizontal and vertical components (coordinates) of phasors on a phasor plot [3]. From the phasor components, we can obtain the average fluorescence (phase) lifetime of the multi-exponential decay by

\[
\tau = \frac{s}{g \omega} \approx \frac{\sum_i a_i \tau_i}{\sum_i a_i} = \sum_i a_i \tau_i.
\]

The average fluorescence lifetime alone cannot resolve the heterogeneity of multi-exponential decays, as different fluorophore compositions could result in the same average lifetime measurements. The phasor plot, on the other hand, can be used to resolve the heterogeneity because different fluorophore compositions can alter the phasor components even if the average lifetime might be unaltered.

Section S2. System calibration

A fluorescence sample with a known lifetime (e.g., \(10^{-3} \text{ M}\) coumarin 6 in methanol with a lifetime of 2.30 ns [4]) is required for system calibration. To compensate for the wavelength dependency of the PMT’s sensitivity, the calibration should be repeated for each excitation wavelength that will be used. Note that while the fluorescence lifetime of a fluorophore is independent of excitation wavelengths, in intravital imaging experiments, different excitation wavelengths could excite different fluorophores with distinct lifetime values, thus resulting in different lifetime measurements. Once the calibration is complete, the system is ready for imaging, and no more calibration is required. First, the relations between the phase shifts \(\phi\) and the phase shifters' bias voltages \(V_b\), i.e., \(\phi(V_b)\), should be calibrated for all four signal paths. Considering a fluorescence sample with a known lifetime \(\tau\) (e.g., \(\tau = 2.3\) ns for coumarin 6 in methanol) and assuming that its concentration is \(c\), for each bias voltage \(V_b\), Eq. (S9) becomes

\[
\overline{V}_{\phi}(V_b) = \frac{P^2 M \overline{E}}{T} \frac{1}{\sqrt{1 + (\overline{\tau} \omega)^2}} \sin \left[ \phi(V_b) + \arctan(\overline{\tau} \omega) \right] + O_m.
\]

By changing \(V_b\) and measuring \(\overline{V}_{\phi}\), we can obtain a curve, \(\overline{V}_{\phi}(V_b)\), with maximal and minimal values:

\[
\overline{V}_{\phi,\text{max}} = \frac{P^2 M \overline{E}}{T} \frac{1}{\sqrt{1 + (\overline{\tau} \omega)^2}} + O_m,
\]

\[
\overline{V}_{\phi,\text{min}} = \frac{P^2 M \overline{E}}{T} \frac{-1}{\sqrt{1 + (\overline{\tau} \omega)^2}} + O_m.
\]

We then have
Thus, from Eq. (S18), we can extract the phase calibration curve, \( \varphi(V_b) \), as

\[
\varphi(V_b) = \arcsin \left( \frac{T}{P^2 MC} \sqrt{1 + (\tau \omega)^2} \right) - \arctan(\tau \omega)
\]

Therefore, an arbitrary phase shift \( \varphi \) between 0 to \( 2\pi \) can be introduced to the system by applying the corresponding voltage \( V_b \) from the calibration curve \( \varphi(V_b) \). Fig. S3 shows an example of \( \overline{V}_{IF} \) signals acquired from the four mixers’ IF ports, as well as the phase calibration curve \( \varphi(V_b) \) calculated using Eq. (S21), during the system calibration using a coumarin 6 lifetime standard.

Second, the coefficient \( M/B \) used in Eq. (S16) and the offset \( O_B \) in Eq. (S4) should be obtained during the calibration process. For the same fluorescence sample with a known lifetime \( \tau \), the DC signal from the bias tee, Eq. (S4), becomes

\[
\overline{V}_{DC} = \frac{BP^2 c}{T} + O_B.
\]

The offset \( O_B \) can be obtained by turning off the laser beam \( P = 0 \) while measuring \( \overline{V}_{DC} \). Therefore, based on Eqs. (S20) and (S22), we get

\[
\frac{M}{B} = \frac{\overline{V}_{IF,max} - \overline{V}_{IF,min}}{2(\overline{V}_{DC} - O_B)} \sqrt{1 + (\tau \omega)^2},
\]

which is used for lifetime and phasor measurements.

Note that in Fig. S3 the four mixers have almost identical electrical properties as the calibration curves overlap with each other; therefore, it is valid to assume that the four mixers have identical conversion losses \( M \) and DC offsets \( O_B \). However, while the conversion loss \( M \) is a design specification and can be guaranteed to be identical for the same type of mixers, there is no guarantee that the DC offsets \( O_B \) are the same for all mixers. \[2\] Thus, an additional calibration for the DC offset difference may be needed. We add a subscript \( i \) to denote the signals from the \( i \)-th mixer; the fluorescence signal from the \( i \)-th mixer’s IF port becomes

\[
\overline{V}_{IF,i}(V_b) = \frac{P^2 MC}{T} \frac{1}{\sqrt{1 + (\tau \omega)^2}} \sin \left[ \varphi(V_b) + \arctan(\tau \omega) \right] + O_M + d_i.
\]

where the \( i \)-th DC offset is

\[
O_M = O(P_{RF}) + d_i.
\]

We found through experiments that \( O(P_{RF}) \), as a function of the RF signal’s power \( P_{RF} \), was identical for all four mixers, and that the DC offset difference was due to \( d_i \). Therefore, we can compensate for such difference by calibrating the differences among \( d_i \). To do that, we turn off the laser beam \( P = 0 \) while measuring \( \overline{V}_{IF,i} \) from the four mixers:
\[
\begin{align*}
\bar{V}_{B_1} &= O_{M_1} = O(P_{RF}) + d_1, \\
\bar{V}_{B_2} &= O_{M_2} = O(P_{RF}) + d_2, \\
\bar{V}_{B_3} &= O_{M_3} = O(P_{RF}) + d_3, \\
\bar{V}_{B_4} &= O_{M_4} = O(P_{RF}) + d_4,
\end{align*}
\] (S26)

and we define two new calibration parameters, \(d_{13}\) and \(d_{24}\), as
\[
d_{13} = d_1 - d_3 = \bar{V}_{B_1} - \bar{V}_{B_3}, \\
d_{24} = d_2 - d_4 = \bar{V}_{B_2} - \bar{V}_{B_4}.
\] (S27)

After the calibration, the DC offset differences among the mixers can be compensated by including \(d_{13}\) and \(d_{24}\) in FLIM measurements: Eq. (S12) becomes
\[
\tau = \frac{1}{\omega} \frac{V_{B_1}(0) - V_{B_1}(\pi) - d_{13}}{V_{B_2}(0.5\pi) - V_{B_4}(1.5\pi) - d_{24}},
\] (S28)

and finally, the phasor components [Eq. (S16)] are now
\[
g = \frac{V_{B_1}(0.5\pi) - V_{B_3}(1.5\pi) - d_{13}}{2(V_{DC} - O_p)(M / B)}, \\
s = \frac{V_{B_2}(0) - V_{B_4}(\pi) - d_{24}}{2(V_{DC} - O_p)(M / B)}.
\] (S29)

Note that all the calibration procedures described in this section can be performed easily and completed automatically with our open-source Instant-FLIM-Control program.

