Integrated biophotonics approach for noninvasive and multiscale studies of biomolecular and cellular biophysics

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Abstract. In the crowded cellular milieu, biological processes require coordinated intermolecular interactions, conformational changes, and molecular transport that span a wide range of spatial and temporal scales. This complexity requires an integrated, noninvasive, multiscale experimental approach. Here, we develop a multimodal fluorescence microspectroscopy system, integrated on a single platform, to gain information about molecular interactions and their dynamics with high spatio-temporal resolution. To demonstrate the versatility of our experimental approach, we use rhodamine 123-labeled mitochondria in breast cancer cells (Hs578T), verified using differential interference contrast (DIC) and fluorescence (confocal and two-photon) microscopy, as a model system. We develop an assay to convert fluorescence intensity to actual concentrations in intact, individual living cells, which contrasts with conventional biochemical techniques that require cell lysates. In this assay, we employ two-photon fluorescence lifetime imaging microscopy (FLIM) to quantify the fluorescence quantum yield variations found within individual cells. Functionally driven changes in cell environment, molecular conformation, and rotational diffusion are investigated using fluorescence polarization anisotropy imaging. Moreover, we quantify translational diffusion and chemical kinetics of large molecular assemblies using fluorescence correlation spectroscopy. Our integrated approach can be applied to a wide range of molecular and cellular processes, such as receptor-mediated signaling and metabolic activation. © 2008 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.2952297]

Keywords: biophotonics; mitochondria; fluorescence correlation spectroscopy; rotational diffusion; translational diffusion; fluorescence lifetime imaging microscopy; polarization anisotropy; rhodamine 123.

1 Introduction

Molecule-molecule interactions, conformational dynamics, and diffusion underlie the majority of biological processes in living cells such as cell signaling and metabolic pathways. These biomolecular processes span a wide range of spatial and temporal scales (Fig. 1) inherent in the crowded complex milieu of living cells. Fluorescence-based techniques have become a powerful and noninvasive tool for cell studies with minimal interference with the cell machinery. Different modalities of fluorescence (e.g., confocal and two-photon microscopy) have been used to visualize and interrogate cellular compartments and the interior of biological tissues with diffraction-limited spatial resolution (≈λ/2 nm). Near-field scanning optical microscopy (NSOM), total internal reflection (TIR), and more recently, stimulated emission depletion (STED) have provided a means to image cell organelles with a spatial resolution that extends beyond the diffraction limit. The recent development of single-molecule techniques has reached a new pinnacle, yielding molecular insights into the complexity of enzyme dynamics and chemical kinetics as well as protein folding. From the dynamic perspective, translational diffusion (D_f = 10^{-10} to 10^{-12} cm²/s) has been characterized using fluorescence recovery after photobleaching (FRAP), single particle tracking (SPT), fluorescence correlation spectroscopy (FCS). On a faster time scale, rotational diffusion and excited-state lifetime (10^{-12} to 10^{-8} s) have been used for cell and protein studies due to their sensitivity to both molecular conformation and local environment. Harms et al. have used single-molecule anisotropy imaging for studying lateral and rotational diffusion of fluorescence-labeled lipids on supported lipid bilayers simultaneously. In most cases, however, these techniques are being used separately and on an
individual basis, which makes a comprehensive look at the same biological system from different perspectives challenging.

Mitochondria play a key role in cellular processes such as energy metabolism and apoptosis. In addition, mitochondrial anomalies have been reported to correlate with aging and diseases such as cancer, neurodegenerative diseases, and diabetes. Rhodamine 123 (Rh123) has been widely used as a mitochondrial label with minimal cell toxicity. Rh123 is a lipophilic, photostable fluorophore with a high extinction coefficient ($\varepsilon \approx 7.5 \times 10^{4} \text{ cm}^{-1} \text{ mol}^{-1}$) and fluorescence ($\approx 525 \text{ nm at emission}$) quantum yield ($\approx 0.9$). The high affinity of Rh123 to mitochondria has been attributed to its positive charge and the membrane potential of mitochondria, a discovery that has been exploited in membrane potential studies. Compared with normal cells, cancer cells generally exhibit a relatively high mitochondrial membrane potential, thus having a high affinity to the charged Rh123 in the mitochondria. The nature of Rh123 interaction with mitochondria is not fully understood, even though this fluorescence label has been widely used for tracking and electrophysiological assessment of mitochondria.

Here, we describe an integrated, noninvasive, fluorescence-based approach that enables us to perform comprehensive studies on biomolecular and cellular systems over a wide range of spatial (nanometer to millimeter) and temporal (picosecond to second) resolutions (Fig. 1). This experimental approach involves the integration of fluorescence microscopy [confocal and two-photon (2P)], time-resolved fluorescence lifetime imaging (FLIM), fluorescence polarization anisotropy imaging, and different modalities of fluorescence correlation spectroscopy, all built on a single platform. Fluorescence microscopy and differential interference contrast (DIC) imaging would provide the morphological context and cellular framework in which the molecular processes take place. To convert fluorescence intensity to a concentration image, FLIM is used to account for the variation of fluorescence quantum yield (i.e., lifetime) of a given fluorophore throughout cells/tissues. Multiplexional fluorescence decay of a given fluorophore can be assigned to either 1, different excited electronic states (with minor changes in the molecular structure or surrounding), or 2, different conformations (or species) of the same fluorophore. To address this issue, steady-state anisotropy imaging would correlate the heterogeneity in FLIM with the constraints of cellular environment on the fluorophore. In addition, time-resolved anisotropy measurements enable us to relate different fluorescence lifetimes to structures, based on the hydrodynamic volume and surroundings of the fluorophore, since multiplexional anisotropy decays can be interpreted as segmental mobility (conformational changes) or as multiple species with different volumes due to association (transient or long-lived) with other molecules. Further, biomolecules with large volumes would not rotate during the fluorescence lifetime, and therefore, it will be difficult to determine their sizes. As a result, we employed fluorescence correlation spectroscopy (FCS) to distinguish between conformational changes and the coexistence of multiple diffusing species. Table 1 summarizes these techniques, their temporal and spatial resolution, experimental observables, and the projected molecular information. As a proof of principle, we used human breast cancer cells (Hs578T) labeled with Rh123 as a model system to demonstrate the potential of this integrated, multiscale experimental approach.

