Supporting Information

Stringari et al. 10.1073/pnas.1108161108

SI Materials and Methods

Imaging. Fluorescence lifetime images are acquired with two different microscopes. The first set-up is a two-photon microscope coupled with a Becker and Hickl 830 card (Becker and Hickl). Ti:Sapphire laser system (Spectra-Physics Mai Tai) with 80-MHz repetition rate is used to excite the sample. The laser is coupled with a Zeiss Axiovert S100TV microscope. The scanning system is constituted by a scanning mirror (Cambridge Technology Mirror scanner 6350). A Zeiss 40 × 1.2 NA water-immersion objective is used. For image acquisition, the following settings are used: image size of 256 × 256 pixels, scan speed of 32 μm per pixel. A dichroic filter (700DCSPXR; Chroma Technologies) is used to separate the fluorescence signal from the laser light and the fluorescence is detected by a hybrid detector (HPM-100; Hamamatsu). An additional barrier filter is used to block the near IR light. The second set-up for fluorescence lifetime imaging microscopy (FLIM) is a Zeiss 710 microscope coupled to a Ti:Sapphire laser system (Spectra-Physics Mai Tai) and a ISS A320 FastFLIM. A 40 × 1.2 NA water-immersion objective (Zeiss Korr C-Apochromat) is used. For image acquisition the following settings are used: image size of 256 × 256 pixels or 1,024v × 1,024 pixels and scan speed of 25 μm per pixel. A dichroic filter (690 nm) is used to separate the fluorescence signal from the laser light and the fluorescence. For the acquisition of FLIM images, fluorescence is detected by a photomultiplier (H7422P-40; Hamamatsu) and a 610-nm short-pass filter is placed in front of the detector. FLIM data are acquired and processed by the SimFCS software developed at the Laboratory of Fluorescence Dynamics, University of California at Irvine. The excitation wavelengths used were 900, 880, and 740 nm. All samples are excited at 900 nm if not specified otherwise. An average power of about 5 mW was used to excite the live tissue. FLIM calibration of the system is performed by measuring the known lifetime of the fluorescein with a single exponential of 4.04 ns. FLIM data are collected until 100 counts in the brightest pixel of the image are acquired. Typically the acquisition time was of the order of few seconds.

Solution Preparation. Retinol solution (Retinol all trans, Sigma no. R7632) was prepared in DMSO at a concentration of 1 mg/mL at pH 8.5. Retinoic acid (Sigma no. R2625) solution was prepared in DMSO at a concentration of 0.01M (3 mg/mL) at pH 8.5. 250 μM NADH (Sigma no. N8129) solution was prepared in 100 mM Mops buffer at pH 7. A solution of 250 μM NADH is mixed 1:1 with 1,000 unit/mL lactate dehydrogenase (LDH; Sigma no. L3916). FAD (Sigma no. F6625) is diluted at 2 mg/mL in water at pH 7.4. GFP is diluted in 10 mM Tris buffer at a concentration of 20 nM. Protoporphyrin IX (Sigma no. P8293) is diluted at 1.5 mg/mL in dimethylformamide:methanol (1:1) at pH 7. Collagen matrix is prepared with Collagen Type I (BD Biosciences; 354236) at a concentration of 3.75 mg/mL The phasor location of GFP and collagen were measured at 900 nm. Free and bound NADH and FAD were measured at 740 nm. The phasors of retinol, retinoic acid, and porphyryn IX were measured at 790-nm wavelength.

Phasor Transformation and Resolution of a Mixture of Components. When fluorescence lifetime data are acquired in the time domain, the components g (x-coordinate) and s (y-coordinate) of the phasor plot are given by the following expressions:

$$g_{i,j}(\omega) = \frac{\int_0^\infty I_{i,j}(t)\cos(\omega t)dt}{\int_0^\infty I_{i,j}(t)dt} \quad \text{[S1]}$$

$$s_{i,j}(\omega) = \frac{\int_0^\infty I_{i,j}(t)\sin(\omega t)dt}{\int_0^\infty I_{i,j}(t)dt} \quad \text{[S2],}$$

where the indices i and j identify a pixel of the image and ω frequency ($\omega = 2\pi f$), where f is the laser repetition rate (i.e., 80 MHz in our experiment). All phasor plots are calculated at 80 MHz (i.e., the first harmonic of the laser repetition rate and for some cases for higher harmonics).