**Section S3. SNR analysis of instant FLIM**

We analyze the SNR performance of instant FLIM and compare it with conventional FD-FLIM methods through Monte Carlo simulations and error-propagation analyses [5,6]. These FD-FLIM methods differ in the modulation waveforms of the excitation light, and consequently, the detected fluorescence signals are also different, as shown in Fig. S4A. These differences result in different sensitivity and SNR in lifetime measurements. Here, we use the \(F\)-value, i.e., photon economy, a widely used figure of merit in FLIM, to quantify and compare SNRs of FLIM measurements. It is defined as the ratio of the uncertainties in lifetime (\(\tau\)) and intensity (\(I\)) measurements:
\[
F = \frac{\sigma_{\tau}/\tau}{\sigma_I/I} = \sqrt{\frac{N_{det}}{N_{det}}} \frac{\sigma_{\tau}}{\tau},
\] (S30)

where we denote \(I = N_{det}\), the number of photons detected in a measurement, and use the fact that \(N_{det}\) is shot-noise-limited, i.e., Poisson distributed, such that \(\sigma_I = \sqrt{N_{det}}\). Note that the shot-noise-limited performance is achieved using high-sensitivity PMTs and low-noise amplifiers in a FLIM system. Since the \(F\)-value quantifies the uncertainty in lifetime measurements, a smaller \(F\) means that the lifetime is measured more accurately, hence a better SNR performance. Whereas a smaller \(F\) is desired, it is limited to \(F > 1\) due to shot noise; \(F = 1\) only exists in an ideal shot-noise-limited FLIM system.

We use Monte Carlo simulations and error-propagation analyses to acquire the \(F\)-values of instant FLIM and conventional FD-FLIM methods. The Monte Carlo simulations are performed by dividing each modulation period \(T\) (12.5 ns for 80 MHz modulation) into \(L\) time units \(\Delta t\) [5,6]. In each \(\Delta t\), a random number, \(r\), that is uniformly distributed in \([0, 1]\) is generated and compared with the probability density described by the product of the
fluorescence signal \( F(t) \) and the time unit \( \Delta t \). If \( r \) is larger than \( F(t) \Delta t \), the simulation considers that a fluorescence photon is emitted and recorded by the detector. In the analysis of instant FLIM, \( F(t) \) is identical to Eq. (S1), while in other cases, \( F(t) \) are analytically calculated by convolving the corresponding excitation signal (Fig. S4A) with the impulse response function, \( f(t) \). A modulation period \( T \) is completed after the simulation goes through all \( \Delta t \) in that period; then, another \( T \) is simulated likewise, and the emitted photons are cumulatively recorded by the simulation. For a single lifetime measurement, \( N \) modulation periods are simulated, and the lifetime \( \tau \) is calculated based on the FD-FLIM technique in use. This lifetime measurement process is then repeated for 1,000 times to obtain the statistical properties (mean and variance) and consequently, the \( F \)-value, of the lifetime measurement. A diagram summarizing the Monte Carlo simulation process is shown in Fig. S4B.

The analytical error-propagation analysis is applicable when the lifetime can be written in the following form:

\[
\tau = \frac{U}{V} = \frac{\mu_1 + \sigma_1 Y_1}{\mu_2 + \sigma_2 Y_2} = \frac{\mu_1}{\mu_2} \frac{1 + \delta_1 Y_1}{1 + \delta_2 Y_2}, \tag{S31}
\]

where \( U \) and \( V \) are the random variables used in lifetime calculation with means, \( \mu_1, \mu_2 \), standard deviations, \( \sigma_1, \sigma_2 \), and coefficients of variation, \( \delta_1 = \sigma_1 / \mu_1, \delta_2 = \sigma_2 / \mu_2; Y_1 \) and \( Y_2 \) are auxiliary random variables with zero means and unity variances. In practice, \( \sigma_1^2 \approx E[U^2] \) and \( \sigma_2^2 \approx E[V^2] \) as \( E[U]^2 \) and \( E[V]^2 \) are negligible compared to \( E[U^2] \) and \( E[V^2] \). If the moments of third and higher orders are omitted, Eq. (S31) can be expanded as

\[
\tau = \frac{\mu_1}{\mu_2} \left(1 + \delta_1 Y_1 - \delta_1 \delta_2 Y_1 Y_2 + \delta_2^2 Y_2^2 + L \right). \tag{S32}
\]

We then get the expected value of \( \tau \) as

\[
E[\tau] = \frac{\mu_1}{\mu_2} \left(1 - \rho \delta_1 \delta_2 + \delta_2^2 \right), \tag{S33}
\]

where \( \rho = E[Y_1 Y_2] \) is the correlation coefficient of \( U \) and \( V \). We also have

\[
E[\tau^2] = \left( \frac{\mu_1}{\mu_2} \right)^2 \left(1 + 3 \delta_2^2 - 4 \rho \delta_1 \delta_2 \right). \tag{S34}
\]

Therefore, the variance of \( \tau \) can be calculated as

\[
\sigma_\tau^2 = E[\tau^2] - E[\tau]^2 = \left( \frac{\mu_1}{\mu_2} \right)^2 \left( \delta_1^2 + \delta_2^2 - 2 \rho \delta_1 \delta_2 \right), \tag{S35}
\]

and the \( F \)-value can be calculated with Eq. (S30).

Based on the principle of instant FLIM in Section S1, the fluorescence signal \( F(t) \) can be analytically calculated as

\[
F(t) = \frac{1}{\tau} \delta_\tau(t) * \exp \left( -\frac{t}{\tau} \right) = \frac{1}{\tau} \delta_\tau \exp \left( \frac{x + kT}{\tau} \right) \int_{-\infty}^{x} \exp \left( -\frac{x - \lambda T}{\tau} \right) d\lambda = \frac{1}{\tau} \frac{\exp(-t/\tau)}{1 - \exp(-T/\tau)}, \tag{S36}
\]

where we scale the excitation power and the fluorophore concentration down to \( P = 1 \) and \( c = 1 \) such that on average only one photon is emitted during a modulation period \( T \), i.e., \( \int_0^T F(t) \, dt = 1 \). Thus, \( F(t) \) can be regarded as the probability density function of detecting a
photon, and the mathematical expectation of a random variable $X$ related to detecting a photon is

$$E[X] = \int_0^T X F(t) dt.$$  \hspace{1cm} (S37)

We denote the random processes corresponding to the four mixers' outputs [Eq. (S9)] in instant FLIM as $X_1, X_2, X_3$ and $X_4$. With Eqs. (S6) and (S8), Eq. (S9) becomes

$$V_{\phi}(\varphi) = \frac{1}{T} \int_0^T v_{\phi}(t, \varphi) dt$$

$$= \frac{1}{T} \int_0^T [M \sin(\omega t + \varphi)v_{RF}(t) + O_m] dt$$

$$= \frac{M}{T} \int_0^T \sin(\omega t + \varphi) [F(t) - q_0] dt + O_m$$

$$= \frac{M}{T} \int_0^T \sin(\omega t + \varphi) F(t) dt + O_m,$$  \hspace{1cm} (S38)

where we use the fact that $v_{RF}(t)$ and $q_0$ are the RF and DC parts of $F(t)$ and therefore $v_{RF}(t)$ can be written as $v_{RF}(t) = \overline{F}(t) - q_0$. With Eqs. (S37) and (S38), we can write the random processes as

$$X_1 = \frac{M}{T} \sin(\omega t) + O_m,$$

$$X_2 = \frac{M}{T} \cos(\omega t) + O_m,$$

$$X_3 = -\frac{M}{T} \sin(\omega t) + O_m,$$

$$X_4 = -\frac{M}{T} \cos(\omega t) + O_m.$$  \hspace{1cm} (S39)