2 Materials and Methods

2.1 Cell Culture and Mitochondrial Labeling

The human breast cancer cell line Hs578T and its culture media were obtained from American Type Culture Collection (ATCC, Manassas, Virginia). The media is a mixture of Dulbecco’s Modified Eagle Media (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were cultured in T-75 flasks (BD Biosciences, San Jose, California) in a 37 °C incubator with 5% CO2 and allowed to reach 80 to 90% confluence before passing. The cells were then passed to glass-bottomed petri dishes (MatTek, Ashland, Massachusetts) and incubated overnight. After that, the media was removed and replaced with fresh DMEM containing mitochondrial marker Rh123 (Invitrogen, Chicago, Illinois) at a final concentration of 500 nM. When performing FCS measurements, the incubation concentration was reduced to 100 nM. The labeled cells were subsequently incubated for ~15 min before washing three times with phosphate buffered saline (PBS) (Invitrogen, Grand Island, New York) and adding 2-mL imaging buffer (135-mM NaCl, 5-mM KCl, 1-mM MgCl2, 1.8-mM CaCl2, 20-mM HEPES, and 5-mM glucose). Cell morphology was monitored before and after each measurement using DIC to examine possible cell photodamage. For control experiments, free Rh123 was used in PBS (pH=7.4) at low concentrations (~5 μM).

2.2 Experimental Setup and Data Analysis

A schematic diagram of the multimodal system, housed on a single platform, is described in Fig. 2. The background and data analysis of each technique are briefly discussed.

2.2.1 Confocal microscopy

The confocal microscope consists of an inverted microscope (Olympus, IX81, Melville, New York), 1.2-NA (60 ×) water-immersion objective, a laser scanning unit (FV300, Olympus),
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Table 1 Integrated fluorescence microspectroscopy provides molecular and cellular information on multiple temporal and spatial scales. Different aspects of each technique are summarized.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Spatial resolution</th>
<th>Temporal resolution</th>
<th>Observables</th>
<th>Molecular information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence (confocal and 2P) Microscopy</td>
<td>~250 nm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>~200 ns&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Fluorescence intensity per pixel during laser scanning</td>
<td>• Spatial distribution (2- and 3D) of fluorophores</td>
</tr>
<tr>
<td>Fluorescence polarization imaging</td>
<td>~250 nm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>~200 ns&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Parallel and perpendicular fluorescence intensity recorded simultaneously during laser scanning</td>
<td>• Cell morphology</td>
</tr>
<tr>
<td>2P fluorescence lifetime imaging</td>
<td>~250 nm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>~20 ps&lt;sup&gt;c&lt;/sup&gt;</td>
<td>The probability histogram of the arrival of a single photon per excitation-detection cycle</td>
<td>• Dipole-moment orientation</td>
</tr>
<tr>
<td>Time-resolved fluorescence polarization anisotropy</td>
<td>~250 nm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>~50 ps</td>
<td>Time-resolved fluorescence with parallel and perpendicular polarizations, with respect to the excitation laser pulses</td>
<td>• Excited-state dynamics</td>
</tr>
<tr>
<td>Förster resonance energy transfer (FRET)</td>
<td>~10 nm</td>
<td>~20 ps</td>
<td>Time-resolved fluorescence of a FRET pair (i.e., donor and acceptor)</td>
<td>• Conformation sensitive</td>
</tr>
<tr>
<td>Fluorescence correlation spectroscopy (FCS)</td>
<td>~250 nm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100 ns to &gt;10 s</td>
<td>Fluorescence fluctuations of single molecules diffusing throughout an open observation volume</td>
<td>• Environment sensitive</td>
</tr>
</tbody>
</table>

<sup>a</sup>Diffraction-limited spatial resolution based on excitation wavelength of ~500 nm.
<sup>b</sup>Depending on the resolution and size of each image frame.
<sup>c</sup>Depending on the signal-to-noise ratio and the complexity of the fluorescence decay.

and cw lasers [Fig. 2(a)] with DIC capability. Rh123 was excited using 488 nm from an argon ion laser (Melles Griot, Carlsbad, California), while the fluorescence was collected using a 525/30-BP filter (Chroma, Rockingham, Vermont) and two photomultiplier tubes (PMTs). Data acquisition and analysis were carried out using the Fluoview300 (Olympus) imaging software for 3-D and time-series imaging. The confocal system was modified to allow for fluorescence polarization anisotropy imaging using a newly developed algorithm.

2.2.2 Femtosecond laser system

For 2P microscopy and dynamics studies, femtosecond laser pulses (~120 fs, 76 MHz) were generated using a titanium-sapphire solid state laser system (Mira 900-F, Coherent, Santa Clara, California) pumped by a diode laser (Verdi-10W, Coherent). The fundamental excitation wavelength range (λ<sub>e</sub>: 700 to 1000 nm) was extended to 350 to 500 nm and 500 to 700 nm using a second harmonic generator (SHG 4500, Coherent) and optical parametric oscillator (Mira OPO, Coherent), respectively [Fig. 2(b)]. The laser pulses (1P or 2P) were steered toward either the modified FV300 scanner or the back exit port of the microscope for sample excitation using a high NA objective. For single-point measurements, the repetition rate was reduced to 4.2 MHz using a pulse picker (Mira 9200, Coherent).
2.2.3 Fluorescence lifetime imaging microscopy

Following pulsed excitation, the epifluorescence (1P or 2P) was isolated using a dichroic mirror and filters before being separated into two channels (using either a 50/50 or polarizing beamsplitter) for lifetime, anisotropy, or FRET imaging [Fig. 2(c)]. The epifluorescence polarization was resolved using a Glan-Thompson polarizer per channel prior to being amplified, routed, and then detected using multichannel plate-PMTs (R3809U, Hamamatsu, Hamamatsu City, Japan). The time-correlated single-photon counting histogram was then recorded using the SPC-830 module (Becker and Hickl, Berlin, Germany), which was synchronized with a fast photodiode signal. The measured fluorescence decays at a given pixel can generally be described as a sum of exponentials with time constants ($\tau_i$) and amplitudes ($\alpha_i$):

$$I_{S4\tau}(t,x,y) = \sum_{i=1}^{3} \alpha_i(x,y) \cdot \exp[-t/\tau_i(x,y)].$$

The average fluorescence lifetime is calculated as:

$$\langle \tau_f \rangle = \sum \alpha_i \tau_i / \sum \alpha_i,$$

where the number of exponentials depends on the fluorophore structure and its surroundings. These measurements were carried out using magic-angle (54.7 deg) polarization to eliminate rotational diffusion effects on the measured lifetime. The excited-state fluorescence decay rate ($k_{ir} = 1/\tau_i$) of a given fluorophore is dependent on the radiative ($k_r$) and nonradiative ($k_{nr}$) rates: $k_{ir} = 1/\tau_i = k_r + k_{nr}$. A nonlinear least-square fitting routine (SPCM, Becker and Hickl) was used to analyze fluorescence decays with a deconvolution algorithm. The system response function was either generated by the software (based on the decay rise time) or measured experimentally (using the second harmonic signal from monobasic potassium phosphate crystals).

In 2P FLIM, $256 \times 256$ pixels were used with 64 time bin per pixel (i.e., 259 ps/bin), which naturally leads to a low signal-to-noise ratio and low time resolution per pixel. As a result, FLIM measurements were complemented with comparative studies with a 1P excitation laser that was strategically parked (i.e., single point) on a region of interest (e.g., mitochondrion). Another alternative is to scan the 2P laser pulses over a region of interest (e.g., a single cell), which we refer to as pseudo-single point measurements. In either case, the fluorescence signal was stored in 1024 channels (12.2 ps/channel) and the fitting quality was evaluated using the residual and $\chi^2 (<1.3)$.