The phasor transformations of FLIM data acquired in the frequency domain at an angular modulation frequency ω are:

$$g_{ij}(\omega) = m_{i,j}\cos\phi_{i,j} \quad \text{[S3]}$$

$$s_{ij}(\omega) = m_{i,j}\sin\phi_{i,j} \quad \text{[S4],}$$

where $m_{i,j}$ and $\phi_{i,j}$ are the modulation and the phase of the emission with respect to the excitation. Estimations of the lifetime in terms of the phase and modulation can be performed in each pixel by the following formulas (1, 2):

$$\tau_\phi = \frac{1}{\omega}tan(\phi) \quad \text{[S5]}$$

$$\tau_m = \frac{1}{\omega}\sqrt{\left(\frac{1}{m^2}-1\right)} \quad \text{[S6]}$$

In the case of a single exponential decays the two lifetimes obtained by the phase and by the modulation with Eqs. S5 and S6 are equal, but for a multiexponential lifetime system the apparent lifetimes are different.

In the phasor plot if the decay is a single exponential $I(t) = Ae^{-t/\tau}$, the coordinates are given by:

$$g(\omega) = \frac{1}{1+(\omega\tau)^2} \quad \text{[S7]}$$

$$s(\omega) = \frac{\omega\tau}{1+(\omega\tau)^2} \quad \text{[S8],}$$

where τ is the lifetime of the decay and ω is the laser frequency. There is a direct relationship between a phasor location and lifetime. Every possible lifetime can be mapped into this universal representation of the decay (phasor plot). All possible single exponential lifetimes lie on the "universal circle," defined as the semicircle going from point (0, 0) to point (1, 0), with radius 1/2. Point (1, 0) corresponds to $\tau = 0$, and point (0, 0) to $\tau = \infty$. In the phasor coordinates the single lifetime components add directly because the phasor follows the vector algebra. A mixture of two distinct single lifetime components, each of which lie separately on the single lifetime semicircle, does not lie on the semicircle. All of the combinations of two single exponential components must be along the line joining the two lifetime points. In a system with many single lifetime components, the phasor coordinates g and s are described as:

$$g(\omega) = \sum_k \frac{h_k}{1 + (\omega\,\tau_k)^2} \quad \textbf{[S9]}$$

$$s(\omega) = \sum_k \frac{h_k\,\omega\,\tau_k}{1 + (\omega\,\tau_k)^2} \quad \textbf{[S10],}$$

where h_k is the intensity weighted fractional contribution of the single-exponential component with lifetime τ_k. The phasor location of the mixture of single-lifetimes is the intensity-weighted average of the contributions of each single-lifetime that lie separately on the semicircle.

In general, in a system with multiple fluorescent components like a tissue, the overall decay is a phasor that is the sum of the independent phasors of each fluorescence component:

$$G(\omega) = \sum_n f_n g_n(\omega) \quad \textbf{[S11]}$$

$$S(\omega) = \sum_n f_n s_n(\omega) \quad \textbf{[S12],}$$

where f_n is the fractional contribution of each component characterized by the phasor coordinates g_n and s_n. Two molecular species with multiexponential decay are identified by two specific points in the phasor plot inside the semicircle. All possible weighting of the two molecular species give phasors distributed along a straight line joining the phasors of the two species. In the case of three molecular species, all of the possible combinations are contained in a triangle where the vertices correspond to the phasor of the pure species. The phasor plot of an n-component mixture will be contained in a polygon with n-vertices located in the position of the phasor of each contributing component. The calculation of the fractional intensities f_{n} of different fluorescence components that contribute to the signal is performed by a linear estimation on the system described by Eqs. **S11** and **S12** by graphically resolving the sum of phasors.

Multiple Harmonic Phasor Analysis. Phasor transformation of data both acquired in the time domain or frequency domain can be performed at higher harmonics of the fundamental laser frequency ω. We analyze the same FLIM data with phasor transformations at the second and third harmonic ($\omega = n\omega_0$ with $n = 2, 3$) of the fundamental laser repetition angular frequency ($\omega_0 = 2\pi f$), where f is the laser repetition rate (i.e., 80 MHz). For each frequency at which the FLIM data are transformed, we obtain a different phasor histogram. Multiharmonic phasor analysis of FLIM images can separate tissue components that have a similar phasor location but arise from different lifetime distribution, as shown in Figs. S4 and S5. The sensitivity of components separation with higher harmonics analysis is greater for the short lifetime component, such as collagen.

Median Filter. A median filter assigns to one pixel the median value of a 3 × 3 surrounding matrix. This kind of filter does not change the spatial resolution of the image. The median filter is applied to the G and S matrices, so that the features of the images seen in intensity are not weighted. Therefore, we maintain the correlation between neighbor pixels based on lifetime values rather than on intensity features.