Since Eq. (S12) follows the form of Eq. (S31), we have $U = V_{\phi}(0) - V_{\phi}(\pi)$ and $V = \omega [V_{\phi}(0.5\pi) - V_{\phi}(1.5\pi)]$. Considering that $N_{\text{det}}$ photons are detected in a lifetime measurement, based on Eqs. (S3) and (S39), we get the means of $U$ and $V$ as

$$\mu_1 = N_{\text{det}} E[X_1 - X_2] = -2N_{\text{det}} M \text{ Im}[q_1] = N_{\text{det}} \frac{2M}{T} \frac{\tau \omega}{1 + \tau^2 \omega^2},$$

$$\mu_2 = \omega N_{\text{det}} E[X_2 - X_4] = 2\omega N_{\text{det}} M \text{ Re}[q_1] = \omega N_{\text{det}} \frac{2M}{T} \frac{1}{1 + \tau^2 \omega^2}. $$  \hspace{1cm} (S40)

The variances and correlation coefficients of $U$ and $V$ can also be obtained as

$$\sigma_1^2 = N_{\text{det}} E[(X_1 - X_2)^2] = N_{\text{det}} \frac{2M^2}{T} [q_0 - \text{ Re}[q_2]] = N_{\text{det}} \frac{2M^2}{T^2} \frac{4 \tau^2 \omega^2}{1 + 4 \tau^2 \omega^2},$$

$$\sigma_2^2 = \omega^2 N_{\text{det}} E[(X_2 - X_4)^2] = \omega^2 N_{\text{det}} \frac{2M^2}{T^2} [q_0 + \text{Re}[q_2]] = \omega^2 N_{\text{det}} \frac{2M^2}{T^2} \frac{2 + 4 \tau^2 \omega^2}{1 + 4 \tau^2 \omega^2},$$

$$\rho \sigma_1 \sigma_2 = \omega N_{\text{det}} E[(X_1 - X_2)(X_2 - X_4)] = -\omega N_{\text{det}} \frac{2M^2}{T^2} \text{ Im}[q_2] = \omega N_{\text{det}} \frac{2M^2}{T^2} \frac{2 \tau \omega}{1 + 4 \tau^2 \omega^2}. $$  \hspace{1cm} (S41)

Then, from Eq. (S35), the standard deviation of $\tau$ is
Fig. S4

Consequently, we get the $F$-value of instant FLIM as

$$F = \frac{\sigma_T}{\tau} = \left(1 + \tau^2 \omega^2\right)^{\frac{1 + 2 \tau^2 \omega^2}{N_{\text{det}}(1 + 4 \tau^2 \omega^2)}}.$$  \hfill (S43)

which is identical to the $F$-value of a two-photon (2P) FD-FLIM when the excitation is modulated as a Dirac comb [6]; this is expected as instant FLIM uses a femtosecond pulse excitation in a 12.5 ns (80 MHz) modulation period, which can be seen as a Dirac comb.

We compare the SNR performances of instant FLIM and conventional FD-FLIM methods [6], including 2P FD-FLIM with sinusoidal (1.0, 0.5 modulation degrees) and periodic square wave (0.2, 0.5 duty cycles) modulations, as well as one-photon (1P) FD-FLIM with sinusoidal modulation (1.0 modulation degree), by plotting their simulated (symbols) and analytical (curves) $F$-values in Fig. S4C. Since the $F$-value is only dependent on the product of the modulation frequency and the fluorescence lifetime [5], i.e., $\omega \tau$, and the modulation frequency for instant FLIM is fixed at 80 MHz, for a fair comparison, we fix the modulation frequency of all the methods to 80 MHz and vary the lifetime from 0 to 4.5 ns when we investigate the $F$-values. The analytical results are in good agreement with the ones obtained from Monte Carlo simulations. Our instant FLIM method has the smallest $F$-value ($F = 1$), which is the shot noise limit and the best possible SNR performance a FLIM method can achieve. In comparison, the 2P sinusoidal (1.0, 0.5 modulation degrees) and periodic square wave (0.2, 0.5 duty cycles) modulations have their best SNR performances at $F = 2.62, F = 4.07, F = 1.44,$ and $F = 2.89$, respectively; the conventional 1P sinusoidal (1.0 modulation degree) case has its best $F$-value at $F = 3.67$. To illustrate the SNR difference among these FD-FLIM methods, we select four targeted lifetime values (0.375 ns, 1.625 ns, 2.875 ns, a 4.125 ns) and plot the traces of 1,000 measurements of their values using different FD-FLIM methods from the Monte Carlo simulations (Fig. S4D). A lifetime histogram and the standard deviation ($\sigma_T$) of the 1,000 lifetime measurements are plotted next to each trace. In all four cases, the instant FLIM measurements have the smallest $\sigma_T$ compared to conventional FD-FLIM methods. In summary, through Monte Carlo simulations and error-propagating analyses, instant FLIM has been demonstrated to be superior in SNR performance compared with conventional FD-FLIM techniques; in some cases, instant FLIM can approach the shot noise limit ($F = 1$), the best possible SNR performance of a FLIM measurement. This is achieved by the efficient utilization of the 80 MHz femtosecond pulsed laser in instant FLIM.

Section S4. Instant FLIM with adaptive optics

Adaptive optics (AO) can be employed in instant FLIM to reduce the optical aberrations induced by the imaging system or the sample, and potentially improve the imaging quality and extend the penetration depth [7]. We implemented AO as an optional module in instant FLIM by introducing a deformable mirror to the setup. To reduce the system complexity and cost, we employed sensorless AO, in which a wavefront sensor was not required, and indirect wavefront sensing based on an optimization metric was used. Using the optimization algorithms described below, the AO module iteratively adjusts the parameters of the deformable mirror to improve the image-based optimization metric.

Depending on the user’s choice in the Instant-FLIM-Control program, the optimization metric, $V$, can be the mean pixel value or the normalized variance of the image, where the normalized variance is defined as the variance of all the pixel values divided by the square of the mean pixel value. We employed Zernike modes to describe the optical aberrations as well as the aberration compensation applied to the deformable mirror. The optimization algorithms are performed in an $N$-dimensional aberration space generated by a base:
\[ \zeta = [z_1, z_2, L, z_N]^T, \]  
where \( z_1, z_2, \ldots, z_N \) are Zernike polynomials. The deformable mirror (Thorlabs DMP40-P01) we used supports up to 12 Zernike modes \( (Z_2^2, Z_2^0, Z_2^2, Z_3^3, Z_3^3, Z_3^1, Z_3^2, Z_4^4, Z_4^2, Z_4^0, \) \( Z_4^2, Z_4^2) \) in the optimization. The aberration compensation, \( \Phi \), can then be described in the aberration space spanned by \( \zeta \):

\[ \Phi = \alpha^T \zeta = \sum_{n=1}^{N} a_n z_n, \]  
where

\[ \alpha = [a_1, a_2, L, a_N]^T \]

are the Zernike coefficients in which \( a_j \) represents the contribution of \( z_j \) to \( \Phi \). In practice, an optimization algorithm iteratively monitors the image-based metric \( V \) and updates the Zernike coefficients \( \alpha \) that control the wavefront shaping elements (mirror segments) on the deformable mirror; after multiple iterations, if the AO optimization is successful, the metric \( V \) will be increased and the imaging quality will be improved.