2.2.4 Converting fluorescence intensities to concentration images

We have developed a protocol for converting fluorescence intensity images to quantitative concentration of a fluorophore. In this protocol, the 2P FLIM and intensity images of stained cells were recorded simultaneously. Under the same experimental conditions of excitation and detection, the free fluorophore (Rh123 in this case) with known concentration was measured in solution for system calibration. The time-averaged 2P fluorescence ($\lambda_{ir}$) from a given pixel $(x,y)$, $F_{2p}(x,y)$, is defined as:

$$F_{2p}(x,y) = \Phi(x,y) g_P(x,y) \sigma_{2p}(\lambda_x) I_P(\lambda_x)/2f_p\tau_p,$$

where $\Phi(x,y)$ and $C(x,y)$ are the fluorescence quantum yield and concentration of a fluorophore at a given pixel. The fluorescence signal also depends on the detection efficiency [$g_P(x,y)$], the 2P excitation cross section [$\sigma_{2p}(\lambda_x)$] of the fluorophore, and the squared intensity of the excitation laser pulses $I_P(\lambda_x)$. The excitation pulse width ($\tau_p$), repetition rate ($f_p$), and temporal profile ($g_P$) can be determined for a modeled laser system. The fluorescence quantum yield, however, is linearly proportional to the fluorescence lifetime ($\tau_f$) in a given pixel: $\Phi(x,y) = k_e \tau_f(x,y)$, assuming a constant radiative rate ($k_e$) that can be calculated using steady-state spectroscopy. As a result, the dependence of 2P fluorescence signal per pixel on both fluorophore concentration and lifetime should be accounted for such that:

$$F_{2p}(x,y) = \Phi(x,y) g_P(x,y) \sigma_{2p}(\lambda_x) I_P(\lambda_x)/2f_p\tau_p.$$
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\[
(F_{2P}(x,y)) = \tau_p(x,y)\psi(\lambda_p, \lambda_e)C(x,y).
\]  

(3)

The system parameter \( \psi = k_0 \xi(\lambda_p) \sigma_{2P}(\lambda_e)g_pG(\lambda_e)/2f_p\tau_p \) can be determined experimentally albeit challenging. The system parameter, however, can be cancelled out in relative measurements when the free fluorophore (in solution) of known concentration \( C_{sol}(x,y) \) and labeled cells are measured under the same experimental conditions such that:

\[
C_{cell}(x,y) = \frac{F_{2P}(x,y)}{F_{sol}(x,y)} \frac{C_{sol}(x,y)}{\frac{G(x,y)}{G(0)}}. \]

(4)

Accurate calculations of the concentration image \( C_{cell}(x,y) \) in living cells would require careful analysis of the background signal (± the standard deviation) to ensure a nonzero denominator for any pixel in Eq. (4). In addition, the photosensitivity of polarized excitation must also be accounted for, especially for imaging restricted biomolecules in a highly organized environment such as biomembranes. This protocol would also benefit from further modification to account for possible changes of the radiative rate constant (i.e., steady-state spectroscopy) and reduction of fluorescence intensity (or lifetime) due to quenching of aggregated fluorophores in crowded cell compartments.

2.2.5 Molecular order and fluorescence polarization anisotropy

During the excited state lifetime, molecules rotate with characteristic times that depend on their hydrodynamic volumes and immediate surroundings. Both the rotational diffusion and dipole-moment orientation distribution can be measured using fluorescence polarization anisotropy imaging. At any given pixel coordinates \( (x,y) \), the fluorescence polarization anisotropy \( r(t,x,y) \) can be calculated from two simultaneously measured fluorescence intensity images of parallel \( I_\parallel(t,x,y) \) and perpendicular \( I_\perp(t,x,y) \) polarizations, such that:

\[
r(t,x,y) = \frac{[I_\parallel(t,x,y) - I_\parallel(t,x,y)] - G \cdot [I_\perp(t,x,y) - I_\perp(t,x,y)]}{[I_\parallel(t,x,y) - I_\parallel(t,x,y)] + 2G \cdot [I_\perp(t,x,y) - I_\perp(t,x,y)]},
\]

(5)

where the G factor accounts for the bias of the polarization detection. For 1P steady-state anisotropy imaging, the G factor can be estimated by comparing steady-state anisotropy of reference fluorophores in a spectrophotometer and in the confocal system. For time-resolved anisotropy, the G factor can be estimated using the tail-matching approach of a small molecule with a long excited state lifetime (e.g., fluorescein) and a similar emission spectra. The background of parallel \( I_\parallel(t,x,y) \) and perpendicular \( I_\perp(t,x,y) \) images was estimated in an area away from the cell boundaries (or the baseline of corresponding fluorescence decay) and subtracted prior to calculating the corresponding anisotropy decays. Based on the molecular and cellular complexity, time-resolved anisotropy decays can generally be described as:

\[
r(t,x,y) = \sum_{i=1}^{3} \beta_i(x,y) \cdot \exp[-t/\varphi(x,y)],
\]

(6)

where the sum of pre-exponential factors \( (\beta_i) \) equals the initial anisotropy \( (r_0) \). A fluorophore tethered to a large macromolecule would undergo a segmental motion on a fast time scale, while the overall rotational time \( (\varphi) \) would become too slow with respect to the fluorescence lifetime (Fig. 1). In this case, the corresponding pre-exponential factor for the relatively immobile fraction is called a residual anisotropy \( (r_m) \). Time-resolved fluorescence polarization anisotropy decays were analyzed using OriginPro 7 (OriginLab, Northampton, Massachusetts) without deconvolution with the system response function [full-width at half maximum (FWHM) ~50 ps]. The initial anisotropy depends on the orientation angle \( (\delta) \) between the absorbing and emitting dipoles, such that:

\[
r_0(x,y) = \frac{2\alpha}{2\alpha + 3} \left[ 3 \cos^2 \delta(x,y) - 1 \right],
\]

(7)

where \( \alpha \) is the number of excitation photons (=1 for 1P and =2 for 2P), and the maximum theoretical value of \( r_0 \) is 0.4 for 1P and 0.57 for 2P. High NA objectives can also introduce optical depolarization effects, i.e., smaller steady-state anisotropy. The rotational diffusion time \( (\varphi) \) of a spherical molecule (which is a valid approximation for Rh123), depends on its hydrodynamic volume \( (V) \) and surrounding viscosity \( (\eta) \):

\[
\varphi(x,y) = \frac{\eta(x,y)V(x,y)}{k_BT},
\]

(8)

where \( T \) and \( k_B \) are temperature (298 K) and the Boltzmann constant, respectively. In some cases, it is difficult to separate the viscosity and size effects on the observed rotational time and, as a result, the apparent hydrodynamic volume may be defined as \( V_{ap}(x,y) = V(x,y)/\varphi_0 \), normalized by the viscosity of water \( (\eta_0) \) at room temperature (0.0089 P). Because the excited state lifetime represents the observation window for real-time monitoring of the rotational diffusion of a fluorophore, this technique is limited to molecules with relatively small volumes.