1. Spencer RD, Weber G (1969) Measurements of subnanosecond fluorescence lifetimes with a crosscorrelation phase fluorometer. *Ann N Y Acad Sci* 158:361–376.

2. Jameson DM, Hall RD, Gratton E (1984) The measurement and analysis of heterogeneous emissions by multifrequency phase and modulation fluorometry. *Appl Spectrosc Rev* 20(Issue 1):55–106.

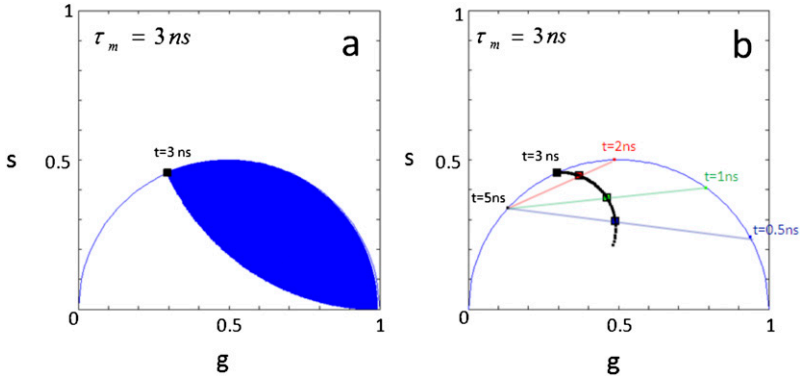

Fig. S1. Resolving power of the phasor plot with respect to average lifetime and classic multiexponential fitting. (*A*) The blue area in the phasor plot represent all possible combinations of two single lifetimes components t_1 and t_2 that give rise to an average lifetime $\tau_m = f_1 t_1 + f_2 t_2$ of 3 ns (i.e., the same average lifetime can be obtained with different combination of two components). Instead, the phasor representation can separate tissue components with the same average lifetime but that are characterized by different lifetime distributions. (*B*) Three molecular species with average lifetime of 3 ns are represented in the phasor plot. The red species has 5-ns and 2-ns components, the green species 5 ns and 1 ns, and the blue species 5 ns and 0.5 ns. The 5-ns exponential component is common to all three species. If you want to resolve a mixture of these molecular species by the classic multiexponential fitting, it is impossible to assign the fractional intensity of the 5-ns exponential to any specific species.

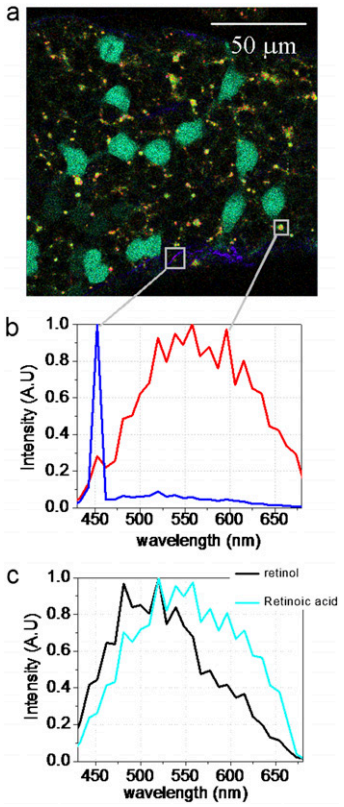

Fig. S2. Spectral image of seminiferous tubule. (*A*) Spectral image excited at 900 nm of the same field of view of the FLIM image of Fig. 2. The colors of the image are spectrally coded. The blue pixels correspond to the second harmonic generation signal acquired at 450 nm. Two different regions of interest are selected. (*B*) Emission spectra measured in the two regions of interest. (*C*) Emission spectra measured from pure retinol and retinoic acid.