In the Instant-FLIM-Control program, we use three AO algorithms to optimize \( V \) and imaging quality. The first one is a simple max search algorithm that sequentially searches through each Zernike coefficient, from \( a_1 \) to \( a_N \), until a maximized \( V \) is found for each one of them. For example, to find the optimal value for \( a_j \), the algorithm keeps all the other Zernike coefficients unaltered while changing \( a_j \) from -1 to 1 with a step size defined by the user, e.g., 0.2. This is valid due to the orthogonality of the Zernike modes. The optimal \( a_j \) is found at the value where \( V \) is maximized from \(-1 < a_j < 1\). This algorithm is easy and straightforward, but it is time-consuming: for a step size of 0.2, full optimization of 12 Zernike modes requires the acquisition of 120 raw images. However, this is also the most robust and reliable algorithm, as it guarantees that a global optimum or its proximity can be found, whereas the other algorithms are likely to find a local optimum.

The second algorithm is a quadratic search algorithm [8] which approximates the metric \( V \) of the aberration \( \Phi \) as a quadratic function of the Zernike coefficients \( \alpha \):

\[ V(\Phi) \approx V_0 - \sum_{n=1}^{N} c_n a_n^2, \]

where \( V_0 \) and \( c_n \) are constants and the metric has a paraboloidal shape around its maximum. The goal of the optimization algorithm is to find the coordinates of \( \alpha \) that maximizes \( V \). This process can be decomposed into \( N \) independent one-dimensional parabolical optimization problems for each Zernike coefficient \( a_n \), as Eq. (S47) can be written as

\[ V(\Phi) \approx \left( V_0 - \sum_{n=1}^{N} c_n a_n^2 \right) - c_{e_n} a_n^2. \]

The optimal \( a_n \) that maximizes \( V \) can then be calculated by three measurements of \( V \), i.e., \( V(\Phi_0), V(\Phi_0 + bz_n) \), and \( V(\Phi_0 - bz_n) \) corresponding to \( a_n = a_{n0}, a_n = a_{n0} + b, \) and \( a_n = a_{n0} - b \), respectively, where \( \Phi_0 \) is the initial aberration, \( a_{n0} \) is the initial value of \( a_n \) (\( a_{n0} = 0 \) by default), and \( b \) is the bias amplitude used to search the parabolic maximum. With the three metrics obtained from the three raw images, the optimal \( a_n \), i.e., \( a_{n0} \), can be estimated using parabolic maximization as

\[ a_{n0} = a_{n0} + \frac{V(\Phi_0 + bz_n) - V(\Phi_0 - bz_n)}{2V(\Phi_0) - V(\Phi_0 + bz_n) - V(\Phi_0 - bz_n)} \frac{b}{2}. \]
In the algorithm, this one-dimensional optimization process is repeated for $N$ times to calculate the optimal Zernike coefficients $a_{1x}, a_{2x}, \ldots, a_{Nx}$ that jointly maximizes $V$. Whereas each process requires three measurements, the metric for the initial image $V(\Phi_0)$ is common; therefore, to correct for $N$ Zernike coefficients, the algorithm merely requires $2N + 1$ raw images. For our deformable mirror supporting 12 Zernike modes, the optimization needs only 25 raw images. Compared to the other AO optimization algorithms, the quadratic search one is the fastest and requires the least number of raw images to be captured. However, it is not always reliable as the approximation assumption in Eq. (S47) is only accurate for small aberration amplitudes, which may not be satisfied in practice. Therefore, this algorithm is recommended only if the speed is an essential requirement for AO optimization.

The third algorithm in the program is based on the stochastic parallel gradient descent (SPGD) method [9]. In each SPGD iteration, after the metric $V$ is acquired from a raw image, the Zernike coefficients in $\alpha$ are perturbed in parallel by a small non-zero stochastic value. In our program, the value is randomly chosen from [-0.05, -0.025, 0.025, 0.05]. During the $m$-th iteration, the Zernike coefficients applied to the $m+1$-th iteration is calculated as

$$a_{\alpha}(m+1) = a_{\alpha}(m) - \eta[V(\Phi + \delta \zeta) - V(\Phi)]\delta,$$

(S50)

where the superscripts in brackets are the indices of the iteration, $\eta$ is the learning rate, $\Phi$ is the aberration before the iteration, $\zeta$ is the Zernike base described in Eq. (S44), and $\delta$ is an array consisting of the non-zero stochastic values. The learning rate $\eta$ is negative in our case (-0.01 by default) as the optimization is to maximize $V$. If $\eta$ is chosen properly, the metric $V$ will be increased after each iteration. The algorithm is stopped when a maximal $V$ is found or the specified steps of iterations are reached. Compared to the previous algorithms, the SPGD method updates the Zernike coefficients in parallel and it could acquire the optimal coefficients more accurately. However, due to the stochastic nature of the method, the algorithm usually takes much more iterations, hence imaging time, than the other methods, and it could also easily fall into a local maximum. As a complement to the other algorithms, the SPGD method is recommended when high precision is preferred in determining the optimal Zernike coefficients.

To demonstrate the performance of instant FLIM when the AO module is added, we acquired through-skull 3D FLIM stacks of the intact brain in a living mouse with and without AO. We used the simple max search algorithm for the AO optimization. With AO, the instant FLIM system was able to generate 3D intensity, lifetime, and phasor labeled stacks of the living mouse brain, through the skull, with a penetration depth up to 300 μm (Fig. S7 and Movie S2). Note that the excitation power of the laser was gradually increased from 8.53 mW to 21.13 mW during the imaging; the penetration depth limit was achieved when further increasing the power could not increase the signal level. We then imaged the animal with the same imaging condition but without the AO module. Specifically, we added a folding mirror in front of the AO module to reflect the laser beam directly into the two-photon microscope. As shown in Fig. S7, when the AO module was not used, we could only achieve a penetration depth of 130 μm. In comparison, the penetration depth for instant FLIM with a well-optimized AO module (300 μm) was more than double of that without AO.

**Section S5. Super-resolution instant FLIM with GSOS**

We show that the instant FLIM system can be used to generate super-resolution FLIM images based on the recently demonstrated generalized stepwise optical saturation (GSOS) technique [10]. By linear combining $M$ FLIM images obtained with different excitation powers in the complex domain, a new FLIM image with a $\sqrt{M}$-fold increase in spatial resolution can be obtained. Because the linear combination in GSOS is applied to the complex Fourier coefficients, $q_k$, of the fluorescence, $F(t)$, we rewrite Eq. (S5) such that $q_k$ is explicitly shown in the equation:
\[ v_{\text{IF}}(t) = 2 \sum_{k \in Z} \text{Re}\{q_k \exp(ik\omega t)\} = 2 \sum_{k \in Z} \text{Re}\{q_k\} \cos(k\omega t) - 2 \text{Im}\{q_k\} \sin(k\omega t). \]  

(S51)

Following the analog signal processing in instant FLIM, the output signals of the mixers [Eq. (S9)] become

\[
V_\text{m}(\varphi) = \frac{1}{T} \int_0^T v_{\text{IF}}(t, \varphi) dt
= \frac{1}{T} \int_0^T [M \sin(\omega t + \varphi)v_{\text{RF}}(t) + O_M] dt
= \frac{M}{T} [\text{Re}\{q_1\} \sin(\varphi) - \text{Im}\{q_1\} \cos(\varphi)] + O_M.
\]  

(S52)

Consequently, after introducing the phase shifts, the measured mixer outputs [Eq. (S10)] are

\[
V_\text{m}(0) = -\frac{M}{T} \text{Im}\{q_1\} + O_M,
V_\text{m}(0.5\pi) = \frac{M}{T} \text{Re}\{q_1\} + O_M,
V_\text{m}(\pi) = \frac{M}{T} \text{Im}\{q_1\} + O_M,
V_\text{m}(1.5\pi) = -\frac{M}{T} \text{Re}\{q_1\} + O_M.
\]  