2.2.6 Fluorescence correlation spectroscopy

FCS is a powerful tool for investigating translational diffusion and chemical kinetics that cause concentration (i.e., fluorescence) fluctuations of single molecules as they diffuse throughout an open observation volume. To extend our observation time for monitoring translational diffusion and association kinetics, we have integrated different modalities of FCS into our experimental platform (Fig. 1). The epifluorescence was isolated from the excitation light using a dichroic mirror (690SP) and a filter (525/50BP) prior to being separated into two channels (using either 50/50 or polarizing beamsplitters). Fluorescence fluctuation signal from an open observation volume (~10^{-15} L) was then focused on an optical fiber (50 μm diam), which acted as a confocal pinhole prior to detection by one (autocorrelation) or two (cross-correlation) avalanche photodiodes (APDs, SPCM CD-2969,
The signals were then correlated using an external multiple-tau-digital correlator (ALV/6010-160, Langen/Hessen, Germany). The system was routinely calibrated using a photosatable fluorophore, rhodamine green (Invitrogen), with known diffusion coefficient ($D_T \sim 2.8 \times 10^{-6}$ cm$^2$/s).

The autocorrelation function $G(\tau)$ of fluorescence fluctuation is defined as

$$G(\tau) = \frac{1}{N} \left[ \frac{\delta F(t) \cdot \delta F(t+\tau)}{\langle F(t) \rangle^2} \right] ,$$

where $F(t)$ is the fluorescence intensity at a given time $t$, and $G(\tau)$ is the corresponding fluorescence fluctuation. The autocorrelation for a single diffusing species in a 3-D Gaussian observation volume is given by

$$G_D(\tau_{x,y,z}) = \frac{1}{N} \left[ \frac{\delta F(t) \cdot \delta F(t+\tau_{x,y,z})}{\langle F(t) \rangle^2} \right] .$$

where $N$ is the average number of molecules with a residence diffusion time of $\tau_0$ and an axial-to-lateral dimension ratio (or structure parameter) of $S=a/\sigma_0$. For spherical fluorophores under 1P excitation, the diffusion coefficient $D_T = \sigma_0^2/4 \tau_0$ = $k_BT/4\pi\sigma_0^2$, where $k_B$ and $a$ are Boltzmann constant and the radius of the diffusing fluorophore, respectively. In contrast to rotational diffusion ($D_R = 1/6\phi k_BT/3\pi\eta V$), the translational diffusion time using FCS depends on $\sqrt{V}$, and therefore, is less sensitive to the molecular hydrodynamic volume.

3 Results and Discussion

3.1 Mitochondrial Distribution and Movement in the Context of Cell Morphology

DIC and confocal fluorescence images of the Rh123-stained human breast cancer cell line MCF-7 revealed a relatively large nucleus-to-cell-size ratio [Figs. 3(a)-3(c)], which agrees with previous reports on other cancer cell lines, such as human gastric carcinoma cells. This increase of nucleus-to-cytoplasm ratio in cancer cells has already been used by pathologists as a qualitative indicator for malignancy. The representative DIC [Fig. 3(a)] and confocal [Fig. 3(b)] images show the cell morphology (while undergoing mitosis) and mitochondrial distribution where Rh123 is localized. The observed perinuclear mitochondrial distribution in cancer cells has been observed previously in other cancer cell lines such as breast cancer cells (MCF-7) and human lung carcinoma cells (A549), which is attributed to ATP supply for detoxification and high motility in these carcinoma cells.

For time-dependent measurements on a single mitochondrion, it is essential to assess the mobility of mitochondria such that we can optimize our data acquisition time, which can be as long as several minutes. A time sequence (up to 90 s) of mitochondrial imaging is also shown in Figs. 4(a)-4(j), away from the nucleus (0.1 frame/s), for tracking the movements of single mitochondrion. A video of the mitochondrial movement [Video 1] is provided as supplementary material (37 frames, 10 s/Frame). A rough estimate of the lateral diffusion coefficient of a single mitochondrion can be calculated using a basic single particle tracking algorithm,
especially for a small and round mitochondrion (e.g., $D_T \sim 1.16 \times 10^{-11} \text{cm}^2/\text{s}$ and an average velocity of $\sim 0.016 \mu\text{m}/\text{s})$. For visualization purposes, the trajectory of a single mitochondrion is also shown [Fig. 4(k)]. In these rough calculations, we assumed a Brownian motion ($\langle r^2 \rangle = 4D_T t$), which may not be a valid approximation.

### 3.2 Excited State Dynamics of Rh123 are Sensitive to Mitochondrial Environment

#### 3.2.1 Two-photon fluorescence lifetime imaging spectroscopy

Typical 2P fluorescence intensity and lifetime images of Rh123-labeled mitochondria in a single cancer cell are shown in Figs. 5(a) and 5(b), respectively. These images are recorded using 960-nm excitation (76 MHz, ~3 mW at the sample) and a collection time of up to 120 s. The pseudocolor of FLIM images (256×256 pixels, 64 time channels/pixel, ~259 ps/channel) represents the average lifetime per pixel of Rh123 throughout the cell. The fluorescence of mitochondrial Rh123 decays as a single exponential with an average lifetime of $3.1 \pm 0.5 \text{ ns}$ ($n=5$), which is shorter than free Rh123 in solution (3.7 ± 0.3 ns, $n=3$), measured under the same conditions. The corresponding pixel-lifetime histogram [Fig. 5(c)] shows a broad lifetime distribution due to the heterogeneity of the mitochondrial environment. The observed single-exponential fluorescence decay of Rh123 is in agreement with Scheenenburger et al., who studied Rh123-stained BKEz-7 endothelial cells using a combination of one-photon FLIM and total-internal reflection techniques. However, the reported fluorescence lifetime of Rh123 is slightly shorter (2.7 ± 0.3 ns) in BKEz-7 endothelial cells (using 5 and 10 $\mu\text{M}$ for 30-min incubation). As the dye concentration was increased to 50 and 100 $\mu\text{M}$, the authors reported biexponential decays with a fast decay component of $0.55 \pm 0.1 \text{ ns}$ (amplitude fraction $0.4 \pm 0.2$). Further reduction of Rh123 fluorescence lifetime was reported in the BKEz-7 mitochondria. These differences with our Rh123 fluorescence lifetime in cancer cells can be attributed to the low concentration used in our studies, which would minimize possible homo fluorescent energy transfer (FRET). In addition, the sensitivity of this lipophilic fluorophore to the mitochondrial membrane potential, which would depend on the cell line, should not be ruled out. Finally, the use of magic-angle polarization in our studies would eliminate rotational effects on the measured excited state dynamics of Rh123. These FLIM results demonstrate the sensitivity of Rh123 fluorescence lifetime (i.e., quantum yield) to the cellular environment, and therefore, must be accounted for before converting 2P intensities to accurate concentration images of the dye (see next).