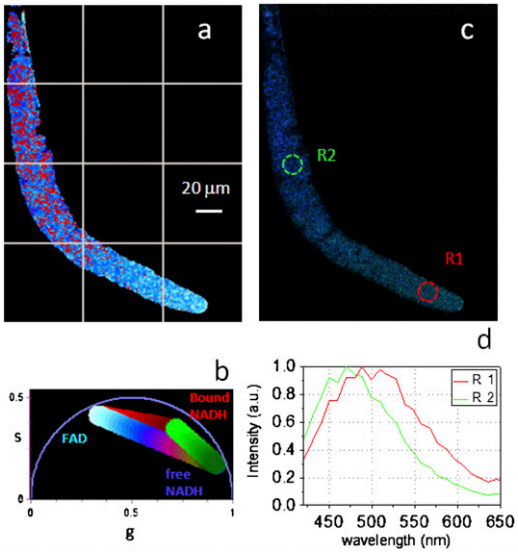

Fig. S3. Metabolite gradients in *Caenorhabditis elegans* germ line. (*A*) Phasor color map of the relative concentrations of FAD, free NADH, and NADH bound to lactate dehydrogenase (LDH) in the same *C. elegans* germ line of Fig. 4*A* that is excited at 740 nm. Pixels in the images are colored according to palette shown in the phasor plot (*B*). (*B*) Phasor plot selection using linear cluster that represent all possible relative concentrations of pure FAD, free NADH, and NADH bound to LDH. (*C*) Spectral image excited at 740 nm of the same field of view of *C. elegans* germ line of Fig. 4*A*. The colors of the image are spectrally coded. The region of interest R1 is selected in the mitotic region and the region of interest R2 is selected in the transition zone of the germ line. (*D*) Emission spectra measured in two regions of interest R1 and R2. The emission spectrum of R1 has a peak at 510 nm, and the R2 emission spectrum has a peak at 470 nm.

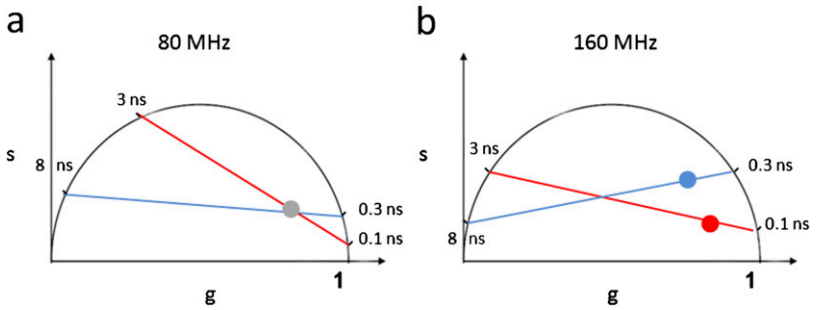

Fig. S4. Multiharmonic phasor representation. (*A*) Phasor plot at the first harmonic, (80 MHz). The gray spot represents two species with two different lifetime distributions. One is a combination of 0.1 ns and 3 ns and the other is a combination of 0.3 ns and 8 ns. (*B*) Phasor plot at the second harmonic, (i.e., at 160 MHz). The same species with the same combination of lifetime are here represented. The species that is a combination of 0.1 and 3 ns is represented in red, and the one that is combination of 0.3 and 8 ns is represented in blue.

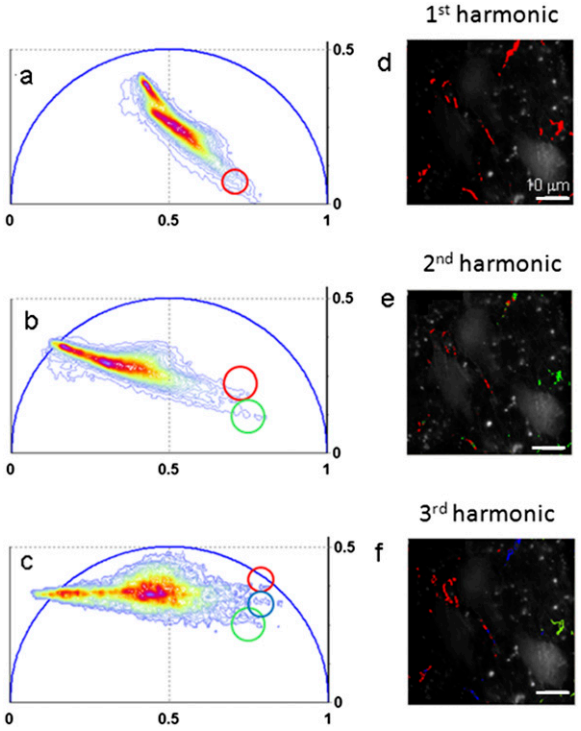

Fig. S5. Multiharmonic phasor analysis separates different lifetime distribution in living tissue. (*A*) Phasor plot of the FLIM images at right calculated at the first harmonic. The red color selects the collagen phasor cluster. (*B*) Phasor plot of the same FLIM images calculated at the second harmonic. The phasor distribution has two separated clusters selected by the red and the green colors. (*C*) Phasor plot of the same FLIM images calculated at the third harmonic. The phasor distribution has three separated clusters selected by the red, the green and the blue colors. (*D*–*F*) Phasor color maps of the FLIM images analyzed with the first harmonic (*D*), second harmonic (*E*), and third harmonic (*F*). Pixels are highlighted with the same color corresponding to the clusters in plot (*A*–*C*). (Scale bars, 10 μm.)