(S53)

Then, the differences among them [Eq. (S11)] are directly proportional to the real and imaginary parts of the Fourier coefficient:

\[
V_\text{m}(\pi) - V_\text{m}(0) = \frac{2M}{T} \text{Im}\{q_1\},
V_\text{m}(0.5\pi) - V_\text{m}(1.5\pi) = \frac{2M}{T} \text{Re}\{q_1\}.
\]  

(S54)

Therefore, the magnitude and phase of the complex Fourier coefficient, \(q_1\), can be extracted as

\[
|q_1| \approx \sqrt{[V_\text{m}(\pi) - V_\text{m}(0)]^2 + [V_\text{m}(0.5\pi) - V_\text{m}(0.5\pi)]^2},
\angle q_1 = \arctan \left[ \frac{V_\text{m}(\pi) - V_\text{m}(0)}{V_\text{m}(0.5\pi) - V_\text{m}(0.5\pi)} \right],
\]  

(S55)

which are then combined as a complex value, \(q_1 = |q_1| \exp(i\angle q_1)\).

In GSOS with \(M\) steps (M-GSOS), the measurement is repeated for \(M\) times with \(M\) different excitation powers, \(I_{01}, I_{02}, \ldots, I_{0M}\), where \(I_{01} < I_{02} < \cdots < I_{0M}\). Hence \(M\) images consisting of these complex Fourier coefficients, \(q_{1,1}, q_{1,2}, \ldots, q_{1,M}\), are obtained based on the measurements described above [Eqs. (S53)-(S55)]. The GSOS linear combination is then applied to these complex images,

\[
q_{1,M\text{-GSOS}} = \sum_{m=1}^{M} c_m q_{1,m},
\]  

(S56)

where the linear combination coefficients, \(c_m\), are calculated based on the excitation powers, \(I_{01}, I_{02}, \ldots, I_{0M}\). Here, we give the general expression to calculate \(c_m\):
where $N$ is the number of excitation photons needed for a fluorophore to emit one photon ($N = 1$ for 1PEF, $N = 2$ for 2PEF). The result of the GSOS linear combination is a new image ($q_{1,M\text{-GSOS}}$) with complex pixel values, where its magnitude, $|q_{1,M\text{-GSOS}}|$, is a super-resolution image with a $\sqrt{M}$-fold increase in resolution over the diffraction limit, and its phase, $\angle q_{1,M\text{-GSOS}}$, is identical to that of the original Fourier coefficient, $\angle q_1$ [10]. From the phase, a fluorescence lifetime image can be obtained by

$$\tau = -\frac{1}{\omega} \tan(\angle q_{1,M\text{-GSOS}}) = -\frac{1}{\omega} \tan(\angle q_1).$$  

(S58)

As a result, by linear combining $M$ complex images obtained with an instant FLIM system using the GSOS principle, we can obtain a super-resolution FLIM image where its intensity, $|q_{1,M\text{-GSOS}}|$, is enhanced by a factor of $\sqrt{M}$ and its lifetime information, $\tau = -\tan(\angle q_{1,M\text{-GSOS}})/\omega$, is preserved.