#### 3.2.2 Single-point, time-resolved fluorescence

To overcome the low signal-to-noise ratio and temporal resolution in FLIM, single-point fluorescence decays were also measured on both free Rh123 in solution (pH 7.4) and mitochondrial Rh123 in living cells [Fig. 5(d), Table 2]. When one-photon laser pulses (488 nm, 4.2 MHz, a few $\mu\text{W}$) were strategically focused on an apparent Rh123-stained mitochondrion in live cells, the 1P fluorescence decayed as a biexponential with $\tau_1 = 2.38 \pm 0.19 \text{ ns}$, $\tau_2 = 0.42 \pm 0.05$, and $\tau_2 = 3.75 \pm 0.09 \text{ ns}$, $\tau_2 = 0.58 \pm 0.05$ [Fig. 5(d)]. The estimated average lifetime of 3.2 ± 0.1 ns ($n=8$) of these decays are consistent with the previously mentioned 2P FLIM studies (3.1 ± 0.3 ns; $n=5$). These decay parameters are significantly different from free Rh123 in solution, where a single exponential was observed with a fluorescence lifetime of 3.9 ± 0.2 ns. The measured fluorescence lifetime of Rh123 in solution (PBS, pH 7.4) is in agreement with the literature value. Further, Ferguson et al. reported a high fluorescence quantum yield (~0.96) for free Rh123, with a negligible yield (0.02) for the triplet intersystem crossing in aqueous solution.

The presence of two decay components of mitochondrial Rh123 suggests the existence of either two different emitting species (e.g., two conformations of the fluorophore or different local environments) or excited electronic states. There is some evidence suggesting that the cell uptake of Rh123 is sensitive to the membrane potential, while its toxicity correlates with the inhibition of both F0F1-ATPase and the respiratory function. As a result, it was hypothesized that a fraction of the Rh123 uptake was likely to exist in the mitochondrial matrix for F0F1-ATPase inhibition. Due to the dynamic nature of mitochondria and their relative sizes, it is possible that our observation volume may also sample a fraction of fluorophores in the cytosol or on the glass surface. However, these fractions are likely to be negligible under our experimental conditions.

The heterogeneity of fluorescence lifetime (i.e., quantum yield) throughout the cell is used to convert fluorescence intensity images to accurate concentration distributions (Sec. 3.3). Further, we conducted both steady-state and time-resolved fluorescence polarization imaging to elucidate the structural basis that underlies the biexponential decay of mitochondrial Rh123 (Sec. 3.4).

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**Table 2** A summary of the fitting parameters of time-resolved fluorescence (magic angle detection) of Rh123 in mitochondria of cancer cells. These single-point, one-photon measurements were carried out using 480-nm laser pulses (4.22 MHz), which were strategically focused on regions of interest (i.e., mitochondria), and the fluorescence emission was detected using 525/50BP filter ($n=8$). The corresponding fluorescence decay of free Rh123 in solution is also shown for comparison.

<table>
<thead>
<tr>
<th>Environment</th>
<th>$\alpha_1$</th>
<th>$\tau_1$ (ns)</th>
<th>$\alpha_2$</th>
<th>$\tau_2$ (ns)</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td>0.42(5)*</td>
<td>2.4(2)</td>
<td>0.58(5)</td>
<td>3.75(9)</td>
<td>3.2(1)</td>
</tr>
<tr>
<td>PBS (pH 7.4)</td>
<td>1</td>
<td>3.9(2)</td>
<td>—</td>
<td>—</td>
<td>3.9(2)</td>
</tr>
</tbody>
</table>

*The number in parentheses represents the standard deviation in the last digit of a given fitting parameter.
Fig. 5 2P intensity, FLIM, and lifetime distribution histogram of Rh123-labeled mitochondrial reflect a heterogeneous environment in a single cancer cell. The 2P fluorescence images (256 x 256 pixels) were recorded simultaneously using 960-nm excitation, 120-s acquisition time, with 259 ps/channel. (a) A combination of these 2P fluorescence intensity and (b) FLIM images are used to calculate the concentration distribution of Rh123 throughout the intact, living cell without the need for cell lysates. In addition, the 2P FLIM image provides molecular information concerning how the cell environment may influence the fluorescence properties of the fluorophore. (c) The lifetime-pixel histogram reflects the heterogeneity of the mitochondria distribution with an estimated average lifetime of 3.04 ns. Under the same experimental conditions, the fluorescence lifetime of free Rh123 in PBS (pH 7.4) was measured (arrow) at a known concentration. (d) Comparative time-resolved fluorescence of free and mitochondria-bound Rh123 was also measured using the single-point approach for better signal-to-noise ratio. The fluorescence of mitochondrial Rh123 decays as a biexponential with \( \tau_1 = 2.14 \) ns, \( \alpha_1 = 0.32 \), \( \tau_2 = 3.58 \) ns, and \( \alpha_2 = 0.68 \). In contrast, free Rh123 in solution decays as a single exponential with an estimated lifetime of 3.97 ns.
3.3 Concentration Imaging Reveals High Affinity of Rh123 to Mitochondria

To achieve accurate concentrations of biomolecules in living cells, it is essential to quantify fluorescence signal dependence on the fluorophore concentration, fluorescence quantum yield (i.e., lifetime), and the detection efficiency of the imaging system. 2P FLIM measurements of free and mitochondrial Rh123 will enable us to quantify the fluorescence intensity and lifetime distribution throughout a living cell. The relative detection efficiency (or system parameter) can be determined absolutely or cancelled out in relative measurements using free Rh123 in solution with known concentrations.

As shown in Fig. 6, the estimated maximal concentration of Rh123 is up to 30 times higher than the stock solution (0.5 µM), which indicates a high affinity of this lipophilic marker to the mitochondrial membrane. Previous studies have shown that the Rh123 specificity and strong affinity to mitochondria are due to the ionic interaction between this lipophilic marker and the mitochondrial inner membrane in live cells. Unlike most of the rhodamine derivatives, Rh123 is positively charged, which results in its strong attraction to the negatively charged mitochondrial matrix and the high potential gradient across the inner membrane. Above the background signal, the average accumulation of Rh123 per pixel is 2.7 ± 0.9 µM (n=5), which is about five-fold larger than the initial Rh123 incubation concentration (0.5 µM). One must emphasize that the previously estimated mitochondrial Rh123 concentration should be considered a lower limit due to the optical selectivity of polarized excitation of large, randomly distributed molecules.