Instant FLIM can be used to generate super-resolution 3D FLIM stacks. Fig. S8 shows a side-by-side comparison between a diffraction-limited (DL) and a super-resolution (two-step GSOS) 3D FLIM stacks of a fixed mouse brain. The DL stack was acquired with an instant FLIM system at an excitation power of 12.31 mW. The two-step GSOS stack, on the other hand, was generated by linear combining two DL instant FLIM stacks, $q_{1,1}$ and $q_{1,2}$, measured at $I_1 = 12.31$ mW and $I_2 = 13.44$ mW, respectively. The GSOS linear combination coefficients were calculated according to Eq. (S57) ($N = 2$ for 2PEF): $c_1 = 1$ and $c_2 = -I_0^1/I_0^2 = -0.8389$. The two-step GSOS stack, $q_{1,2\text{-GSOS}}$, was then generated based on Eq. (S56), i.e., $q_{1,2\text{-GSOS}} = c_1 q_{1,1} + c_2 q_{1,2} = q_{1,1} - 0.8389 q_{1,2}$; its intensity, $|q_{1,2\text{-GSOS}}|$, and fluorescence lifetime, $\tau = -\tan(\angle q_{1,2\text{-GSOS}})/\omega$, are plotted as brightness and hue, respectively, to construct the 3D FLIM stack. The resolution improvement of the GSOS stack compared to the DL stack can be seen in Fig. S8 (Movie S3), where the line profiles of the normalized intensity values at the positions of the white arrowheads show the features that are better resolved in the GSOS stack.
Fig. S1. Detailed diagram and photos of an instant FLIM system. (A) Detailed diagram of the system configuration. NDF, neutral density filter; $\lambda/2$, half-wave plate; PBS, polarizing beam splitter; $\lambda/4$, quarter-wave plate; DM, deformable mirror; M, mirror; PMT, photomultiplier tube; TA, transimpedance amplifier; DAQ, data acquisition; LPF, low pass filter; LNA, low-noise amplifier; PS, phase shifter. Three sets of components are grouped together and highlighted as system modules. The two-photon microscope module is a conventional two-photon laser scanning microscope setup; the optional adaptive optics module can improve the imaging quality and penetration depth; the analog signal processing module is the essential part of an instant FLIM system. (B) Photo of an implementation of the optional adaptive optics module. (C) Photo of an implementation of the conventional two-photon microscope module. (D) Photo of an implementation of the instant FLIM analog signal processing module.
Fig. S2. Analog signal processing in instant FLIM. (A) Diagram showing how analog signals are processed in instant FLIM. The signals are labeled with numbers and their typical waveforms are shown in (B). (B) Waveforms of the analog signals under real experimental conditions: excitation wavelength, 920 nm; sample, coumarin 6 in methanol; excitation power, 3.6 mW. These signals are: 1, $v_{ref}(t)$, the reference signal from the mode-locked Ti:sapphire laser; 2, $v_{80MHz}(t)$, the pure 80 MHz signals after low pass filtering, amplifying, and splitting the reference signal; 3-6, $v_{LO}(t, \phi)$, the phase-shifted reference signals directed to the mixers’ LO ports; 7, $v_{RF}(t)$, the low pass filtered, amplified, and split PMT signals directed to the mixers’ RF ports; 8, $v_{IF}(t, \phi)$, one of the mixed signals generated on the mixers’ IF ports; 9-12, $V_{IF}(\phi)$, the low pass filtered (DC) mixed signals that are measured by a DAQ card. Unit, volts.
Fig. S3. Instant FLIM system calibration using a lifetime standard. (A) Raw calibration curves acquired from a lifetime standard (1 mM coumarin 6 in methanol) by changing the bias voltage ($V_b$, stepping from 0 to 10V) applied to the four phase shifters and measuring the voltages ($V_{IF}$) on the four mixers' IF ports. A set of calibration curves should be measured for each excitation wavelength that will be used for imaging. Here we show the curves for excitation wavelengths at 780 nm, 800 nm, 860 nm, and 920 nm. Unit, volts. (B) Phase calibration curves, $\varphi(V_b)$, generated using Eq. (S21) based on the raw data in (A). Unit, radians.
Fig. S4. SNR analysis of instant FLIM and conventional FD-FLIM techniques. (A) Excitation and fluorescence waveforms of the six FD-FLIM techniques in comparison: instant FLIM, this work; Sin (1.0), two-photon (2P) sinusoidal (1.0 modulation degree) modulation; Sin (0.5), 2P sinusoidal (0.5 modulation degree) modulation; Square (0.2), 2P periodic square wave (0.2 duty cycle) modulation; Square (0.5), 2P periodic square wave (0.5 duty cycle) modulation; 1P-FLIM, one-photon (1P) sinusoidal (1.0 modulation degree) modulation. Modulation period, 12.5 ns (80 MHz). (B) Diagram summarizing the Monte Carlo simulation process. (C) F-values as a function of fluorescence lifetimes with modulation frequency fixed at 80 MHz for different FD-FLIM methods. The symbols and curves are results of the numerical Monte Carlo simulation and the analytical error-propagation analysis, respectively. (D) Simulated lifetime measurements extracted from four different lifetime values in the Monte Carlo simulations for different FD-FLIM methods. The histograms as well as the standard deviations of the lifetime measurements are also shown.
Fig. S5. Overview of the Instant-FLIM-Control and Instant-FLIM-Analysis software. (A) “System calibration” module calibrates the instant FLIM system using a lifetime standard. “Fast scan” module allows fast acquisition (>1 frames per second using galvo scanners) of two-photon intensity images, which can be used to locate the focal position, to find a ROI, and to generate image-based metrics for the AO optimization algorithms. “Intensity imaging”, “Lifetime imaging”, and “Phasor plots” modules simultaneously acquire and instantaneously generate intensity images, lifetime images, and phasor plots. “3D and 4D measurements” module configures 3D and 4D instant FLIM measurements and displays the results. “GSOS (super-resolution FLIM)” module permits two-step GSOS super-resolution FLIM using two instant FLIM images of the same field-of-view captured under different excitation powers. “Adaptive optics” module optimizes the Zernike coefficients applied to the deformable mirror using the three AO optimization algorithms described in Section S4. (B) Screenshot of the Instant-FLIM-Analysis program where the 3D intensity and phasor stacks of a living Tg(sox10:megfp) zebrafish embryo (2 dpf) acquired with an instant FLIM system were imported and analyzed. (C) Maximized z-projections of the exported 3D stacks segmented by the phasor labels (top) and the phasor clusters (bottom, K=5) shown in (B).
Fig. S6. Instant FLIM with phasor labeling and phaser clustering techniques. (A) Demonstration of the phasor labeling technique, where ROIs are manually drawn on the phasor plot of the fixed BPAE cells to label different cellular structures shown on the right. (B) Demonstration of the phasor clustering technique, where the phasors of the fixed BPAE cells are automatically clustered using the K-means clustering algorithm (K=3) to denote different cellular structures shown on the right. (C) Two-photon fluorescence intensity and lifetime images of MDA-MB-231-EGFP cells under 18 different temperatures acquired with an instant FLIM system. (D) Application of the phasor labeling technique to the instant FLIM results of the MDA-MB-231-EGFP cells in (C). (E) Application of the phasor clustering technique (K=18) to the instant FLIM results of the MDA-MB-231-EGFP cells in (C). As temperature increases, the GFP lifetimes decrease and the phasors shift from bottom-left to top-right of the phasor plot. Scale bar, 20 μm.
Fig. S7. Instant FLIM with adaptive optics enabling through-skull in vivo lifetime imaging of intact mouse brains. (A) In vivo two-photon fluorescence intensity and lifetime maximized x-projection 3D images of an intact Cx3cr1-GFP/+ mouse brain acquired with an instant FLIM system without (top) and with (bottom) the adaptive optics (AO) module. (B) Skull-excluded maximized z-projections (top) and skull-included 3D reconstructions (bottom) of the in vivo intensity, lifetime, and phasor labeled 3D stacks of the intact mouse brain acquired with an instant FLIM system with the AO module. The microglia were labeled with EGFP, and the blood vessels were injected with Texas Red-Dextran. The excitation power was gradually increased from 8.53 mW to 21.13 mW to compensate for the signal loss as the imaging depth increased. Scale bar, 20 μm.
Fig. S8. Super-resolution instant FLIM enabled by GSOS. (A) 3D fluorescence lifetime stacks of a fixed Cx3cr1-GFP/+ mouse brain (microglia labeled with EGFP, blood vessel injected with Dextran 594) captured by an instant FLIM system conventionally and combined with the two-step GSOS technique. The conventional diffraction-limited (DL) stack was imaged at a power of 12.31 mW, and the GSOS stack was generated by linear combining two stacks imaged at 12.31 mW and 13.44 mW, respectively. (B) Maximized z-projections of the 3D stacks in (A). Insets: magnified views. (C) Maximized x-projections of the 3D stacks in (A). Insets: magnified views. The horizontal lines are scanning artifacts caused by the galvo scanners. (D) Line profiles of the normalized intensity values at the positions of the white arrowheads in the DL and GSOS stacks in (B) and (C). Norm., normalized. Scale bars, 20 μm.
Table S1. Acquisition parameters for all data.