3.4 Mitochondrial Environment is Restrictive to Rh123 Rotational Diffusion

Fluorescence anisotropy (both steady state and time resolved) has been widely used in biochemical and biophysical studies of conformational changes associated with intermolecular interactions, such as protein-protein, DNA-protein, and cell signaling. Anisotropy imaging also enables us to discuss fluorescence lifetime heterogeneity in FLIM measurements in terms of both structural conformations and restriction of the cell environment.

3.4.1 Steady-state fluorescence polarization anisotropy imaging

Steady-state fluorescence anisotropy imaging provides a spatial visualization of the dipole-moment distribution of fluorophores and their orientation order with respect to laser polarization. The steady-state fluorescence polarization anisotropy images of Rh123-stained cancer cells were constructed using parallel- and perpendicular-polarization images (either confocal or 2P) that were recorded simultaneously. The anisotropy image of each pixel was calculated using Eq. [5] with background subtractions. Typical confocal 1P fluorescence images with parallel and perpendicular polarizations (using 488 nm, cw) are shown in Fig. 7(a) and 7(b). The calculated anisotropy image is shown in Fig. 7(c) with a color code bar for the initial anisotropy per pixel. Since the polarization-analyzed images were recorded with a high NA objective (1.2 NA, 60X, water immersion), the apparent steady-state anisotropy per pixel is convoluted with a possible objective-induced depolarization effect. The wide range of average anisotropy per pixel reveals a heterogeneous molecular environment with different degrees of restriction on the fluorophore rotational mobility (or tumbling). The average steady-state anisotropy per single cancer cell is 0.23 ± 0.04 (n=7), which is significantly smaller than the theoretical maximum (r0=0.4 for 1P). The observed anisotropy images with low average anisotropy indicate competing depolarization effects that may include 1. high NA objective, 2. fluorescence resonance energy transfer (e.g., homo-FRET), and 3. a percentage of free Rh123 in a nonrestrictive mitochondrial environment. However, the average value is misleading due to the heterogeneous cell environment, as revealed by the 2-D anisotropy image [Fig. 7(c)]. Using Eq. [7] the anisotropy per pixel can be converted to the angle (δ) between absorbing and emitting dipole of Rh123 molecules. The corresponding average angle for the 1P average anisotropy per single cell is  δ=33 deg ± 4 deg (n=7).

To the best of our knowledge, these steady-state anisotropy results of Rh123-stained mitochondria in live cells are the first to be reported. Previously, Sidwani, Holowka, and Baird have studied the steady-state fluorescence anisotropies (an average of ~0.13) of NBD-PE-labeled lipid bilayers in a cuvette. More recently, changes in the anisotropy (r0) ~ 0.1 of DiI-C18-labeled plasma membrane under different conditions of extensive cross-linking of IgE-receptor (FcεRI) in RBL-2H3 mast cells have been reported, compared with (r0) = 0.30 ± 0.01 for DiI-C12-labeled fluid giant unilamellar vesicles (GUVs). However, the lipid markers used in those studies have different chemical structures, and therefore, intercalation mechanisms with the lipid bilayer. In addition,
the inner membrane of mitochondria is known to have higher membrane potential than the plasma membrane in living cells or synthetic lipid bilayers such as GUVs, which explains the labeling specificity of Rh123 to mitochondria. As a result, we conclude that Rh123 experiences a more restrictive environment in mitochondria, which we further examine using time-resolved fluorescence polarization anisotropy.

3.4.2 Time-resolved, one-photon-fluorescence polarization anisotropy

To elucidate the structural conformation that underlies Rh123 interactions with mitochondria, we have conducted time-

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*Fig. 7* A typical 1P polarization anisotropy image of Rh123-stained mitochondria in a live, intact cell reveals heterogeneous restriction of the fluorescent marker. (a) Parallel and (b) perpendicular 1P fluorescence (525/30 nm) polarization images were recorded simultaneously using 488-nm excitation. These images are used in calculating (c) the anisotropy image where each pixel represents the initial anisotropy \( r_0(x, y) \) of the fluorophore Rh123. The different background of parallel and perpendicular images were calculated and subtracted prior to anisotropy image calculations. (d) Complementary time-resolved fluorescence anisotropy decays of free and mitochondria-bound Rh123 are shown. Rh123 in mitochondria exhibit biexponential anisotropy decays (for example, \( \phi_1 = 1.82 \text{ ps} \) \( \beta_1 = 0.07 \), \( \phi_2 = 37 \text{ ns} \), and \( \beta_2 = 0.24 \)). The fitting quality can be further improved by adding a minor (~0.025) fast component (~100 ps). The observed complex rotational diffusion reveals a heterogeneous environment that exerts variable constraints on the fluorophore mobility. In contrast, the anisotropy of free Rh123 (PBS, pH 7.4) decays as a single exponential with a rotational time of ~120 ps, which is consistent with its molecular weight. The fitting parameters of time-resolved anisotropy are summarized in Table 3.
resolved anisotropy measurements in living cells where the laser pulses (480 nm, 4.2 MHz) were strategically focused on a single mitochondrion. Time-resolved anisotropy of mitochondrial Rh123 decays as a multieponential [Fig. 7(d)]. This represents a complex pattern of rotational motion (tumbling). On average (n=6), Rh123 in the mitochondrial compartment exhibits a biexponential decay with \( \varphi_1 = 2.2 \pm 0.6 \text{ ns} \) (\( \beta_1 = 0.08 \pm 0.01 \)) and \( \varphi_2 = 48 \pm 17 \text{ ns} \) (\( \beta_2 = 0.23 \pm 0.01 \)), in addition to a minor fast component \( \sim 100 \text{ ps} \) and an amplitude of \( \sim 0.03 \). The overall initial anisotropy \( r_0 = 0.32 \pm 0.01 \) and fitting parameters as summarized in Table 3. Since the rotational diffusion time is limited by the fluorescence lifetime \( \sim 3.7 \text{ ns} \) of the fluorophore, the slower component will be less accurate for large (or more restricted) molecules. Using Eq. 8 and the average rotational diffusion time of 36.2 ns, the apparent hydrodynamic volume of mitochondrial Rh123 is \( \sim 167 \text{ nm}^3 \) (i.e., \( \sim 3.4 \text{ nm radius} \)). In contrast, the fluorescence polarization anisotropy of free Rh123 exhibits a single exponential decay with a fast rotational time (\( \sim 120 \text{ ps} \)) in PBS (pH 7.4) at room temperature, which yield a hydrodynamic volume of \( \sim 0.55 \text{ nm}^3 \) (i.e., \( \sim 0.51 \text{ nm radius} \)). Such comparison suggests that the apparent hydrodynamic radius of mitochondrial Rh123 is roughly seven times larger than the free label.