<table>
<thead>
<tr>
<th>Figure</th>
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<th>Excitation wavelength (nm)</th>
<th>Excitation power (mW)</th>
<th>Image size (pixel/voxel)</th>
<th>Pixel size (mm)</th>
<th>Voxel depth (μm)</th>
<th>Time interval (s)</th>
<th>Notes</th>
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<tbody>
<tr>
<td>Figs. 1B, S6A, S6B</td>
<td>Fixed BPAE cells, mitochondria labeled with MitoTracker Red, F-actin with Alexa Fluor 488, nuclei with DAPI</td>
<td>12 (Average x20)</td>
<td>800</td>
<td>8.95</td>
<td>560 x 560</td>
<td>100</td>
<td>N/A</td>
<td>N/A</td>
<td>2D fixed sample</td>
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<td>Fig. 1D</td>
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<td>800</td>
<td>9.39</td>
<td>360 x 360</td>
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<td>Fig. 2A</td>
<td>Fixed Cx3cr1-GFP+ mouse brain, microglia labeled with EGFP, blood vessel injected with Dextran 594</td>
<td>12</td>
<td>920</td>
<td>8.53 to 21.13</td>
<td>540 x 540 x 350</td>
<td>200</td>
<td>0.5</td>
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<td>3D fixed sample</td>
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<td>Fig. 2B, S7, Movie S2</td>
<td>Live Cx3cr1-GFP+ mouse, microglia labeled with EGFP, blood vessel injected with Texas Red-Dextran</td>
<td>12</td>
<td>920</td>
<td>8.53 to 21.13</td>
<td>AO: 360 x 360 x 600</td>
<td>No AO: 360 x 360 x 260</td>
<td>300</td>
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<td>Fig. 2D</td>
<td>Fixed BPAE cells, mitochondria labeled with MitoTracker Red, F-actin with Alexa Fluor 488, nuclei with DAPI</td>
<td>12 (Average x20)</td>
<td>800</td>
<td>Step1: 8.95</td>
<td>Step2: 10.61</td>
<td>560 x 560</td>
<td>100</td>
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<td>N/A</td>
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<td>Fig. 3B, left</td>
<td>Live Tg(pu1:gfp) zebrafish, microglia labeled with EGFP</td>
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<td>920</td>
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<td>360 x 360 x 60</td>
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<td>Fig. 3B, right</td>
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<td>12</td>
<td>920</td>
<td>18.43</td>
<td>360 x 360 x 30</td>
<td>300</td>
<td>1</td>
<td>N/A</td>
<td>3D live animal</td>
</tr>
<tr>
<td>Fig. 3F</td>
<td>Live Tg(pu1:gfp) zebrafish, microglia labeled with EGFP</td>
<td>12</td>
<td>920</td>
<td>12.31</td>
<td>360 x 360 x 30</td>
<td>300</td>
<td>1</td>
<td>900</td>
<td>4D live animal</td>
</tr>
<tr>
<td>Figs. 4B</td>
<td>Live Cx3cr1-GFP+ mouse, microglia labeled with EGFP, blood vessel injected with Texas Red-Dextran</td>
<td>12</td>
<td>920</td>
<td>12.31</td>
<td>520 x 520 x 20 x 15</td>
<td>200</td>
<td>2</td>
<td>120</td>
<td>4D live animal</td>
</tr>
<tr>
<td>Figs. 4C, 4F</td>
<td>Live Cx3cr1-GFP+ mouse, microglia labeled with EGFP, blood vessel injected with Texas Red-Dextran</td>
<td>12</td>
<td>920</td>
<td>12.31</td>
<td>360 x 360 x 20 x 3</td>
<td>200</td>
<td>2</td>
<td>120</td>
<td>4D live animal</td>
</tr>
<tr>
<td>Fig. 5B, Movie S4</td>
<td>Live Tg(pu1:gfp) zebrafish, microglia labeled with EGFP, injury induced by 800 nm laser at 43.50 mW for 1 s</td>
<td>12</td>
<td>920</td>
<td>11.05</td>
<td>560 x 560 x 30</td>
<td>49</td>
<td>300</td>
<td>1.5</td>
<td>900</td>
</tr>
<tr>
<td>Fig. 5F</td>
<td>Live Tg(pu1:gfp) zebrafish, microglia labeled with EGFP, injury induced by 800 nm laser at 43.50 mW for 20 s</td>
<td>12</td>
<td>920</td>
<td>11.05</td>
<td>560 x 560 x 30</td>
<td>49</td>
<td>300</td>
<td>1.5</td>
<td>900</td>
</tr>
<tr>
<td>Figs. 6B, Movie S5</td>
<td>Live Cx3cr1-GFP+ mouse, microglia labeled with EGFP, blood vessel injected with Texas Red-Dextran, injury induced by 800 nm laser at 43.50 mW for 120 s</td>
<td>12</td>
<td>920</td>
<td>12.31</td>
<td>520 x 520 x 20 x 29</td>
<td>200</td>
<td>2</td>
<td>120</td>
<td>4D live animal</td>
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<tr>
<td>Figs. 6C, 6F</td>
<td>Live Cx3cr1-GFP+ mouse, microglia labeled with EGFP, blood vessel injected with Texas Red-Dextran, injury induced by 800 nm laser at 43.50 mW for 120 s</td>
<td>12</td>
<td>920</td>
<td>12.31</td>
<td>300 x 300 x 20 x 24</td>
<td>200</td>
<td>2</td>
<td>120</td>
<td>4D live animal</td>
</tr>
<tr>
<td>Figs. S6C-S6E</td>
<td>Live MDA-MB-231-EGFP cells imaged at a temperature between 18.1 and 46.7 °C</td>
<td>12 (Average x10)</td>
<td>920</td>
<td>8.53</td>
<td>360 x 360</td>
<td>200</td>
<td>N/A</td>
<td>N/A</td>
<td>2D live cells</td>
</tr>
<tr>
<td>Fig. S8, Movie S3</td>
<td>Fixed Cx3cr1-GFP+ mouse brain, microglia labeled with EGFP, blood vessel injected with Dextran 594</td>
<td>12 (Average x5)</td>
<td>920</td>
<td>Step1: 12.31</td>
<td>Step2: 13.44</td>
<td>540 x 540 x 118</td>
<td>150</td>
<td>0.5</td>
<td>N/A</td>
</tr>
<tr>
<td>Movie S6</td>
<td>Live Cx3cr1-GFP+ mouse, microglia labeled with EGFP, blood vessel injected with Texas Red-Dextran, injury induced by 800 nm laser at 43.50 mW for 120 s</td>
<td>12</td>
<td>920</td>
<td>12.31</td>
<td>300 x 300 x 20 x 6</td>
<td>200</td>
<td>2</td>
<td>120</td>
<td>4D live animal</td>
</tr>
<tr>
<td>Movie S7</td>
<td>Live Cx3cr1-GFP+ mouse, microglia labeled with EGFP, blood vessel injected with Texas Red-Dextran, injury induced by 800 nm laser at 43.50 mW for 120 s</td>
<td>12</td>
<td>920</td>
<td>12.31</td>
<td>530 x 530 x 20 x 14</td>
<td>200</td>
<td>2</td>
<td>120</td>
<td>4D live animal</td>
</tr>
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### Table S2. Parts and price list for instant FLIM.

<table>
<thead>
<tr>
<th>Vendor</th>
<th>Part number</th>
<th>Qty</th>
<th>Price</th>
<th>Description and link</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Instant FLIM components</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ThorLabs</td>
<td>EF502</td>
<td>4</td>
<td>$69.86</td>
<td>Low Pass Filter (LPF), DC-100 kHz, BNC Connector</td>
</tr>
<tr>
<td>Mini-Circuits</td>
<td>BLP-90+</td>
<td>2</td>
<td>$35.45</td>
<td>Low Pass Filter (LPF), DC-81 MHz, BNC Connector</td>
</tr>
<tr>
<td>Mini-Circuits</td>
<td>ZX60-P103LN+</td>
<td>6</td>
<td>$69.95</td>
<td>Low Noise Amplifier (LNA), 50-3000 MHz, SMA Connector</td>
</tr>
<tr>
<td>Mini-Circuits</td>
<td>ZSC-4-3+</td>
<td>2</td>
<td>$56.95</td>
<td>4-Way Power Splitter, 0.25-250 MHz, BNC Connector</td>
</tr>
<tr>
<td>Mini-Circuits</td>
<td>JSPHS-150+</td>
<td>8</td>
<td>$34.95</td>
<td>Phase Shifter (PS), 100-150 MHz, Surface Mount</td>
</tr>
<tr>
<td>Mini-Circuits</td>
<td>TB-152+</td>
<td>8</td>
<td>$29.95</td>
<td>Board to Surface Mount Phase Shifters, SMA Connector</td>
</tr>
<tr>
<td>Mini-Circuits</td>
<td>ZAD-3H+</td>
<td>4</td>
<td>$50.95</td>
<td>Level 17 Frequency Mixer, 0.05-200 MHz, BNC Connector</td>
</tr>
<tr>
<td>Mini-Circuits</td>
<td>ZFBT-282-1.5A+</td>
<td>1</td>
<td>$59.95</td>
<td>Bias Tee, 10-2800 MHz, SMA Connector</td>
</tr>
<tr>
<td><strong>Total cost:</strong></td>
<td></td>
<td></td>
<td>$1,666.89</td>
<td></td>
</tr>
</tbody>
</table>