Partikian et al. investigated the rotational diffusion of green fluorescent protein (GFP) in the mitochondrial matrix using time-resolved anisotropy.\(^{10}\) The authors reported that the rotational times of GFP in both the mitochondrial matrix and buffer solution are basically the same.\(^{11}\) However, Rh123 is a much smaller fluorophore, and yet exhibits a much slower rotational diffusion component inside cells than in solution (\( \sim 120 \text{ ps} \)). Moreover, the large population fraction (\( \sim 73\% \)) of the slow Rh123 suggests that Rh123 is predominately restricted to the mitochondrial inner membrane. The minor fast rotational component (<100 ps) is likely due to free Rh123 in the mitochondrial matrix (since free Rh123 in the cytosol is negligible) or segmental mobility within a small wobbling angle. Assigning the intermediate rotational time (\( \sim 3 \text{ ns} \)), however, to a well-defined conformation is not straightforward using only these results. One possibility is that Rh123 intercalates to the mitochondria via two different mechanisms. First, the majority (\( \sim 73\% \)) of Rh123 is attached to the inner membrane of mitochondria in the presence of high membrane potential. To be more specific, Rh123 may be attached to F1,F0-ATPases, causing inhibition of its activities, as proposed by Modica-Napoliato and Singh.\(^{12}\) Second, a small fraction (\( \sim 27\% \)) of the fluorescent label, which is positively charged, may exist in a relatively restricted environment either on the inner membrane or in the space between the inner and outer membranes of the mitochondria. These multiple conformations may explain the observed multieponential fluorescence decays using high temporal resolution (i.e., single-point measurements). The root-square of residual-to-initial (\( r_{\text{res}} \)) anisotropy ratio (i.e., order parameter) is 0.82 \( \pm 0.03 \) for Rh123 in mitochondria, which indicates a significant degree of orientation constraint on the fluorophore dipole moment.\(^{10}\) This argument is consistent with the previous studies on Rh123 inhibition of oxidative phosphorylation via the disruption of electron transport chain in the inner membrane of mitochondria.\(^{11}\)

### Table 3 Fitting parameters of time-resolved fluorescence polarization anisotropy of Rh123 in the mitochondria of cancer cells

<table>
<thead>
<tr>
<th>Environment</th>
<th>( \beta_1 )</th>
<th>( \varphi_1 ) (ns)</th>
<th>( \beta_2 )</th>
<th>( \varphi_2 ) (ns)</th>
<th>( \beta_3 )</th>
<th>( \varphi_3 ) (ns)</th>
<th>( r_0 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td>0.024(4)(^a)</td>
<td>0.08(3)</td>
<td>0.07(1)</td>
<td>2.2(4)</td>
<td>0.23(2)</td>
<td>42(4)</td>
<td>0.32(1)</td>
</tr>
<tr>
<td>PBS (pH 7.4)</td>
<td>0.28(3)</td>
<td>0.11(1)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

\(^a\)The number in parentheses represents the standard deviation in the last digit of a given fitting parameter.

### 3.5 Translational Diffusion of Mitochondrial Rh123 as Probed Using FCS

To further explore translational diffusion of Rh123 in mitochondria, we performed fluorescence autocorrelation analysis using FCS with an extended observation time scale up to seconds (with \( \sim 200 \text{ ns} \) resolution). The fluorescence fluctuation autocorrelation of rhodamine green (RhG), which has a lateral diffusion coefficient \( D_{\text{RhG}} = 2.8 \times 10^{-6} \text{ cm}^2/\text{s} \) was measured to calibrate our FCS setup with an underfilled objective (1.2 NA, 60\( \times \), water immersion) and a 50-\( \mu \text{m} \) fiber as the confocal pinhole. The excited-state lifetime of Rh123 (\( \sim 3.7 \text{ ns} \) in solution, \( \sim 3.1 \text{ ns} \) in cells from the FLIM measurement before) and the absorption cross section at 488-nm excitation (\( \sim 1.15 \times 10^{-16} \text{ cm}^2 \)) suggest a saturation intensity of \( \sim 614 \text{ kW/cm}^2 \) for cellular studies and \( \sim 442 \text{ kW/cm}^2 \) in solution. As a result, all FCS measurements reported here were carried out in the linear-excitation intensity regime (typically, 200 \( \mu \text{W/cm}^2 \)) to avoid cellular photodamage or other photophysical processes (e.g., photobleaching, intersystem crossing, and saturation) that would complicate data interpretation. Under such a low excitation intensity, the fluorescence fluctuation autocorrelation function of free rhodamine green (N ~19 molecules) in solution is best described by diffusion alone (i.e., Eq. 9) with an estimated diffusion time of \( \tau_D = 0.106 \pm 0.002 \text{ ms} (n=3) \) at room temperature. Based on the measured diffusion time of RhG and the axial-to-lateral dimension (or structural parameter, \( S \sim 10.8 \)), an observation volume of \( \sim 1.87 \mu\text{m}^3 \) (for 3-D Gaussian) was calculated. Under the same experimental conditions, the stained cells were first imaged (or visualized using the microscope eye piece) to select a region of interest (e.g., a mitochondrion) for FCS measurements where the laser was strategically focused. Since FCS is a single-molecule technique that requires only a few molecules in the observation volume at any given time,
the incubation concentration of the stock Rh123 was reduced (~100 nM, ~15 min) for FCS compared with other fluorescence measurements. Further photobleaching was usually necessary before an autocorrelation function could be observed. As a result, it is not clear if the estimated observation volumes in solution and in restrictive environments that require photobleaching (e.g., mitochondria) would be exactly the same.

Having an average dimension of ~1 to 2 μm in diameter, a single mitochondrion is unlikely to move considerably during the acquisition time (100 to 300 s) of FCS measurements due to its projected small diffusion coefficient according to our own estimate (~1.12 × 10^{-11} cm^2/s) and other literature values (~5 × 10^{-12} cm^2/s). However, our assumption of a Brownian diffusion may not be accurate considering the crowded and complex cellular environment. Moreover, mitochondria in cells keep undergoing fusion and fission46 and therefore changing their shapes and sizes.

Typical autocorrelation curves of Rh123 in solution and mitochondria are shown in Fig. 8. Free Rh123 exhibits a single diffusion coefficient of ~3.3 × 10^{-6} cm^2 s^{-1} in solution (at room temperature) with an estimated brightness (⟨F⟩/N) of ~4.35 × 10^3 photon sec^{-1} molecule^{-1}. The autocorrelation of cytosolic Rh123 fluorescence (i.e., away from mitochondria) can also generally be described as a single diffusing species with a comparable diffusion coefficient as that of free Rh123 in solution (N ~ 3 molecules, D_F ~ 3.6 × 10^-8 cm^2 s^{-1}). In contrast, the autocorrelation function of mitochondrial Rh123 is best described as two diffusing species (or the same species in two different microenvironments), such that

\[ G_f(\tau, x, y) = \left[ (1 - f)G_{D_1}(\tau, x, y) + f \cdot G_{D_2}(\tau, x, y) \right], \] (10)

where \( f \) is the fraction of the second diffusing species and \( G_{D_i}(x, y) \) is the corresponding autocorrelation function of the \( i \)th species at pixel coordinates \( (x, y) \) inside a cell image. The estimated diffusion coefficients of these species are \( D_{D_1} = 2.3 \times 10^{-6} \text{ cm}^2/\text{s} \) and \( D_{D_2} = 1.1 \times 10^{-7} \text{ cm}^2/\text{s} \), where the amplitude fraction of the slower component is ~82%. The diffusion coefficients of both diffusing species in mitochondria (Table 4) have large standard deviations, which indicate the complex sampling environment inside the observation volume. Further, these diffusion coefficients yield an apparent hydrodynamic radius of ~1.1 and ~22.3 nm for the fast and slow components, respectively. Importantly, the slow laterally diffusing mitochondrial Rh123 is ~6 times larger than the rotating equivalent, which perhaps suggests a cluster formation on the FCS time scale. Previous studies have suggested that Rh123 is likely to partition into the mitochondrial inner membrane, where the intermembrane region (i.e., the space between the inner and outer membranes) of mitochondria is roughly 18 to 20 nm in thickness. This provides a number of possible scenarios for the interpretation of our FCS results. Furthermore, any measurement on the inner membrane of mitochondria has the propensity to sample fluorophores from the cytosol and/or the mitochondrial matrix.51 Pastikan et al. reported translational diffusion of GFP in mitochondrial matrix compartments using FRAP with a reported diffusion coefficient of ~2–3 × 10^{-7} cm^2/s. These results were attributed to a relatively uncrowded mitochondrial matrix. In this case, we assign the fast component to free Rh123 diffusing in the mitochondrial matrix. However, sampling fluorophores from the cytosol or imaging buffer should not be ruled out, considering the observation volume (~1.87 μm^3) and the single-molecule sensitivity of FCS. The slow component can be assigned to the translational diffusion of Rh123 in the inner membrane of mitochondria, which is expected to be a more restrictive region than the mitochondrial matrix. Previous re-

![Fig. 8](https://www.spiedigitallibrary.org/journals/Journal-of-Biomedical-Optics/vol.13/issue4/41315-12/fig8)

**Fig. 8** FCS measurements reveal distinct and heterogeneous autocorrelation functions of Rh123-labeled cancer cells. Mitochondrial Rh123 (●) diffuses much slower, and the autocorrelation functions are best described as two diffusing species (or the same species in different microenvironments): \( \tau_{D_1} = 0.12 \text{ ms} \) (18%) and \( \tau_{D_2} = 2.65 \text{ ms} \) (82%). The fluorescence fluctuation autocorrelation of free Rh123 (PBS, pH 7.4; ○) can be described as a single diffusing species with a diffusion time of ~0.08 ms.

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**Table 4** A summary of fluorescence correlation spectroscopy results of rhodamine-123 in solution and living cancer cells. The fluorescence fluctuation autocorrelation of mitochondrial Rh123 is described using two diffusion species with fast and slow diffusion rates. The apparent diffusion coefficients in cellular Rh123 were calculated relative to that of RhG in solution at room temperature (~2.8 × 10^{-6} cm^2/s). The molecular brightness (number of fluorescence photons per molecule per second) of Rh123 is also shown as a function of the surrounding environment.

<table>
<thead>
<tr>
<th>Region sampled</th>
<th>( D_1 ) (×10^{-6} cm^2 s^{-1})</th>
<th>( D_2 ) (×10^{-6} cm^2 s^{-1})</th>
<th>( f ) (%)</th>
<th>Brightness (photon s^{-1} molecule^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (pH 7.4) (n=3)</td>
<td>3.3(3) (^a)</td>
<td>--</td>
<td>--</td>
<td>6(2) × 10^3</td>
</tr>
<tr>
<td>Mitochondria (n=6)</td>
<td>1.3 (8)</td>
<td>7 (3)</td>
<td>75 (16)</td>
<td>0.8(3) × 10^3</td>
</tr>
</tbody>
</table>

\(^a\)The number in parentheses represents the standard deviation in the last digit of a given fitting parameter.
search has assigned Rh123 interactions with the mitochondrial inner membrane. Apart from the apparent differences in translational diffusion coefficients, there is also a notable decrease in molecular brightness \((D_F/N)\) from free solution to Rh123 in mitochondria (Table 4), which is due to the observed reduction of Rh123 fluorescence lifetime in mitochondria (see before).

4 Conclusions

In this report, we describe an integrated biophotonics approach, built on a single platform, which we use for multi-scale biophysical studies of Rh123 to probe the heterogeneity of the mitochondrial environment in living cells. This experimental approach allows for imaging the cell morphology and mitochondrial distribution using DIC and fluorescence (confocal and two-photon) microscopy at a diffraction-limited spatial resolution \((\sim \lambda/2)\). The sensitivity of the excited-state fluorescence lifetime \((10^{-12} \text{ to } 10^{-8} \text{ s})\) imaging microscopy of Rh123 enables us to quantify the mitochondrial environment effects on the fluorescence quantum yield. The same FLIM approach can also be used to investigate molecule-molecule interactions (e.g., time-resolved FRET), as well as for converting fluorescence intensity to concentration image of living cells without the need to destroy them as in conventional biochemical techniques. Fluorescence polarization anisotropy imaging and rotational diffusion \((10^{-10} \text{ to } 10^{-7} \text{ s})\) provide molecular insights into the structural conformation, volume, and surroundings of a given fluorophore in living cells. The integrated FCS capability extends our observation time to investigate translational diffusion \((10^{-6} \text{ to } 10 \text{ s})\) and chemical kinetics of equilibrated reactions with single-molecule sensitivity. As a result, FCS would enable us to distinguish between conformational changes (that may be transient) and the existence of multiple species with varying volumes. While each method may be quite complex and would require a specialized user (protocol), the interdisciplinary trend in graduate student education would likely help in implementing the proposed approach for multiscale studies. We also acknowledge that some of these measurements will be difficult to carry out simultaneously due to the time consumed in switching between different exit ports. Yet we believe that it will be possible to quantify functionally driven changes in the structural conformation and surroundings using our integrated approach in inherently complex systems. These modalities will provide researchers with a unique opportunity to assess functionally driven, multiscale changes in molecular and cellular biology under controlled stimulation and toward model building. In addition, this integrated approach can be applied to a wide range of molecular and cellular processes such as receptor-mediated signaling. Ultimately, the capability of characterizing the state of molecular assemblies and dynamics within the context of cell morphology may lead to biomedical cell diagnosis and to the high-throughput screening of therapeutic drugs, with single-molecule sensitivity. Currently, we are using the same experimental approach for biomembranes studies (both in an engineered model system and in vivo), energy metabolism, and protein-protein interactions.

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