**Cables and adapters**

<table>
<thead>
<tr>
<th>Vendor</th>
<th>Part number</th>
<th>Qty</th>
<th>Price</th>
<th>Description and link</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mini-Circuits</td>
<td>141-24BM+</td>
<td>4</td>
<td>$19.45</td>
<td>24'' Coaxial Cable, BNC Connector</td>
</tr>
<tr>
<td>Mini-Circuits</td>
<td>141-18BM+</td>
<td>18</td>
<td>$18.45</td>
<td>18'' Coaxial Cable, BNC Connector</td>
</tr>
<tr>
<td>Mini-Circuits</td>
<td>086-6BM+</td>
<td>6</td>
<td>$15.75</td>
<td>6'' Coaxial Cable, BNC Connector</td>
</tr>
<tr>
<td>Mini-Circuits</td>
<td>086-2SM+</td>
<td>8</td>
<td>$10.75</td>
<td>2'' Coaxial Cable, BNC Connector</td>
</tr>
<tr>
<td>Mini-Circuits</td>
<td>SF-BF50+</td>
<td>6</td>
<td>$3.95</td>
<td>SMA-F to BNC-F Adapter</td>
</tr>
<tr>
<td>Mini-Circuits</td>
<td>SM-BF50+</td>
<td>12</td>
<td>$3.95</td>
<td>SMA-M to BNC-F Adapter</td>
</tr>
<tr>
<td>Mini-Circuits</td>
<td>SM-BM50+</td>
<td>6</td>
<td>$3.95</td>
<td>SMA-M to BNC-M Adapter</td>
</tr>
<tr>
<td><strong>Total cost:</strong></td>
<td></td>
<td></td>
<td>$685.20</td>
<td>Note: cheaper cables and adapters are available from other vendors</td>
</tr>
</tbody>
</table>

Note: cheaper cables and adapters are available from other vendors.
Table S3. Comparison of instant FLIM and TCSPC.

<table>
<thead>
<tr>
<th></th>
<th>Instant FLIM</th>
<th>TCSPC [1]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Principle</td>
<td>Multiplexed mixing of phase shifted analog signals</td>
<td>Accumulative recording of photon arrival events</td>
</tr>
<tr>
<td>Acquisition speed</td>
<td>Determined by the scanner (&lt;10 μs/pixel for galvo; ~1 μs/pixel for resonant and polygon)</td>
<td>Determined by the scanner (&lt;10 μs/pixel for galvo; ~1 μs/pixel for resonant and polygon)</td>
</tr>
<tr>
<td>Data processing time</td>
<td>~ms (real-time)</td>
<td>CPU: several minutes</td>
</tr>
<tr>
<td>(for 1-Megapixel images)</td>
<td></td>
<td>GPU: a few seconds [1]</td>
</tr>
<tr>
<td>Lifetime calculation</td>
<td>Matrix operations</td>
<td>Decorrelation and curve-fitting</td>
</tr>
<tr>
<td>Memory and bandwidth requirement (for 1 μs pixel dwell time)</td>
<td>~20 MB/s</td>
<td>~1 GB/s [11]</td>
</tr>
<tr>
<td>Real-time streaming</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Access to full fluorescence decay curves</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>SNR/sensitivity</td>
<td>$F$-value &gt; 1</td>
<td>$F$-value = 1</td>
</tr>
<tr>
<td>Maximal in vivo FLIM imaging depth demonstrated</td>
<td>~300 μm (through skull)</td>
<td>~130 μm (vertebra removed spinal cord) [12]</td>
</tr>
<tr>
<td>Longest 4D (time-lapse 3D) in vivo FLIM demonstrated</td>
<td>12 hours</td>
<td>2 hours [12]</td>
</tr>
<tr>
<td>Hardware cost</td>
<td>&lt;$2,500</td>
<td>~$25,000 (SPC-160, Becker &amp; Hickl)</td>
</tr>
<tr>
<td>Software cost</td>
<td>Free</td>
<td>~$1,500 (SPC-Image, Becker &amp; Hickl)</td>
</tr>
</tbody>
</table>
Movie S1. Instant FLIM imaging of a fixed mouse brain. Slices and 3D reconstructions of two-photon fluorescence intensity, lifetime, and phasor labeled stacks of a fixed Cx3cr1-GFP/+ mouse brain (microglia labeled with EGFP; blood vessel injected with Dextran 594) measured with an instant FLIM system. The stacks were captured from the surface to the depth of 175 μm inside the tissue.

Movie S2. Through-skull in vivo instant FLIM imaging of intact mouse brains with adaptive optics. Slices and 3D reconstructions of two-photon fluorescence intensity and lifetime stacks of a living Cx3cr1-GFP/+ mouse brain acquired with an instant FLIM system without (left) and with (right) an adaptive optics (AO) module. The “No AO” and “AO” stacks were captured from the surface of the skull to 130 μm and 300 μm inside the brain, respectively. The excitation power was gradually increased from 8.53 mW to 21.13 mW to compensate for the signal loss as the imaging depth increased.

Movie S3. Super-resolution instant FLIM imaging of a fixed mouse brain. 3D reconstructed fluorescence lifetime stacks of a fixed Cx3cr1-GFP/+ mouse brain (microglia labeled with EGFP; blood vessel injected with Dextran 594) captured by an instant FLIM system conventionally (left) and combined with the two-step GSOS technique (right). The stacks were captured from the surface to the depth of 59 μm inside the tissue. The diffraction-limited (DL) conventional stack was imaged at a power of 12.31 mW, and the GSOS stack was generated by linear combining two stacks imaged at 12.31 mW and 13.44 mW, respectively.

Movie S4. 4D in vivo instant FLIM imaging of an injured zebrafish brain. 4D reconstructions of the two-photon fluorescence intensity, lifetime, and lifetime surface rendering stacks of the microglia in a living Tg(pu1:gfp) zebrafish brain at 4 dpf acquired with an instant FLIM system. The 4D stacks were captured every 15 minutes and the totally imaging session was 12 hours long. A laser injury was induced at t = 30 minutes by an 800 nm laser at 43.50 mW lasting for 1 second. The site of the injury is denoted with dashed yellow circles. The dynamics as well as the lifetime changes of the zebrafish microglia responding to the injury can be observed with the 4D movie.

Movie S5. 4D in vivo instant FLIM imaging in an injured mouse brain. 4D reconstructions of the two-photon fluorescence intensity and lifetime stacks of the microglia in a living Cx3cr1-GFP/+ mouse brain acquired with an instant FLIM system. A laser injury was induced at t = 4 minutes by an 800 nm laser at 43.50 mW lasting for 120 seconds. The site of the injury is denoted with dashed yellow circles. The dynamics and the lifetime changes of the mouse microglia responding to the injury can be seen with the 4D movie.

Movie S6. 4D in vivo lifetime and phasor imaging in injured mouse brains. Two-photon fluorescence intensity and lifetime maximized z-projections of the 4D stacks as well as their phasor plots for each time point of the microglia in a living Cx3cr1-GFP/+ mouse brain acquired with an instant FLIM system. A laser injury was induced at t = 0 by an 800 nm laser with a power of 43.50 mW lasting for 120 s. The sites of the injury are denoted with dashed yellow circles. The stacks were captured at 8 different injury sites from one of two mice.

Movie S7. Phasor labeling and phasor clustering techniques applied to 4D in vivo instant FLIM stacks of an injured mouse brain. Reconstructed 4D stacks of the microglia in a living Cx3cr1-GFP/+ mouse brain segmented with the phasor labeling (left) and phasor clustering (K=5) (right) techniques. A laser injury was induced at t = 0 by an 800 nm laser with a power of 43.50 mW lasting for 120 s. The site of the injury is denoted with dashed yellow circles. The cellular structures segmented by the phasor labels or clusters all responded to the injury.

References